(54) **METHOD FOR PRODUCING HIGHLY ACTIVE CELLOBIOHYDROLASE AND HIGHLY ACTIVE CELLOBIOHYDROLASE**

VERFAHREN ZUR HERSTELLUNG HOCHAKTIVER CELLOBIOHYDROLASE UND HOCHAKTIVER CELLOBIOHYDROLASE

PROCÉDÉ DE PRODUCTION DE CELLOBIOHYDROLASE HAUTEMENT ACTIF ET CELLOBIOHYDROLASE HAUTEMENT ACTIF

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(56) References cited:
**EP-A1- 2 357 227   JP-A- 2010 046 034
US-A1- 2013 309 729**

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Description

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The present invention relates to a saccharifying enzyme for biorefinery applications. In particular, the present invention relates to a method for producing cellobiohydrolase with improved activity, and cellobiohydrolase with high activity.

Description of the Related Art

[0002] Various modifications of proteins have been performed using transgenic techniques. Also for enzymes, attempts to improve the enzyme activity such as enhancement in activity or increase in expression level have been made. As a method for enhancing the enzyme activity, a method of modifying an amino acid, introducing a mutation into the amino acid at the active center where an enzyme binds to a substrate, and selecting a highly active enzyme has been employed.
[0003] In many instances, the substrate binding site of an enzyme is exemplified by a key and a keyhole. Meanwhile, some of enzymes that act on a polymer substrate such as cellulose are known to have a relatively long substrate binding site with a tunnel shape or a groove shape into which the polymer substrate is fitted, though they are limited to only a part of the enzymes.
[0004] Exoglucanase that is an enzyme configured to cut the cellulose chain at the end produces cellobiose as a reaction product, and is called cellobiohydrolase. In the case of cellobiohydrolase, the active center has a tunnel shape, as described above, and the cellobiose cut by the enzyme reaction leaves from the opposite side of the tunnel. Therefore, the enzyme reaction proceeds without the cellobiohydrolase separating from the cellulose chain.
[0005] Attempts to improve the enzyme activity of cellobiohydrolase have been made in the past, and several mutants have been already reported. For example, a mutant relating to Talaromyces (see the specification of U.S. Patent No. 8790894) and a mutant of cellobiohydrolase derived from Phanerochaete (see Japanese Patent Laid-Open No. 2010-046034) are known.

SUMMARY OF THE INVENTION

[0006] However, enzymes with higher efficiency have been desired in the field of biorefmery, and the aforementioned conventional arts lack a sufficient effect of improving the activity. Accordingly, it is required to produce a cellobiohydrolase mutant with higher activity.
[0007] Further, a processive enzyme that catalyzes a reaction while holding the polymer substrate like cellobiohydrolase has a substrate binding site extending over a wide range. Therefore, the effects of many amino acids remain unstudied. Accordingly, it is an object of the present invention to obtain cellobiohydrolase with higher activity by investigating substitutional effects at the sites of unstudied amino acids.
[0008] A method for producing cellobiohydrolase with improved activity of the present invention includes: substituting, with alanine, asparagine that is the amino acid at position 62 from an N-terminal of cellobiohydrolase shown in SEQ ID NO: 1, or asparagine at position corresponding to position 62 of SEQ ID NO: 1 in an amino acid sequence of cellobiohydrolase having homology with the cellobiohydrolase shown in SEQ ID NO: 1.
[0009] As a result of analysis by alanine scanning, which is one way to specify a site that affects protein functions, cellobiohydrolase with high activity could be obtained by substituting asparagine that is the amino acid at position 62 of cellobiohydrolase shown in SEQ ID NO: 1 with alanine. This amino acid residue is located within the tunnel structure of the cellobiohydrolase.
[0010] Accordingly, it is presumed that, when cellobiohydrolase has homology with the cellobiohydrolase shown in SEQ ID NO: 1, cellobiohydrolase with high activity would be obtained likewise by substituting asparagine at an equivalent position to the position 62 with alanine.
[0011] Further, cellobiohydrolase with improved activity of the present invention includes: a mutation by substitution, with alanine, of asparagine that is the amino acid at position 62 from an N-terminal of cellobiohydrolase shown in SEQ ID NO: 1, or asparagine at the position corresponding to position 62 of SEQ ID NO: 1 in an amino acid sequence of cellobiohydrolase having homology with the cellobiohydrolase shown in SEQ ID NO: 1.
[0012] The cellobiohydrolase in which the asparagine at position 62 in the sequence shown in SEQ ID NO: 1 is substituted with alanine has high activity. Accordingly, cellobiohydrolase with higher activity can be obtained by combination with a mutant obtained by substituting asparagine at position 62 or asparagine at a position corresponding to the position 62 with alanine, or an amino acid substitution that includes such substitution and that is known to strengthen the enzyme activity.

