

(12) STANDARD PATENT
(19) AUSTRALIAN PATENT OFFICE

(11) Application No. **AU 2019277550 B2**

(54) Title
Trichoderma filamentous fungus mutant strain and method for producing protein

(51) International Patent Classification(s)
C12N 1/15 (2006.01) **C12P 19/14** (2006.01)
C12N 9/42 (2006.01) **C12P 21/02** (2006.01)
C12N 15/09 (2006.01)

(21) Application No: **2019277550** (22) Date of Filing: **2019.05.30**

(87) WIPO No: **WO19/230860**

(30) Priority Data

(31) Number	(32) Date	(33) Country
2018-105256	2018.05.31	JP

(43) Publication Date: **2019.12.05**

(44) Accepted Journal Date: **2025.03.06**

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(56) Related Art
ZHANG, WEIXIN ET AL.: Two Major Facilitator Superfamily Sugar Transporters from Trichoderma reesei and Their Roles in Induction of Cellulase Biosynthesis, JBC, vol. 288, no. 46, 2013, pages 32861 - 32872, Diego Martinez et al., "Genome sequencing and analysis of the biomass-degrading fungus Trichoderma reesei (syn. Hypocrea jecorina)," Nature Biotechnology, 2008, vol. 26, No. 5, pp. 553-560.

DESCRIPTION

TITLE OF INVENTION: *TRICHODERMA* FILAMENTOUS FUNGUS MUTANT STRAIN
AND METHOD FOR PRODUCING PROTEIN

5

TECHNICAL FIELD

[0001]

The present invention relates to a mutant strain of a filamentous fungus of the genus *Trichoderma*, the mutant strain having an enhanced protein-producing ability, and to a protein
10 production method using the mutant strain.

BACKGROUND ART

[0002]

Filamentous fungi of the genus *Trichoderma* are known for having a high protein-
15 producing ability, and studies have heretofore been made on protein production using the
filamentous fungi. Filamentous fungi of the genus *Trichoderma* are used especially for
producing a cellulase classified as a saccharifying enzyme among proteins using cellulose,
lactose, cellobiose, or the like as an inducer.

[0003]

20 Genetic modifications are being investigated in order to further increase cellulase
production amount, in particular to enhance the production amount of β -glucosidase, which
accounts for a small proportion of the saccharifying enzymes. Such investigations include
overexpression or deletion of a factor which controls cellulase production.

[0004]

25 Non-Patent Literature 1 describes that a filamentous fungus of the genus
Trichoderma was modified by reducing the function of Cre1, which is a transcription factor
repressing cellulase production among the factors controlling cellulase production, thereby
acquiring a mutant strain of the filamentous fungus of the genus *Trichoderma* which has a
high cellulase-producing ability.

30 [0005]

Meanwhile, it is known that genetic modifications result in a decrease in cellulase
production amount. Non-Patent Literature 2 describes that in cases when *Trichoderma*
reesei in which a sugar transporter thereof has been deleted is used together with lactose or

cellulose as an inducer, cellulase production amount decreased.

CITATION LIST

NON-PATENT LITERATURE

5 [0006]

Non-Patent Literature 1: Juliano P, Single nucleotide polymorphism analysis of a *Trichoderma reesei* hyper-cellulolytic mutant developed in Japan, Bioscience, Biotechnology, and Biochemistry, Volume 77, 2013, Issue 3, P534-543

10 Non-Patent Literature 2: Porciuncula Jde, Identification of Major Facilitator Transporters Involved in Cellulase Production during Lactose Culture of *Trichoderma reesei* PC-3-7, Biosci Biotechnol Biochem. 77, 1014-1022 (2013).

SUMMARY OF INVENTION

TECHNICAL PROBLEM

15 [0007]

As described above, a protein-production-controlling transcription factor in a filamentous fungus of the genus *Trichoderma* has been identified, but this is considered to be merely a part of the control mechanism. Then, an object of the present invention is to acquire a mutant strain of a filamentous fungus of the genus *Trichoderma* which has an enhanced protein-producing ability by making a search for a novel mechanism controlling protein production of the filamentous fungus of the genus *Trichoderma*, and to provide a protein production method using the mutant strain of the filamentous fungus of the genus *Trichoderma*.

25 SOLUTION TO PROBLEM

[0008]

The present inventors thought that if a gene of a filamentous fungus of the genus *Trichoderma* which, when modified, can bring about an increase in protein production can be specified, then the amount of proteins that can be produced by the filamentous fungus of the genus *Trichoderma* can be further increased. The inventors diligently made investigations and, as a result, have discovered that improvements in protein production property and β -glucosidase production property can be attained by cultivating a mutant strain of a filamentous fungus of the genus *Trichoderma*, the mutant strain having been reduced, by a

genetic modification, in the function of a polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2. The present invention has been accomplished based on this finding.

[0009]

5 More specifically, the present invention includes the following (1) to (6).

(1) A mutant strain of a filamentous fungus of the genus *Trichoderma*, having a reduced function of a polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2.

10 (2) The mutant strain of a filamentous fungus of the genus *Trichoderma* according to (1), in which at least 413th and succeeding amino acid residues from the N-terminal side in the amino acid sequence represented by SEQ ID NO: 2 is deleted.

(3) A method of producing a protein, the method including a step of cultivating the mutant strain of a filamentous fungus of the genus *Trichoderma* according to (1) or (2).

15 (4) A method of producing a cellulase, the method including a step of cultivating the mutant strain of a filamentous fungus of the genus *Trichoderma* according to (1) or (2).

(5) The method of producing a cellulase according to (4), the method including a step of cultivating the mutant strain of a filamentous fungus of the genus *Trichoderma* according to (1) or (2) in a culture medium including one or more kinds of inducers selected from the group consisting of lactose, cellulose, and xylan.

20 (6) A method of producing a sugar from a cellulose-containing biomass, the method including:

step a of producing a cellulase by cultivating a *Trichoderma reesei* mutant strain having a reduced function of a polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2; and

25 step b of saccharifying the biomass by using the cellulase obtained in the step a.

ADVANTAGEOUS EFFECTS OF INVENTION

[0010]

30 The mutant strain of a filamentous fungus of the genus *Trichoderma* which has been reduced in the function of a polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2 has improved protein-producing ability as compared with the filamentous fungus of the genus *Trichoderma* in which the function of the polypeptide has not been reduced. Furthermore, especially in the case where the produced protein is cellulases, an

unexpected effect that the cellulases have various improved specific activities is also obtained.

DESCRIPTION OF EMBODIMENTS

[0011]

5 The present invention is characterized in that a mutation is introduced into a parent strain of a filamentous fungus of the genus *Trichoderma*, which is a microorganism originally having an excellent protein-producing ability, to thereby further enhance the protein-producing ability. Consequently, the parent strain of a filamentous fungus of the genus *Trichoderma* to be used in the present invention is not limited to wild strains, and mutant
10 strains of a filamentous fungus of the genus *Trichoderma* which have been improved so as to have an increased protein-producing ability can also be favorably used as the parent strain. For example, a mutant strain having an improved protein production property by performing a mutation treatment with a mutagen, UV irradiation, etc. can be utilized as the parent strain of a mutant strain of a filamentous fungus of the genus *Trichoderma*. Specific examples of
15 mutant strains usable as the parent strain include: *Trichoderma parareesei* (ATCC MYA-4777), which is an ancestor to *Trichoderma reesei*; QM6a strain (NBRC31326), QM9123 strain (ATCC24449), QM9414 strain (NBRC31329), PC-3-7 strain (ATCC66589). QM9123 strain (NBRC31327), RutC-30 strain (ATCC56765), CL-847 strain (Enzyme, Microbiol. Technol. 10, 341-346 (1988)), MCG77 strain (Biotechnol. Bioeng. Symp. 8, 89 (1978)), and
20 MCG80 strain (Biotechnol. Bioeng. 12, 451-459 (1982)), which are known mutant strains derived from *Trichoderma reesei*; and strains derived from these. QM6a strain, QM9414 strain, and QM9123 strain are available from NBRC (NITE Biological Resource Center), and PC-3-7 strain and RutC-30 strain are available from ATCC (American Type Culture Collection).