[0013] The cellobiohydrolase with improved activity of the present invention has a sequence shown in SEQ ID NO: 2. The cellobiohydrolase shown in SEQ ID NO: 2 has specific activity 12 times that of a wild type enzyme.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014]

FIG. 1 is a view schematically showing the three-dimensional structure of cellobiohydrolase and showing the respective positions of amino acid residues;
FIG. 2 is a graph showing a relative activity ratio of mutants of cellobiohydrolase to the wild type by alanine scanning; and
FIG. 3 is a graph showing the relative activity ratio to the wild type, when the amino acid at position 62 from the N-terminal is substituted.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0015] The crystal structure of cellobiohydrolase has been already known. A site where cellobiohydrolase interacts with cellulose as a substrate is surrounded by a long loop, which is called a tunnel structure. The active center which binds to the cellulose and where a degradation reaction occurs is located within the tunnel structure. Accordingly, a highly active mutant is considered to be obtained by obtaining an enzyme having a mutation of an amino acid within the tunnel structure.

[0016] However, since the tunnel structure extends over a wide range, it is difficult to analyze all the amino acids that affect the activity by binding to the substrate. Therefore, amino acids that are predicted to be inside the tunnel structure based on the three-dimensional structure, and that may highly possibly affect the activity by inhibiting the movement of cellulose as the substrate are selected to be analyzed by alanine scanning. First, in order to introduce a mutation, a cellobiohydrolase gene was isolated, and a vector for expression in Aspergillus oryzae was constructed.

(1) Construction of vector that expresses cellobiohydrolase in Aspergillus oryzae (Genomic DNA extraction of Acremonium cellulolyticus)

[0017] Acremonium cellulolyticus strain H1 (FERM BP-11508, which will be hereinafter abbreviated as "strain H1") was inoculated on a PDB agar medium (a plate medium obtained by adding 1.5% (mass/volume) agarose to a PDA medium (BD Difco, PDA broth)) and was cultured at 30°C for one week. The obtained fungi were cut out together with the agar to a diameter of 5 mm and were inoculated on the PDA medium, followed by shaking culture at 30°C and 130 rpm. The resultant culture was centrifuged at 15,000 rpm for 10 minutes, thereby recovering the fungi. Further, washing of the recovered fungi with the PDA medium was repeated twice to obtain a fungal sample.

[0018] Beads were placed in a 2 mL plastic tube containing the fungal sample, and were subjected to milling by a desktop bead mill (Shake Master, manufactured by Bio Medical Science) for 90 seconds three times, thereby allowing the fungal sample to be in powder form. Thereafter, DNA was extracted using Nucleon (manufactured by Amersham plc).

(DNA cloning of wild type cellobiohydrolase of Acremonium cellulolyticus)

[0019] Using primers 1 and 2 shown in SEQ ID NOS: 3 and 4 (see Table 1) and using the obtained genomic DNA as a template, the sequence encoding wild type cellobiohydrolase was amplified by PCR. As DNA polymerase, KOD-plus (manufactured by TOYOBO CO., LTD.) was used. After one cycle of PCR at 94°C for 2 minutes was performed, 30 cycles of PCR at 96°C for 20 seconds, subsequently at 60°C for 30 seconds, and further at 72°C for 5 minutes were performed. The obtained PCR product was purified using a QIAquick PCR purification kit (manufactured by Qiagen N.V.). The amino acid sequence of the obtained wild type cellobiohydrolase of Acremonium cellulolyticus is shown in SEQ ID NO: 1.

(Preparation of pBR-enoAP-agdAT-niaD)

[0020] A vector, pBR-enoAP-agdAT-niaD, in which nitrate reductase gene niaD derived from Aspergillus oryzae, the terminator region of agdA gene derived from Aspergillus oryzae, and the promoter region of enoA gene derived from Aspergillus oryzae are incorporated into Escherichia coli plasmid, pBR322, was produced. Expression of the nitrate reductase gene niaD makes it possible to select fungi into which plasmids that enable the growth of Aspergillus oryzae even in the presence of nitric acid are introduced. A preparation method will be briefly described below.

[0021] First, plasmids into which the genes of nitrate reductase gene niaD derived from Aspergillus oryzae are incor-

porated are constructed. Aspergillus oryzae RIB40 strain (NBRC number: 100959, which will be hereinafter abbreviated as "RIB40 strain") was obtained from the National Institute of Technology and Evaluation. Using primers 3 and 4 shown in SEQ ID NOS: 5 and 6 and using the genomic DNA of RIB40 strain as a template, DNA of nitrate reductase gene niaD was amplified by PCR.

[0022] The amplified niaD DNA was purified by digestion with AvaI and NdeI, which was thereafter inserted into the AvaI and NdeI sites of pBR322 to construct pBR-niaD.

[0023] Then, the terminator region of agdA gene derived from Aspergillus oryzae was incorporated into pBR-niaD.

[0024] Using primers 5 and 6 shown in SEQ ID NOS: 7 and 8 and using the genomic DNA of RIB40 strain as a template, DNA in the terminator region of agdA gene derived from Aspergillus oryzae (which may be hereinafter referred to as "agdA terminator") was amplified by PCR.

[0025] The obtained PCR-amplified product was digested with restriction enzymes SalI and AvaI, and the digested product was inserted into the SalI and AvaI sites of pBR-niaD to obtain plasmid pBR-agdAT-niaD.

[0026] Then, the promoter region of enoA gene derived from Aspergillus oryzae was incorporated into the obtained pBR-agdAT-niaD.

[0027] Using primers 7 and 8 shown in SEQ ID NOS: 9 and 10 and using the genomic DNA of RIB40 strain as a template, DNA in the promoter region of enoA gene derived from Aspergillus oryzae (which may be hereinafter referred to as "enoA promoter") was amplified by PCR.

[0028] The obtained PCR-amplified product was digested with restriction enzymes NheI and SalI, which was inserted into the NheI and SalI sites of pBR-agdAT-niaD to obtain plasmid pBR-enoAP-agdAT-niaD.

[Table 1]

Primer number	Nucleotide sequence 5' → 3'	SEQ ID NO:	Remarks
1	tcctccaagt tacccatgtc tgccttgaac tctttc	3	Cellobiohydrolase
2	cgcttcgtcg acccccctaca aacattgaga gtagtaaggg	4	Cellobiohydrolase
3	atgctcggga gctttggatt tcctacgtct tc	5	niaD
4	atgcatatgt cgagagtgtt gtgtgggtca acg	6	niaD
5	atggtcgacg aagcgtaaca ggatagccta gac	7	agdA Terminator
6	atgcccgaga gtaacccatt cccggttctc tag	8	agdA Terminator
7	atggctagca gatctcgcgg cagggttgac	9	enoA Promoter
8	atggtcgacc ccgggtaact tggaggacgg aagaaaagag	10	enoA Promoter

(Incorporation of wild type cellobiohydrolase DNA into pBR-enoAP-agdAT-niaD)

[0029] The pBR-enoAP-agdAT-niaD was digested with a restriction enzyme SmaI to obtain SmaI restriction fragment of pBRenoAP-agdAT-niaD.

[0030] The sequence encoding wild type cellobiohydrolase was cloned into the SmaI fragment using an In-Fusion (R) HD Cloning Kit (manufactured by Clontech Laboratories, Inc.) to obtain plasmid pBR-enoAP-CBH-agdAT-niaD (vector for CBH expression in Aspergillus oryzae).

(Incorporation of mutant cellobiohydrolase DNA into pBR-enoAP-agdAT-niaD)

[0031] Mutant cellobiohydrolase was produced by PCR of two fragments, one of which is from the 5' end of cellobio-hydrolase gene to the mutation site and the other of which is from the mutation site to the 3' end of the gene. The construction was achieved by incorporating the obtained two fragments of DNA into pBR-enoAP-agdAT-niaD. At this time, since the primer containing the mutation site is designed to anneal, the mutant cellobiohydrolase gene is continuous from the 5' end to the 3' end while having the same amino acid sequence as the wild type cellobiohydrolase except for having the mutation site when it is incorporated into pBR-enoAP-agdAT-niaD.

Example 1

(Production of alanine substitution mutants)

[0032] Based on the three-dimensional structure of cellobiohydrolase of Acremonium cellulolyticus, amino acids at 30

sites located within the tunnel structure of cellobiohydrolase derived from acremonium having the amino acid sequence shown in SEQ ID NO: 1 were substituted with alanine, and the activity was analyzed to select amino acids that lead to an improvement in enzyme activity. Of the 30 sites of substitution, activity improvement as compared to wild type cellobiohydrolase was seen in 6 sites.