25 [0012]

 The polypeptide consisting of an amino acid sequence represented by SEQ ID NO: 2 is a polypeptide possessed by filamentous fungi of the genus *Trichoderma*, and in the National Center for Biotechnology Information, this polypeptide is registered also as a predicted protein (EGR44419) that *Trichoderma reesei* QM6a strain has. The polypeptide
30 consisting of the amino acid sequence represented by SEQ ID NO: 2 is a polypeptide whose function is not known, but Conserved Domain Architecture Retrieval Tool of National Center for Biotechnology Information discloses that the 26th to 499th amino acid residues from the N-terminal side have a sugar (and other) transporter domain. It is presumed from the

disclosure that the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2 participates at least in sugar transportation between the inside and the outside of the fungus bodies. In the present invention, the wording “reduced in the function of the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2” means that a gene encoding EGR44419 has a mutation.

[0013]

In the present invention, a decrease in the function of the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2 indicates a state in which a base sequence encoding the amino acid sequence represented by SEQ ID NO: 2 has undergone a mutation, resulting in a decrease in or the elimination of the function of the polypeptide.

Furthermore, also included in decreases in the function of the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2 is the case where a base sequence other than a base sequence encoding the amino acid sequence represented by SEQ ID NO: 2 has undergone a mutation and this has resulted in a decrease in or the elimination of the expression of the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2. A mutation of a base sequence is caused by substitution, deletion, insertion, or duplication of a base, etc.

[0014]

Specific examples of the gene encoding the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2 include the base sequence represented by SEQ ID NO: 1.

[0015]

Examples of methods for reducing the function of the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2 include a method of introducing a mutation that causes a total deletion of a sugar (and other) transporter domain, a partial deletion of a sugar (and other) transporter domain, a change in the conformation of a sugar (and other) transporter domain, or a total deletion of the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2. It is possible to reduce the function of the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2, also by introducing a mutation that causes a decrease in or the elimination of the expression of the polypeptide.

[0016]

The wording “deletion of a sugar (and other) transporter domain” means a total or

partial loss of the domain, a change of the whole or some of the domain into different amino acid(s), or a combination of them. More specifically, that wording means that the sequence identity to the amino acid sequence of the sugar (and other) transporter domain becomes 80% or less, preferably 50% or less, more preferably 20% or less, more preferably 10% or less, more preferably 5% or less, more preferably 3% or less, more preferably 1% or less, and most preferably 0%.

[0017]

In the present invention, specific examples of the case where the function of a polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2 is reduced by a mutation, such as deletion, substitution, or addition, that has occurred in the amino acid sequence located in a sugar (and other) transporter domain include a frame shift mutation in the base sequence represented by SEQ ID NO: 1 in which 11 bases have been inserted into the 1,415th position. It is presumed that the mutation causes the translation to end at the 419th position in the amino acid sequence represented by SEQ ID NO: 2 and this has shortened the amino acid sequence constituting the sugar (and other) transporter domain, resulting in a decrease in the original function.

[0018]

The total deletion of a sugar (and other) transporter domain, the partial deletion of a sugar (and other) transporter domain, and the total deletion of a polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2 are attained by causing a gene sequence encoding the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2 to undergo a frame shift or stop codon mutation due to base deletion, insertion, substitution, etc.

[0019]

The decrease or elimination of the expression of a polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2 is attained by causing a mutation to the promoter or terminator region of the gene encoding the amino acid sequence represented by SEQ ID NO: 2. In general, the promoter and terminator regions correspond to a region of hundreds of bases in length before and after the gene participating in transcription. Specific examples of the base sequence containing a promoter and a terminator which participate in transcription of the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2 include the base sequence represented by SEQ ID NO: 7.

[0020]

For introducing such genetic mutations, use can be made of existing genetic mutation methods such as a mutation treatment with a mutagen known to one skilled in the art or with UV irradiation, etc., gene recombination such as homologous recombination using a selection marker, and a mutation by a transposon.

5 [0021]

The mutant strain of a filamentous fungus of the genus *Trichoderma* of the present invention has an enhanced protein-producing ability as compared with the filamentous fungus of the genus *Trichoderma* in which the function of the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2 has not been reduced. In the case where the mutant strain of a filamentous fungus of the genus *Trichoderma* of the present invention is cultivated, an increased protein concentration is obtained as compared with a culture solution of the filamentous fungus of the genus *Trichoderma* in which the function of the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2 has not been reduced. Furthermore, in the case where the protein is an enzyme, the enzyme has increased specific activity. Here, the protein concentration increase rate or enzyme specific activity increase rate is not particularly limited as long as the concentration or the specific activity has increased, but the increase rate is preferably 20% or more.

15 [0022]

Besides having been reduced in the function of the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2, the mutant strain of a filamentous fungus of the genus *Trichoderma* of the present invention may have a gene mutation which brings about an improvement in protein production amount. Specific examples thereof include a gene mutation which reduces the function of a polypeptide represented by SEQ ID NO: 8. The polypeptide consisting of an amino acid sequence represented by SEQ ID NO: 8 is a polypeptide possessed by *Trichoderma reesei*, and in the National Center for Biotechnology Information, this polypeptide is registered as predicted protein EGR50654 that *Trichoderma reesei* QM6a strain has. The polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 8 is a polypeptide whose function is not known, but Conserved Domain Architecture Retrieval Tool of National Center for Biotechnology Information discloses that the 95th to 277th amino acid residues from the N-terminal side have "Middle domain of eukaryotic initiation factor 4G domain" (hereinafter referred to as "MIF4G domain") and that the 380th to 485th amino acid residues from the N-terminal side have MA-3 domain. The two domains, MIF4G and MA-3, are known for having the function of binding to DNAs or

RNAs (Biochem. 44, 12265-12272 (2005); Mol. Cell. Biol. 1, 147-156 (2007)). It is presumed from those disclosures that the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 8 at least has the function of binding to a DNA and/or an RNA. [0023]

5 Specific examples of genes encoding the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 8 include the base sequence represented by SEQ ID NO: 9. Examples of the gene mutation which reduces the function of EGR50654 include a total deletion of the MIF4G domain and/or MA-3 domain possessed by EGR50654, a partial deletion of the MIF4G domain and/or MA-3 domain, and a gene mutation which changes the
10 configuration relationship between the MIF4G domain and the MA-3 domain. Furthermore, the function of the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 8 can be reduced also by introducing a mutation which decreases or eliminates the expression of the polypeptide. Specific examples of the deletion of the function of the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 8 include a
15 mutation in the base sequence represented by SEQ ID NO: 9 which deletes any of the 1,039th to 1,044th bases.

[0024]

The present invention further relates to a protein production method including a step of culturing the mutant strain of a filamentous fungus of the genus *Trichoderma*, the mutant
20 strain having been reduced in a function of a polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2.

[0025]

The protein to be produced in the present invention is not particularly limited, but proteins to be secreted from the fungus body can be efficiently produced. Enzymes are
25 preferred among these. More preferred are saccharifying enzymes such as cellulases, amylases, invertases, chitinases, and pectinases. Still more preferred are cellulases.

[0026]

Cellulases that can be produced in the present invention include various hydrolases, which include enzymes having a decomposition activity against xylan, cellulose, and
30 hemicellulose. Specific examples thereof include cellobiohydrolase (EC 3.2.1.91), which produces cellobiose by hydrolyzing cellulose chains, endoglucanase (EC 3.2.1.4), which hydrolyzes cellulose chains from central portions thereof, β -glucosidase (EC 3.2.1.21), which hydrolyzes cellooligosaccharide and cellobiose, xylanase (EC 3.2.1.8), which is characterized

by acting on hemicellulose and, in particular, on xylan, and β -xylosidase (EC 3.2.1.37), which hydrolyzes xylooligosaccharide. As stated above, whether the mutant strain of a filamentous fungus of the genus *Trichoderma* of the present invention has an enhanced protein-producing activity is ascertained by ascertaining an improvement in cellulase specific activity by
5 ascertaining that the specific activity of any of those hydrolases has improved. The cellulases produced in the present invention have an improved activity of, in particular, β -glucosidase among those enzymes.

[0027]

The β -glucosidase specific activity is determined by the following method. First, a
10 10 μ L of an enzyme dilution is added to 90 μ L of 50 mM acetate buffer containing 1 mM p-nitrophenyl- β -glucopyranoside (produced by Sigma-Aldrich Japan), and the mixture is allowed to react at 30°C for 10 minutes. Then, 10 μ L of 2 M sodium carbonate is added and mixed well to stop the reaction, and the increase in absorbance at 405 nm is measured. Finally, release of 1 μ mol of p-nitrophenol per minute is defined as 1 U of activity, and the
15 specific activity is calculated by dividing it by the protein amount.

[0028]

The β -xylosidase specific activity is determined by the following method. First, a
10 μ L of an enzyme dilution is added to 90 μ L of 50 mM acetate buffer containing 1 mM p-nitrophenyl- β -xylopyranoside (produced by Sigma-Aldrich Japan), and the mixture is allowed
20 to react at 30°C for 30 minutes. Then, 10 μ L of 2 M sodium carbonate is added and mixed well to stop the reaction, and the increase in absorbance at 405 nm is measured. Finally, release of 1 μ mol of p-nitrophenol per minute is defined as 1 U of activity, and thus the specific activity is calculated.

[0029]

The cellobiohydrolase specific activity is determined by the following method.
25 First, a 10 μ L of an enzyme dilution is added to 90 μ L of 50 mM acetate buffer containing 1 mM p-nitrophenyl- β -lactopyranoside (produced by Sigma-Aldrich Japan), and the mixture is allowed to react at 30°C for 60 minutes. Then, 10 μ L of 2 M sodium carbonate is added and mixed well to stop the reaction, and the increase in absorbance at 405 nm is measured.
30 Finally, release of 1 μ mol of p-nitrophenol per minute is defined as 1 U of activity, and the specific activity is calculated by dividing it by the protein amount.

[0030]

The culture medium composition in the cultivating step is not particularly limited as

long as it is a culture medium composition where *Trichoderma reesei* can produce a protein, and a known culture medium composition for microbe of the genus *Trichoderma* can be employed. As a nitrogen source, use can be made, for example, of polypeptone, bouillon, CSL, or soybean cake. An inducer for protein production may be added to the culture
5 medium.

[0031]

In the case of producing cellulases by the present invention, the mutant strain can be cultivated in a culture medium containing one or more inducers selected from the group consisting of lactose, cellulose, and xylan. For introducing cellulose or xylan, biomass
10 containing cellulose or xylan may be added as an inducer. Specific examples of the biomass containing cellulose or xylan include not only plants such as seed plant, pteridophyte, bryophyte, algae, and water plant, but also waste building materials. The seed plants are classified into gymnosperms and angiosperms, and both can be used favorably. The angiosperms are further classified into monocotyledons and dicotyledons. Specific
15 examples of the monocotyledons used preferably include bagasse, switchgrass, napier grass, erianthus, corn stover, corncob, rice straw, and wheat straw, and specific examples of the dicotyledons used preferably include beet pulp, eucalyptus, oak, and white birch.

[0032]

As for the biomass containing cellulose or xylan, a pretreated product may be used.
20 The pretreatment method is not particularly limited, but, for example, known methods such as acid treatment, sulfuric acid treatment, dilute sulfuric acid treatment, alkali treatment, hydrothermal treatment, subcritical treatment, fine grinding treatment, and steaming treatment can be used. Pulp may be used as the pretreated biomass containing cellulose or xylan.

[0033]

25 Non-Patent Literature 2 describes that in cases when a mutant strain obtained by deleting a sugar transporter from *Trichoderma reesei* is cultivated, use of lactose as an inducer results in a decrease in cellulase production amount. However, in cases when the mutant strain of a filamentous fungus of the genus *Trichoderma* of the present invention is cultivated using lactose as an inducer, not only an improvement in protein production amount is attained
30 but also various cellulase specific activities are improved.

[0034]

Methods for cultivating the mutant of a filamentous fungus of the genus *Trichoderma* of the present invention are not particularly limited, and the mutant can be

cultivated, for example, by liquid culture using a centrifuge tube, a flask, a jar fermenter, a tank, etc. or solid culture using a plate, etc. *Trichoderma reesei* is preferably cultivated under aerobic conditions, and among these culture methods, submerged culture of performing the culture by using a jar fermenter or while aerating and stirring in a tank is preferred. The aeration rate is preferably approximately 0.1-2.0 vvm, more preferably 0.3-1.5 vvm, particularly preferably 0.5-1.0 vvm. The culture temperature is preferably approximately 25-35°C, more preferably 25-31°C. The pH condition during culture is preferably pH 3.0 to 7.0, more preferably pH 4.0 to 6.0. As for the culture period, the culture is performed under conditions allowing for protein production until a recoverable amount of proteins are accumulated. Usually, the culture period is approximately 24-240 hours, more preferably 36-192 hours.

[0035]

Methods for recovering a protein contained in the culture solution where the mutant of a filamentous fungus of the genus *Trichoderma* has been cultivated are not particularly limited, but the protein can be recovered by removing the fungus bodies of the filamentous fungus of the genus *Trichoderma* from the culture solution. Examples of methods for removing the fungus bodies include centrifugation, membrane separation, and filter press.

[0036]

Furthermore, in the case where the culture solution in which the mutant of the filamentous fungus of the genus *Trichoderma* has been cultivated is used as a protein solution without removing the fungus bodies therefrom, the culture solution is preferably treated so that the fungus bodies of the filamentous fungus of the genus *Trichoderma* cannot grow therein. Examples of treatment methods for preventing the fungus bodies from growing include heat treatment, chemical treatment, acid/alkali treatment, and UV treatment.

[0037]

In the case where the protein is an enzyme, the culture solution from which the fungus bodies have been removed or which has been treated so that the fungus body cannot grow as stated above, can be used directly as an enzyme solution.

[0038]

The cellulases obtained by cultivating the mutant strain of a filamentous fungus of the genus *Trichoderma* of the present invention, in which the function of a polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2 has been reduced, are high in various cellulase specific activities, in particular in the specific activity of β -

glucosidase, as compared with cellulases obtained by cultivating the filamentous fungus of the genus *Trichoderma* in which the function of the polypeptide has not been reduced.

Consequently, the cellulases can efficiently decompose cellulose-containing biomass to give a saccharified solution having a high glucose concentration, making it possible to obtain a larger quantity of sugar. Methods for saccharifying the cellulose-containing biomass to produce sugar are not particularly limited. The saccharification reaction may be performed in a batchwise method or a continuous method.