[0033] An improvement, even if slight, in the activity by alanine substitution was seen in lysine (K) at position 203, asparagine (N) at position 62, asparagine at position 125, aspartic acid (D) at position 201, arginine (R) at position 289, and aspartic acid at position 281 of the amino acid sequence shown in SEQ ID NO: 1 (the mutants may be respectively referred to as K203A, N062A, N125A, D201A, R289A, and D281A depending on the substituted amino acids and their positions).

[0034] FIG. 1 shows the three-dimensional structure of cellobiohydrolase of Acremonium cellulolyticus obtained by PDB (http://www.rcsb.org/pdb/home/home.do) and the positions of the aforementioned amino acids that showed an improvement in the activity by alanine substitution. The sites that showed an improvement in the activity by alanine substitution exist over the total length of the tunnel structure.

[0035] Table 2 below shows the primers used for producing the aforementioned six mutants. PCR was performed in the same manner as the amplification of the genomic DNA of wild type cellobiohydrolase except that primer 1 used for cloning cellobiohydrolase and primers 9 to 14, and primers 15 to 20 and primer 2 used for cloning cellobiohydrolase described above were used as the respective primer pairs.

[Table 2]

Primer number	Nucleotide sequence 5' → 3'	SEQ ID NO:	Remarks
9	gatgaaggcc aagtcacgag ggcattgaga	11	K203A
10	acgccaggcg gcatctaacg taatggcacc	12	N062A
11	tccgacggcg gaaccggtaa cgaagttcag	13	N125A
12	cttcaaggca cgagggcatt gagagtcaca	14	D201A
13	accaagggcg taagggttga agtcacatcc	15	R289A
14	acatccggca gggtcgcagg taccggcgta	16	D281A
15	gacttggcct tcatcgctgg tcaggccaac	17	K203A
16	gatgccgcct ggcgttgggt ccatggtgtc	18	N062A
17	ggttccgccg tcggatctcg tacttacctg	19	N125A
18	cctcgtgcct tgaagttcat cgctggtcag	20	D201A
19	ccttacgccc ttggtgtcac tgacttctac	21	R289A
20	gaccctgccg gatgtgactt caacccttac	22	D281A

(Production of transformants of Aspergillus oryzae by introduction of vectors for wild type and mutant cellobiohydrolase expression in Aspergillus oryzae)

[0036] Aspergillus oryzae niaD300 strain (niaD deficient strain, obtained from the National Research Institute of Brewing) was transformed with the plasmid pBR-enoAP-CBH-agdAT-niaD into which the aforementioned wild type cellobiohydrolase was incorporated or a plasmid into which a mutation was introduced, by the conventional PEG-calcium method (Mol.Gen.Genet., vol. 218, p. 99 to 104, 1989). The strains which could grow in the Czapek-Dox medium (3% (mass/volume) dextrin, 0.1% (mass/volume) potassium dihydrogenphosphate, 0.2% (mass/volume) potassium chloride, 0.05% (mass/volume) magnesium sulfate, 0.001% (mass/volume) iron sulfate, and 0.3% (mass/volume) sodium nitrate) was selected to obtain transformants (wild type and mutant cellobiohydrolase-transformed Aspergillus oryzae strains).

(Preparation of cellobiohydrolase)

[0037] The produced cellobiohydrolase-transformed Aspergillus oryzae strains were sporulated in the Czapek-Dox medium, and spores were recovered using sterile water. The spores were inoculated into 100 mL of a PD liquid medium (2% (mass/volume) dextrin, 1% (mass/volume) polypeptone, 0.1% (mass/volume) casamino acid, 0.5% (mass/volume) potassium dihydrogenphosphate, 0.05% (mass/volume) magnesium sulfate, and 0.1% (mass/volume) sodium nitrate) contained in a 500 mL erlenmeyer flask to a final spore concentration of 1×10^4/mL. By liquid culture at 30°C for three

days, cellobiohydrolase or cellobiohydrolase having an amino acid mutation was expressed and secreted in the medium. The culture liquid after the culture was used as an enzyme sample. The enzyme sample was investigated by SDS-PAGE analysis, and protein quantitation was conducted thereon for use.