[0039]

Conditions for the saccharification reaction are not particularly limited. The saccharification reaction temperature is preferably in the range of 25-60°C, more preferably in the range of 30-55°C. The saccharification reaction time is preferably in the range of 2-200 hours. The pH in the saccharification reaction is preferably in the range of 3.0-7.0, more preferably in the range of 4.0-6.0. In the case of cellulases derived from the genus *Trichoderma*, the optimal pH for the reaction is 5.0. Furthermore, since the pH changes during the hydrolysis, it is preferred to add a buffer to the reaction solution or to conduct the reaction while keeping the pH constant by using an acid or an alkali.

[0040]

The enzyme composition thus used can be separated and recovered from the saccharified solution obtained by saccharifying cellulose-containing biomass. Methods for separating and recovering the enzyme composition are not particularly limited. Use can be made of a method in which the saccharified solution is filtered with an ultrafiltration membrane or the like to recover the enzyme composition on the non-permeation side. According to need, a step of removing solid matter from the saccharified solution may be conducted before the filtration. The recovered cellulases can again be used for a saccharification reaction.

EXAMPLES

[0041]

The present invention is described specifically below by referring to Examples.

[0042]

<Reference Example 1> Method for Measuring Protein Concentration

A reagent for measuring protein concentration (Quick Start Bradford protein assay, produced by Bio-Rad Laboratories, Inc.) was used. 5 μ L of a diluted filamentous fungus

culture solution was added to 250 μL of the protein concentration measurement reagent returned to room temperature. After leaving the mixture to stand at room temperature for 5 minutes, the absorbance at 595 nm was measured using a microplate reader. Using BSA as a standard, the protein concentration was calculated based on the calibration curve.

5 [0043]

<Reference Example 2> Method for Measuring Specific Activity of Cellulases
(Method for Measuring β -Glucosidase Specific Activity)

10 $10 \mu\text{L}$ of an enzyme dilution was added to $90 \mu\text{L}$ of 50 mM acetate buffer containing 1 mM p-nitrophenyl- β -glucopyranoside (produced by Sigma-Aldrich Japan), and the mixture was allowed to react at 30°C for 10 minutes. Then, $10 \mu\text{L}$ of 2 M sodium carbonate was added and mixed well to stop the reaction, and the increase in absorbance at 405 nm was measured. Release of 1 μmol of p-nitrophenol per minute was defined as 1 U of activity, and the specific activity was calculated by dividing it by the protein amount.

[0044]

15 (Method for Measuring β -Xylosidase Specific Activity)

$10 \mu\text{L}$ of an enzyme dilution was added to $90 \mu\text{L}$ of 50 mM acetate buffer containing 1 mM p-nitrophenyl- β -xylopyranoside (produced by Sigma-Aldrich Japan), and the mixture was allowed to react at 30°C for 30 minutes. Then, $10 \mu\text{L}$ of 2 M sodium carbonate was added and mixed well to stop the reaction, and the increase in absorbance at 405 nm was measured. Release of 1 μmol of p-nitrophenol per minute was defined as 1 U of activity, and the specific activity was calculated by dividing it by the protein amount.

[0045]

(Method for Measuring Cellobiohydrolase Specific Activity)

25 $10 \mu\text{L}$ of an enzyme dilution was added to $90 \mu\text{L}$ of 50 mM acetate buffer containing 1 mM p-nitrophenyl- β -lactopyranoside (produced by Sigma-Aldrich Japan), and the mixture was allowed to react at 30°C for 60 minutes. Then, $10 \mu\text{L}$ of 2 M sodium carbonate was added and mixed well to stop the reaction, and the increase in absorbance at 405 nm was measured. Release of 1 μmol of p-nitrophenol per minute was defined as 1 U of activity, and the specific activity was calculated by dividing it by the protein amount.

30 [0046]

<Reference Example 3> Saccharification Test of Cellulose-Containing Biomass

As biomass to be saccharified, use was made of either wood-derived powdered

cellulose Arbocel (registered trademark) B800 (produced by J. Rettenmaier & Sohne) or bagasse powdered into an average particle diameter of 100 μm . As an enzyme solution, use was made of a filtrate obtained by collecting a 1-mL portion of a culture solution of *Trichoderma reesei* or *Trichoderma reesei* mutant strain, centrifuging the collected culture solution, recovering a supernatant from which the fungus bodies had been removed, and
5 filtrating the supernatant through a 0.22 μm filter. The wood-derived powdered cellulose Arbocel (registered trademark) B800 (produced by J. Rettenmaier & Sohne) is hereinafter often referred to as “Arbocel B800”.

[0047]

10 (Saccharification Reaction)

A saccharification reaction was conducted in the following manner. Into a 2-mL tube were introduced Arbocel (registered trademark) B800 or bagasse powdered into an average particle diameter of 100 μm and a sodium acetate buffer (pH 5.2) so as to result in a final concentration of 0.1 M. Pure water was added in such an amount as to result in a solid
15 concentration at the time of reaction initiation of 8% by weight in the case of using the Arbocel (registered trademark) B800 or of 10% by weight in the case of using the bagasse. The enzyme solution was further added to initiate the reaction under the reaction conditions of 50°C using a heat block rotator. A sample obtained after the saccharification reaction had been conducted for 24 hours was centrifuged for 10 minutes under the conditions of 10,000 \times g
20 and the supernatant was taken out. The saccharification reaction was terminated by adding 1 N sodium hydroxide aqueous solution to the supernatant in an amount of one-tenth the volume of the supernatant. The sugar concentration in the saccharified solution after the termination of the reaction was determined by subjecting the saccharified solution to sugar analysis by the UPLC shown below. As for the enzyme solution to be used in the
25 saccharification reaction, the addition amount thereof was calculated from the protein concentration of the culture solution and the specific activity so as to be suitable for the conditions employed in each of the Examples and Comparative Examples.

[0048]

(Determination of Sugar Concentrations)

30 The saccharified solution was quantitatively analyzed for glucose, xylose, and cellobiose under the following conditions using ACQUITY (registered trademark) UPLC System (Waters).

The quantitative analysis was performed on the basis of calibration curves prepared

with standard samples of glucose, xylose, and cellobiose. Cellobiose concentrations lower than 1 g/L were regarded as below the detection limit.

Column: AQUITY (registered trademark) UPLC BEH Amide 1.7 μm 2.1 \times 100 mm
Column

5 Separation method: HILIC

Mobile phase: mobile phase A: 80% acetonitrile, 0.2% TEA aqueous solution, and mobile phase B: 30% acetonitrile, 0.2% TEA aqueous solution, in accordance with the following gradient. The gradient was a linear gradient reaching the mixing ratio corresponding to the time below.

10 Initiation condition: (A 99.90%, B 0.10%), 2 minutes after initiation: (A 96.70%, B 3.30%), 3.5 minutes after initiation: (A 95.00%, B 5.00%), 3.55 minutes after initiation: (A 99.90%, B 0.10%), 6 minutes after initiation: (A 99.90%, B 0.10%)

Detection method: ELSD (evaporative light scattering detector)

Flow rate: 0.3 mL/min

15 Temperature: 55°C

[0049]

<Example 1>

Preparation of *Trichoderma reesei* QM9414 Mutant Strain I reduced in the function of polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2:

20 A *Trichoderma reesei* mutant strain reduced in the function of the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2 was prepared by preparing a DNA fragment consisting of the gene sequence represented by SEQ ID NO: 3 as a DNA fragment containing a gene encoding the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2 in which the function of the polypeptide consisting of
25 the amino acid sequence represented by SEQ ID NO: 2 had been reduced, and then transforming *Trichoderma reesei* QM9414 strain with the DNA fragment. By this method, a *Trichoderma reesei* mutant strain is obtained in which 11 bases have been inserted into the 1,415th position in SEQ ID NO: 1 to have a polypeptide in which the translation ends at the 419th position in SEQ ID NO: 2. Acetamide and acetamidase (AmdS) gene (amdS) capable
30 of decomposing acetamide were used as selection markers for introducing the DNA fragment. For allowing the DNA fragment consisting of the base sequence represented by SEQ ID NO: 3 to be introduced upstream and downstream of the amdS-containing DNA sequence, a plasmid for mutation introduction was prepared so as to add a portion homologous to the gene

sequence of the *Trichoderma reesei* QM9414 strain.

[0050]

Specifically, a DNA fragment obtained by treating a synthesized DNA fragment shown by SEQ ID NO: 4 with restriction enzymes AflIII and NotI was used as the upstream DNA fragment. In addition, PCR was conducted using genomic DNA extracted in a usual manner from the *Trichoderma reesei* QM9414 strain and oligo DNAs represented by SEQ ID NOs: 5 and 6, and a DNA fragment obtained by treating the resulting amplified fragment with restriction enzymes MluI and SwaI was used as the downstream DNA fragment. The upstream and downstream DNA fragments were introduced into an amdS-inserted plasmid by using restriction enzymes AflIII and NotI and restriction enzymes MluI and SwaI, respectively, to construct a plasmid for mutation introduction. The plasmid for mutation introduction was then treated with restriction enzymes PacI and AscI, and the *Trichoderma reesei* QM9414 strain (NBRC #31329) was transformed with the obtained DNA fragment shown by SEQ ID NO: 3. The manipulations involving the molecular biological technique were performed as described in Molecular cloning, laboratory manual, 1st, 2nd, 3rd (1989). In addition, the transformation was carried out using a standard technique, i.e., a protoplast PEG method, and specifically, was performed as described in Gene, 61, 165-176 (1987). The obtained *Trichoderma reesei* mutant strain was used as QM9414 mutant strain I in the following experiments.

20 [0051]

<Example 2>

Protein Production Test Using QM9414 Mutant Strain I:

(Flask Cultivation)

After spores of QM9414 mutant strain I prepared in Example 1 were diluted with physiological saline to be 1.0×10^7 /mL, 0.1 mL of the diluted spore solution was inoculated into 10 mL of a flask medium shown in Table 1 or 2 which had been placed in a 50 mL baffled flask, and was incubated on a shaker under the conditions of 28°C and 120 rpm for 120 hours. The protein concentration in the culture solution was determined by the method described in Reference Example 1, and the various cellulase specific activities were determined by the methods described in Reference Example 2. The results obtained after the cultivation in the culture medium shown in Table 1 are given in Table 3, and the results obtained after the cultivation in the culture medium shown in Table 2 are given in Table 4.

[0052]

[Table 1]

[Table 1]

	Arbocel B800 (produced by J. Rettenmaier & Sohne)	20 g
	5× Mandel's solution*	200 mL
5	10× Ammonium tartrate solution**	100 mL
	Corn steep liquor	50 g
	Trace element solution***	1 mL
	Tween 80	0.5 mL
	PE-M	1 mL
10		(per 1 L)

* The 5×Mandel's solution contains 7 g/L $(\text{NH}_4)_2\text{SO}_4$, 10 g/L KH_2PO_4 , 2 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 1.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.

** The 10× Ammonium tartrate solution contains 92 g/L ammonium tartrate.

*** The trace element solution contains 0.3 g/L H_3BO_3 , 1.3 g/L $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 5 g/L $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 2 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.4 g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, and 10 g/L ZnCl_2 .

[0053]

[Table 2]

[Table 2]

20	Lactose	20 g
	5× Mandel's solution*	200 mL
	10× Ammonium tartrate solution**	100 mL
	Corn steep liquor	50 g
	Trace element solution***	1 mL
25	Tween 80	0.5 mL
	PE-M	1 mL
		(per 1 L)

[0054]

30 (Collection of Culture Solution)

After 120 hours from the start of cultivation, a 1-mL portion of the culture solution was collected. The collected culture solution was centrifuged under the conditions of 15,000×g and 4°C for 10 minutes to obtain a supernatant. The supernatant was filtrated

through a 0.22 µm filter, and the filtrate was used as a cellulase solution in the following experiments.

[0055]

(Determination of Protein Concentration and Various Cellulase Specific Activities)

5 The protein concentration in the culture solution at 120 hours from the start of cultivation was determined using the technique described in Reference Example 1, and subsequently, the specific activities of the cellulases were determined by the methods described in Reference Example 2. The results are shown in Tables 3 and 4.

[0056]

10 <Example 3>

Preparation of *Trichoderma reesei* QM9414 Mutant Strain II reduced in the function of polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2:

A *Trichoderma reesei* mutant strain reduced in the function of the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2 was prepared by
15 producing a DNA fragment consisting of the gene sequence represented by SEQ ID NO: 10, and then transforming *Trichoderma reesei* QM9414 strain with the DNA fragment. By this method, amdS is inserted between the 435th and 436th bases in SEQ ID NO: 1, and a
Trichoderma reesei mutant strain reduced in the function of SEQ ID NO: 2 is obtained. For allowing the DNA fragment consisting of the base sequence represented by SEQ ID NO: 10
20 to be introduced upstream and downstream of the amdS-containing DNA sequence, a plasmid for mutation introduction was prepared so as to add a portion homologous to the gene sequence of the *Trichoderma reesei* QM9414 strain.

[0057]

Specifically, PCR was conducted using genomic DNA extracted in a usual manner
25 from the *Trichoderma reesei* QM9414 strain and oligo DNAs represented by SEQ ID NOs: 11 and 12, and a DNA fragment obtained by treating the resulting amplified fragment with restriction enzymes AflII and NotI was used as the upstream fragment. In addition, PCR was conducted using genomic DNA and oligo DNAs represented by SEQ ID NOs: 13 and 14, and a DNA fragment obtained by treating the resulting amplified fragment with restriction
30 enzymes MluI and SphI was used as the downstream DNA fragment. The upstream and downstream DNA fragments were introduced into an amdS-inserted plasmid by using restriction enzymes AflII and NotI and restriction enzymes MluI and SphI, respectively, to construct a plasmid for mutation introduction. The plasmid for mutation introduction was

then treated with restriction enzymes AflIII and SphI, and the *Trichoderma reesei* QM9414 strain was transformed with the obtained DNA shown by SEQ ID NO: 10 in the manner as described in Example 1. The obtained *Trichoderma reesei* mutant strain was used as QM9414 mutant strain II in the following experiments.

5 [0058]

<Example 4>

Protein Production Test Using QM9414 Mutant Strain II:

Cultivation was performed by the same operations and conditions as in Example 2 except that QM9414 mutant strain II was used in place of QM9414 mutant strain I prepared in
10 Example 1, and the protein concentration in the culture solution and the various cellulase specific activities were determined. The results are shown in Tables 3 and 4.

[0059]

<Comparative Example 1>

Protein Production Test Using *Trichoderma reesei* QM9414 Strain

15 Cultivation was performed by the same conditions and operations as in Example 2 except that *Trichoderma reesei* QM9414 strain was used in place of QM9414 mutant strain I prepared in Example 1, and the protein concentration in the culture solution and the various cellulase specific activities were determined by the same methods as in Example 2. The results obtained after the cultivation in the culture medium shown in Table 1 are given in
20 Table 3, and the results obtained after the cultivation in the culture medium shown in Table 2 are given in Table 4.