(Measurement of cellulolytic activity)

[0038] As a reactive substrate, microcrystalline cellulose (manufactured by Merck KGaA) was used. Further, a standard curve was created from the measured values of 4-point dilution series (0.5 to 5.0 mM) prepared by appropriately diluting a 10 mM (mmol/L) glucose solution with a 200 mM acetate buffer (pH 5.5).

[0039] First, 40 μL of the 200 mM acetate buffer (pH 5.5) and 50 μL of a 4% (mass/volume) microcrystalline cellulose suspension suspended in the 200 mM acetate buffer were added to a 1.5 mL plastic tube, and they were sufficiently mixed. Thereafter, the temperature thereof was adjusted to 50°C.

[0040] Subsequently, 10 μL of wild type or each mutant (K203A, N062A, N125A, D201A, R289A, or D281A) cellobiohydrolase aqueous solution was added to the tube, thereby starting an enzyme reaction. After a lapse of one hour from the start of the reaction, 100 μL of a DNSA (1.6% (mass/volume) sodium hydroxide, 0.5% (mass/volume) 3,5 dinitrosalicylic acid, and 30% (mass/volume) potassium sodium tartrate tetrahydrate) solution was added thereto and mixed, thereby stopping the reaction. After the reaction was stopped, the mixture was centrifuged (at 15,000 $\times$ g for 5 minutes), and the supernatant was boiled at 100°C for 5 minutes, followed by cooling in ice. Thereafter, 100 μL of the reaction solution was separated from the tube, and the absorbance at 540 nm (A_{540}) of the solution diluted with 100 μL of distilled water was measured. As a blank, a similarly treated sample to which a 20 mM acetate buffer was added instead of the enzyme sample was used. A cellulolytic activity value per unit weight of the enzyme was obtained by calculating a glucose concentration from the measured value of A_{540} and the standard curve, and dividing it by an enzyme concentration obtained by the protein quantitation. FIG. 2 shows the results.

[0041] FIG. 2 shows the results for mutants K203A, N062A, N125A, D201A, R289A, and D281A having improved activity as compared to wild type (WT). In particular, the mutant N062A obtained by substituting asparagine (N) at position 62 with alanine (A) had a specific activity 14 times that of wild type cellobiohydrolase. This activity is significantly high also as compared to the cellobiohydrolase mutants disclosed in the related arts.

Example 2

[0042] Based on the fact that the mutant N062A obtained by substituting asparagine (N) at position 62 with alanine (A) had improved activity as compared to wild type, the amino acid at position 62 was substituted with amino acids having different properties such as hydrophobicity, acidity, and alkalinity, and its activity was measured.

[0043] Table 3 below shows primers used for the production. PCR was performed in the same manner as the amplification of the genomic DNA of wild type cellobiohydrolase except that primer 1 used for cloning the aforementioned cellobiohydrolase and primers 21 to 27, and primers 28 to 34 and primer 2 used for cloning cellobiohydrolase were used as the respective primer pairs.

[Table 3]

Primer number	Nucleotide sequence 5' → 3'	SEQ ID NO:	Remarks
21	acgccaccag gcatctaacg taatggcacc	23	N062W
22	acgccagacg gcatctaacg taatggcacc	24	N062V
23	acgccaggag gcatctaacg taatggcacc	25	N062S
24	acgccactgg gcatctaacg taatggcacc	26	N062Q
25	acgccactcg gcatctaacg taatggcacc	27	N062E
26	acgccacttg gcatctaacg taatggcacc	28	N062K
27	acgccaatgg gcatctaacg taatggcacc	29	N062H
28	gatgcctggt ggcgttgggt ccatggtgtc	30	N062W
29	gatgccgtct ggcgttgggt ccatggtgtc	31	N062V
30	gatgcctcct ggcgttgggt ccatggtgtc	32	N062S
31	gatgcccagt ggcgttgggt ccatggtgtc	33	N062Q
32	gatgccgagt ggcgttgggt ccatggtgtc	34	N062E

(continued)

Primer number	Nucleotide sequence 5' → 3'	SEQ ID NO:	Remarks
33	gatgccaagt ggcgttgggt ccatggtgtc	35	N062K
34	gatgcccatt ggcgttgggt ccatggtgtc	36	N062H

[0044] After plasmids were constructed, cellobiohydrolase-transformed Aspergillus oryzae strains were obtained and mutant enzymes were obtained, in the same manner as in Example 1. Then, the corn stover degrading activity was measured.