[0060]

[Table 3]

[Table 3]

Cultivation with Arbocel B800	Comparative Example 1	Example 2	Example 4
	QM9414 Strain	QM9414 Mutant Strain I	QM9414 Mutant Strain II
Relative value of protein concentration	1	1.5	1.3
Relative value of β -glucosidase specific activity	1	1.9	1.7
Relative value of β -xylosidase specific activity	1	1.3	1.9
Relative value of cellobiohydrolase specific activity	1	1.3	1.1

25

[0061]

[Table 4]

[Table 4]

Cultivation with lactose	Comparative Example 1	Example 2	Example 4
	QM9414 Strain	QM9414 Mutant Strain I	QM9414 Mutant Strain II
Relative value of protein concentration	1	1.7	1.4
Relative value of β -glucosidase specific activity	1	4.3	1.6
Relative value of β -xylosidase specific activity	1	2.5	1.2
Relative value of cellobiohydrolase specific activity	1	3.5	1.1

[0062]

5 The results of Example 2, Example 4, and Comparative Example 1 revealed the following regarding the cultivation in the medium shown in Table 1. When the protein concentration in the culture solution where the *Trichoderma reesei* QM9414 strain had been cultivated was assumed to be 1, the relative value of the protein concentration in the culture solution of QM9414 mutant strain I was 1.5 and the relative value of the protein concentration
10 in the culture solution of QM9414 mutant strain II was 1.3. It was understood from these results that when *Trichoderma reesei* reduced in the function of the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2 is cultivated, the protein production amount can be increased as compared with the case where the function of the polypeptide has not been reduced.

15 [0063]

Furthermore, the culture solutions obtained in Example 2, Example 4, and Comparative Example 1 were examined for cellulase specific activity by the methods described in Reference Example 2. As a result, the following was found. When various cellulase specific activities in the culture solution where *Trichoderma reesei* QM9414 strain
20 had been cultivated were assumed to be 1, the β -glucosidase specific activity was QM9414 mutant strain I: 1.9, and QM9414 mutant strain II: 1.7, the β -xylosidase specific activity was QM9414 mutant strain I: 1.3, and QM9414 mutant strain II: 1.9, and the cellobiohydrolase specific activity was QM9414 mutant strain I: 1.3, and QM9414 mutant strain II: 1.1. It was understood from these results that the cellulases obtained by cultivating the *Trichoderma*
25 *reesei* mutant strains reduced in the function of the polypeptide represented by the amino acid sequence represented by SEQ ID NO: 2 not only attain improved protein production amounts

but also bring about an unexpected effect that an improvement in various cellulase specific activities is attained, as compared with the case where the function of the polypeptide has not been reduced.

[0064]

5 Meanwhile, with respect to the cultivation in the lactose-containing culture medium shown in Table 2, the following was found. When the protein concentration in the culture solution where the QM9414 strain had been cultivated was assumed to be 1, the relative value of the protein concentration in the culture solution of QM9414 mutant strain I was 1.7 and the relative value of the protein concentration in the culture solution of QM9414 mutant strain II
10 was 1.4. It was understood from these results that when *Trichoderma reesei* reduced in the function of the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2 is cultivated, the protein production amount can be increased as compared with the case where the function of the polypeptide has not been reduced.

[0065]

15 Furthermore, the obtained culture solutions were examined for cellulase specific activity by the methods described in Reference Example 2. As a result, the following was found. When various cellulase specific activities in the culture solution where QM9414 strain had been cultivated were assumed to be 1, the β -glucosidase specific activity was QM9414 mutant strain I: 4.3, and QM9414 mutant strain II: 1.6, the β -xylosidase specific
20 activity was QM9414 mutant strain I: 2.5, and QM9414 mutant strain II: 1.2, and the cellobiohydrolase specific activity was QM9414 mutant strain I: 3.5, and QM9414 mutant strain II: 1.1. It was understood from these results that the cellulases obtained by cultivating the *Trichoderma reesei* mutant strains reduced in the function of the polypeptide represented by the amino acid sequence represented by SEQ ID NO: 2 not only attain improved protein
25 production amounts but also bring about an unexpected effect that an improvement in various cellulase specific activities is attained, as compared with the case where the function of the polypeptide has not been reduced. These results revealed that the cultivation of QM9414 mutant strain I, which has been reduced in the function of the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2, in the lactose-containing culture medium
30 attains greater improvements in various cellulase specific activities than the cultivation thereof in the cellulose-containing culture medium.

[0066]

<Example 5>

Saccharification Reaction Test Using Cellulases from QM9414 Mutant Strain I

Using a culture solution collected at 120 hours after initiation of the cultivation of QM9414 mutant strain I obtained in Example 2 in the culture medium shown in Table 1, a saccharification reaction test of cellulose-containing biomass was performed in accordance with the operation and conditions described in Table 5 and Reference Example 3. As the cellulose-containing biomass, use was made of Arbocel (registered trademark) B800 or powdered bagasse. The results thereof are shown in Table 6.

[0067]

[Table 5]

10 [Table 5]

Arbocel B800 or powdered bagasse	80 mg
1 M sodium acetate buffer (pH 5.2)	100 μ L
Enzyme addition amount	0.06 mg (per 1 mL)

15

[0068]

[Table 6]

[Table 6]

		Comparative Example	Example 5
		QM9414 Strain	QM9414 Mutant Strain I
Arbocel B800	Glucose concentration (g/L)	3.3	4.8
	Xylose concentration (g/L)	3.9	4.9
Bagasse	Glucose concentration (g/L)	1.4	1.7
	Xylose concentration (g/L)	2.3	2.5

20 [0069]

<Example 6>

Saccharification Reaction Test 1 Using Cellulases from QM9414 Mutant Strain II

A saccharification reaction test of cellulose-containing biomass was performed in accordance with the operation and conditions described in Reference Example 3 using a culture solution collected at 120 hours after initiation of the cultivation in the culture medium shown in Table 1, of the culture solutions of QM9414 mutant strain II obtained in Example 4.

25

Reaction conditions for the saccharification reaction of Arbocel (registered trademark) B800 are shown in Table 7, and reaction conditions for the saccharification reaction of powdered bagasse are shown in Table 8. The results thereof are shown in Table 9.

[0070]

5 [Table 7]

[Table 7]

Arbocel B800	100 mg
1 M sodium acetate buffer (pH 5.2)	100 μ L
Enzyme addition amount	450 μ L
	(per 1 mL)

10

[0071]

[Table 8]

[Table 8]

15	Powdered bagasse	100 mg
	1 M sodium acetate buffer (pH 5.2)	100 μ L
	Enzyme addition amount	400 μ L
		(per 1 mL)

20 [0072]

[Table 9]

[Table 9]

Supernatant of culture medium from Arbocel B800 cultivation was used		Comparative Example	Example 6
		QM9414 Strain	QM9414 Mutant Strain II
Saccharification of Arbocel B800	Glucose concentration (g/L)	13.0	14.3
	Xylose concentration (g/L)	8.5	9.1
Saccharification of Bagasse	Glucose concentration (g/L)	6.1	6.5
	Xylose concentration (g/L)	4.1	4.1

[0073]

25 <Comparative Example 2>

Saccharification Reaction Test 1 Using Cellulases from *Trichoderma reesei* QM9414 Strain

A saccharification reaction test of cellulose-containing biomass was performed using the same operation and conditions as in Example 5 or 6, except that use was made of a culture solution collected at 120 hours after initiation of the cultivation in the culture medium shown in Table 1, of the culture solutions of *Trichoderma reesei* QM9414 strain obtained in Comparative Example 1. The results thereof are shown in Tables 6 and 9.

[0074]

<Example 7>

Saccharification Reaction Test 2 Using Cellulases from QM9414 Mutant Strain II

A saccharification reaction test of cellulose-containing biomass was performed in accordance with the operation and conditions described in Table 6 and Reference Example 3 using a culture solution collected at 120 hours after initiation of the cultivation in the culture medium shown in Table 2, of culture solutions of QM9414 mutant strain II obtained in Example 4. Reaction conditions for the saccharification reaction of Arbocel (registered trademark) B800 are shown in Table 10, and reaction conditions for the saccharification reaction of powdered bagasse are shown in Table 11. The results thereof are shown in Table 12.

[0075]

[Table 10]

[Table 10]

20	Arbocel B800	100 mg
	1 M sodium acetate buffer (pH 5.2)	100 μ L
	Enzyme addition amount	350 μ L
		(per 1 mL)

25 [0076]

[Table 11]

[Table 11]

	Powdered bagasse	100 mg
	1 M sodium acetate buffer (pH 5.2)	100 μ L
30	Enzyme addition amount	400 μ L
		(per 1 mL)

[0077]

[Table 12]

[Table 12]

Supernatant of culture medium from lactose cultivation was used		Comparative Example	Example 7
		QM9414 Strain	QM9414 Mutant Strain II
Saccharification of Arbocel B800	Glucose concentration (g/L)	5.9	7.2
	Xylose concentration (g/L)	3.4	4.5
Saccharification of Bagasse	Glucose concentration (g/L)	2.9	3.9
	Xylose concentration (g/L)	2.5	3.0

[0078]

5 <Comparative Example 3>

Saccharification Reaction Test 2 Using Cellulases from *Trichoderma reesei* QM9414 Strain

A saccharification reaction test of cellulose-containing biomass was performed using the same operation and conditions as in Example 7, except that use was made of a culture solution collected at 120 hours after initiation of the cultivation in the culture medium shown in Table 2, of the culture solutions of *Trichoderma reesei* QM9414 strain obtained in Comparative Example 1. The results thereof are shown in Table 12.

[0079]

<Conclusions>

The results of Example 5 and Comparative Example 2 revealed the following.

15 With respect to the saccharification reaction of Arbocel (registered trademark) B800 with the culture solutions obtained by the cultivation in the culture medium shown in Table 1, use of the cellulases obtained from the *Trichoderma reesei* QM9414 strain resulted in a saccharified solution having a glucose concentration of 3.3 g/L, whereas use of the QM9414 mutant strain I resulted in a glucose concentration of 4.8 g/L. Furthermore, use of the QM9414 strain
20 resulted in a xylose concentration in the saccharified solution of 3.9 g/L, whereas use of the QM9414 mutant strain I resulted in a xylose concentration of 4.9 g/L.

[0080]

25 With respect to the saccharification reaction of the powdered bagasse, use of the QM9414 strain resulted in a free glucose content of 1.4 g/L, whereas use of the mutant strain I resulted in a free glucose content of 1.7 g/L. Use of the QM9414 strain resulted in a free xylose content of 2.3 g/L, whereas use of the mutant strain I resulted in a free xylose content

of 2.5 g/L.

[0081]

The results of Example 6 and Comparative Example 2 revealed the following.

5 With respect to the saccharification reaction of Arbocel (registered trademark) B800 with the culture solutions obtained by the cultivation in the culture medium shown in Table 1, use of the QM9414 strain resulted in a free glucose content of 13 g/L, whereas use of the QM9414 mutant strain II resulted in a free glucose content of 14.3 g/L. Furthermore, use of the QM9414 strain resulted in a free xylose content of 8.5 g/L, whereas use of the mutant strain II resulted in a free xylose content of 9.1 g/L. With respect to the saccharification reaction of
10 the powdered bagasse, use of the QM9414 strain resulted in a free glucose content of 6.1 g/L, whereas use of the mutant strain II resulted in a free glucose content of 6.5 g/L. Both the QM9414 strain and the mutant strain II gave a free xylose content of 4.1 g/L.

[0082]

The results of Example 7 and Comparative Example 3 revealed the following.

15 With respect to the saccharification reaction of Arbocel (registered trademark) B800 with the culture solutions obtained by the cultivation in the culture medium shown in Table 2, use of the QM9414 strain resulted in a glucose concentration of 5.9 g/L, whereas use of the mutant strain II resulted in a glucose concentration of 7.2 g/L. Furthermore, use of the QM9414 strain resulted in a xylose concentration of 3.4 g/L, whereas use of the mutant strain II
20 resulted in a xylose concentration of 4.5 g/L. With respect to the saccharification reaction of the powdered bagasse, use of the QM9414 strain resulted in a glucose concentration of 2.9 g/L, whereas use of the mutant strain II resulted in a glucose concentration of 3.9 g/L. Use of the QM9414 strain resulted in a xylose concentration of 2.5 g/L, whereas use of the mutant strain II resulted in a xylose concentration of 3.0 g/L. It is understood from these results that
25 the cellulases produced by the mutant strains of a filamentous fungus of the genus *Trichoderma* which have been reduced in the function of the polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2 can produce a larger quantity of sugar than the cellulases produced by the QM9414 strain.

[0083]

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

[0084]

Throughout this specification and the claims which follow, unless the context requires otherwise, the word “comprise”, and variations such as “comprises” and “comprising”, will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

CLAIMS

[Claim 1]

A mutant strain of a filamentous fungus of the genus *Trichoderma*, having a reduced expression of a polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2.

[Claim 2]

The mutant strain of a filamentous fungus of the genus *Trichoderma* according to claim 1, wherein at least 413th and succeeding amino acid residues from the N-terminal side in the amino acid sequence represented by SEQ ID NO: 2 is deleted.

[Claim 3]

A method of producing a protein, the method comprising a step of cultivating the mutant strain of a filamentous fungus of the genus *Trichoderma* according to claim 1 or claim 2.

[Claim 4]

A method of producing a cellulase, the method comprising a step of cultivating the mutant strain of a filamentous fungus of the genus *Trichoderma* according to claim 1 or claim 2.

[Claim 5]

The method of producing a cellulase according to claim 4, the method comprising a step of cultivating the mutant strain of a filamentous fungus of the genus *Trichoderma* according to claim 1 or claim 2 in a culture medium comprising one or more kinds of inducers selected from the group consisting of lactose, cellulose, and xylan.

[Claim 6]

A method of producing a sugar from a cellulose-containing biomass, the method comprising:

step a of producing a cellulase by cultivating a *Trichoderma reesei* mutant strain having a reduced expression of a polypeptide consisting of the amino acid sequence represented by SEQ ID NO: 2; and

step b of saccharifying the biomass by using the cellulase obtained in the step a.

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 Leu Tyr Lys Thr Thr Leu Leu Asn Thr Val Cys Leu Val Ala Gly Leu
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 Ser Ile Phe Phe Phe Gly Tyr Asp Gln Gly Leu Met Gly Gly Val Asn
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 Thr Thr Arg Asp Tyr Ala Glu Arg Met Gly Phe Gly His Trp Asp Glu
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 Asp Gln Asn Ile Val Val Val Asp Lys Pro Leu Leu Gln Gly Gly Ile
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 Val Ala Val Tyr Tyr Leu Pro Gly Thr Leu Cys Gly Cys Leu Leu Gly
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 Gly Trp Leu Gly Asp Arg Tyr Gly Arg Ile Lys Thr Ile Ala Ile Ala
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 Cys Ala Trp Ser Val Cys Ala Ala Ala Leu Gln Ala Ser Ala Met Asn
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 Ala Asn Trp Met Phe Cys Ala Arg Val Leu Asn Gly Val Gly Thr Gly
 130 135 140
 Ile Leu Asn Ala Ile Thr Pro Val Trp Ala Thr Glu Thr Ala Ala His
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 Thr Ser Arg Gly Gln Phe Val Ser Ile Glu Phe Thr Leu Asn Ile Leu
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 Gly Val Val Val Ala Tyr Trp Leu Glu Phe Gly Thr Ser Lys Tyr His
 180 185 190

Asp Asn Thr Ser Ser Phe Ile Trp Arg Phe Pro Val Ala Phe Gln Ile
 195 200 205

Leu Pro Leu Ile Leu Leu Phe Leu Ile Ile Trp Ile Met Pro Glu Ser
 210 215 220

Pro Arg Trp Leu Val Lys Val Gly Arg Glu Glu Glu Ala Arg Phe Ile
 225 230 235 240

Leu Gly Arg Leu Arg Gly Asn Glu Gly Glu Asp Gly Leu Lys Ala Glu
 245 250 255

Ala Glu Tyr Asn Asp Ile Val Asn Ile His Lys Leu Glu Val Asp Thr
 260 265 270

Ala Lys Gln Gln Ser Tyr Phe Ser Met Phe Phe Gly Ile Gly Ser Gly
 275 280 285

Lys Leu His Thr Gly Arg Arg Val Gln Leu Val Ile Trp Leu Gln Ile
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Leu Gln Glu Trp Ile Gly Ile Ala Gly Ile Thr Ile Tyr Gly Pro Glu
 305 310 315 320

Ile Phe Thr Ile Ala Gly Ile Ser Ala Lys Asp Arg Leu Trp Val Ser
 325 330 335

Gly Ile Asn Asn Ile Thr Tyr Met Phe Ala Thr Leu Ile Cys Val Phe
 340 345 350

Thr Ile Asp Arg Ile Gly Arg Arg Trp Thr Leu Tyr Trp Gly Ala Val
 355 360 365

Gly Gln Gly Ile Cys Met Phe Val Ala Gly Gly Leu Ala Arg Ala Thr
 370 375 380

Ile Asn Ala Ser Gly Lys Ala Ser Gln Ser His Ile Gly Gly Ala Ala
 385 390 395 400

Thr Phe Phe Val Phe Leu Tyr Thr Ala Ile Phe Gly Ala Thr Trp Leu
 405 410 415

Thr Val Pro Trp Leu Tyr Pro Ala Glu Ile Phe Pro Leu Gln Val Arg
 420 425 430

Ala Lys Gly Asn Ala Trp Gly Val Val Gly Trp Ser Ile Gly Asn Gly
 435 440 445

Trp Cys Val Leu Leu Leu Pro Thr Ile Phe Lys Ala Leu Asn Glu Lys
 450 455 460

Thr Leu Tyr Ile Phe Gly Ala Val Asn Ala Leu Ser Ile Leu Val Val
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Trp Ala Leu Tyr Pro Glu Ser Asn Gln Arg Thr Leu Glu Glu Met Asp
 485 490 495

Leu Val Phe Ala Ser Asp Ser Ile Trp Ala Trp Glu Ala Glu Arg Asn
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Asn His Gly Val Val Asp Ile Glu Gln Val Ala Glu Pro Lys Glu
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 <212> DNA
 <213> Artificial Sequence

<220>
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<211> 2514
<212> DNA
<213> Artificial Sequence

<220>
<223> DNA fragment for transformation.

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 <213> Artificial Sequence

<220>
 <223> Oligo DNA for PCR.

<400> 5
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<210> 6
 <211> 39
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Oligo DNA for PCR.

<400> 6
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<210> 7
 <211> 3127
 <212> DNA
 <213> Artificial Sequence

<220>
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Lys Ala Glu Tyr Glu Lys Leu Leu Asn Met Arg Ser Gly Gly Thr Tyr
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Ile Pro Pro Ala Arg Leu Arg Ala Leu Gln Ala Gln Ile Thr Asp Lys
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Ser Ser Lys Glu Tyr Gln Arg Met Ala Trp Glu Ala Leu Lys Lys Ser
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Val Pro Glu Leu Phe Gly Glu Asn Leu Val Arg Gly Arg Gly Leu Phe
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Cys Arg Ser Ile Met Lys Ala Gln Ala Ala Ser Leu Pro Phe Thr Pro
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Ile Tyr Ala Ala Met Ala Ala Ile Val Asn Thr Lys Leu Pro Gln Val
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Gly Glu Leu Leu Val Lys Arg Leu Ile Met Gln Phe Arg Lys Gly Phe
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Lys Arg Asn Asp Lys Ala Val Cys Leu Ser Ser Thr Thr Phe Leu Ala
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His Leu Ile Asn Gln Gln Val Gln His Glu Met Leu Ala Gly Gln Ile
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Leu Leu Leu Leu Leu His Lys Pro Thr Asp Asp Ser Val Glu Ile Ala
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Val Gly Phe Cys Lys Glu Val Gly Gln Tyr Leu Glu Glu Met Gln Pro
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Ala Ile Ser Met Ala Val Phe Asp Gln Phe Arg Asn Ile Leu His Glu
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Ser Asp Ile Asp Lys Arg Thr Gln Tyr Met Ile Glu Val Leu Phe Gln
260 265 270

Ile Arg Lys Asp Lys Phe Lys Asp His Pro Ala Ile Lys Glu Glu Leu
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Asp Leu Val Glu Glu Glu Asp Gln Ile Thr His Lys Val Glu Leu Asp
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Gly Glu Ile Asp Val Gln Asp Gly Leu Asn Ile Phe Lys Tyr Asp Pro
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Glu Trp Glu Glu His Glu Glu Ala Tyr Lys Arg Leu Lys Ala Glu Ile
325 330 335

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Asp Glu Ser Ser Glu Asp Glu Glu Asn Glu Glu Thr Lys Ala Met Glu
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Ile Lys Asp Gln Ser Asn Ala Asp Leu Val Asn Leu Arg Arg Thr Ile
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Tyr Leu Thr Ile Met Ser Ser Ala Asp Pro Glu Glu Ala Val His Lys
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Ala Ser Asp Ala Leu Gly Trp His Cys Leu Ser Val Ile His Leu Asn
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Ser Pro Pro Arg Arg Gly Gly Arg Arg Val Ala Ser Arg Ser Arg Ser
 675 680 685

Tyr Ser Ser Gly Ser Ser Arg Ser Pro Pro Pro Arg Asn Arg Gly Arg
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Ala Arg Ser Asn Ser Tyr Ser Ser Tyr Ser Arg Ser Pro Ser Ser Ser
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Pro Arg Arg Gly Arg Asp Ala Asp Ser Ala Ser Pro Pro Pro Arg Arg

725

730

735

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755 760 765

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770 775 780

Pro Ser Arg Ser Arg Ser Pro Pro Pro Ala Ala Arg Gly Arg Arg Gly
785 790 795 800

Ser Tyr Thr Pro Ser Arg Ser Arg Ser Pro Pro Pro Arg Arg Val Arg
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Asp Gly Ser Pro Gly Arg Leu Arg Gly Gly Arg Ser Pro Ser Pro Pro
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Pro Pro Pro Leu Lys Arg Gly Arg Arg Asp Asn
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37

<210> 12

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35

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33