(Measurement of corn stover degrading activity)

[0045] After a saccharification pretreatment was performed by mixing a 2.5% (mass/mass) sulfuric acid aqueous solution with finely milled rice straws as lignocellulosic biomass to a mass ratio of 1:10 and maintaining the mixture at a temperature of 150°C for 10 minutes, the pH was adjusted to 5.8 using a sodium hydroxide aqueous solution, followed by drying at 50°C for one day. Thereafter, the resultant after being finely crushed using a hammer was used as a reactive substrate for activity measurement.

[0046] In the activity measurement, 80 μL of a 200 mM acetate buffer (pH 5.5) and 100 μL of a 4% (mass/volume) corn stover suspension suspended with the 200 mM acetate buffer were added to a 1.5 mL plastic tube, and they were sufficiently mixed. Thereafter, the temperature thereof was adjusted to 50°C.

[0047] Subsequently, 20 μL of wild type or each mutant cellobiohydrolase aqueous solution was added thereto to start an enzyme reaction. After a lapse of one hour from the start of the reaction, the mixture was centrifuged at 15,000 × g and 4°C for 5 minutes. The obtained supernatant was transferred into a new 1.5 mL plastic tube, followed by heat treatment at 95°C for 5 minutes. Thereafter, the supernatant was centrifuged at 15,000 × g and 4°C for 5 minutes. The resultant supernatant was transferred into a new 1.5 mL plastic tube, and thereafter was filtered with a 0.2 μm (13 mm disk) filter. A 0.2 mL filtrate was transferred into a vial, and cellobiose was detected by HPLC measurement. As a standard substance, cellobiose manufactured by Wako Pure Chemical Industries, Ltd. was used, and the sugar concentration was evaluated. In the HPLC measurement, Waters 2695 (manufactured by Nihon Waters K.K.) as a separator, Waters 2414 (manufactured by Nihon Waters K.K.) as an RI detector, and Bio-rad HPX-87H (Bio-Rad Laboratories, Inc.) as a column were used. The measurement was performed using ultrapure water as an eluent in the conditions of a flow rate of 0.6 mL/min, a column temperature of 80°C, and a detector temperature of 40°C. FIG. 3 shows the results.

[0048] An improvement in activity was observed in alanine (A), glutamine (Q), histidine (H) as compared to wild type (asparagine (N)). Alanine had a specific activity 12 times or more that of the wild type (N) even in the case where saccharification was determined with the substrate changed to corn stover.

[0049] Substitution with amino acids having various properties, including hydrophobic amino acids (tryptophan (W) and valine (V)), neutral amino acids (serine (S) and glutamine (Q)), an acidic amino acid (glutamic acid (E)), and basic amino acids (lysine (K) and histidine (H)) was performed, but none of them could show a greater improvement in the activity than the improvement by alanine substitution.

[0050] As a reason why the activity was improved by the substitution with alanine, it is inferred that the steric hindrance when cellulose moves inside the tunnel structure is removed. It is expected that mutants having higher activity would be obtained by further substitution with other mutations that were found to show an improvement in the activity in the present invention, such as lysine at position 203 and arginine at position 289 together with alanine, in addition to the substitution of asparagine at position 62 with alanine.

[0051] As shown in Examples 1 and 2, the cellobiohydrolase mutant in which asparagine at position 62 defined in SEQ ID NO: 2 is substituted with alanine has higher activity as compared to wild type cellobiohydrolase.

[0052] Cellobiohydrolase is an enzyme classified as GH7 of a glycoside hydrolase family. In enzymes belonging to GH7, amino acids located in a tunnel structure are very well conserved across species. Actually, also in Trichoderma reesei and Phanerochaete chrysosporium, the amino acid at a position corresponding to position 62 in SEQ ID NO: 1 is asparagine. Accordingly, it is expected that enzymes with improved activity are obtained by changing asparagine at this position to alanine, not only in cellobiohydrolase shown in SEQ ID NO: 1, but also in cellobiohydrolase shown in homologous amino acid sequences.

[0053] Further, since the tunnel structure is conserved across species, a further improvement in the activity can be expected by introducing other amino acid mutations that have been reported to improve the cellobiohydrolase activity in addition.

SEQUENCE LISTING

[0054]

<110> HONDA MOTOR Co., LTD.

<120> Method for producing highly active cellobiohydrolase and highly active cellobiohydrolase

<130> P48355EP

<140>
<141>

<150> JP 2014-246121
<151> 2014-12-04

<160> 36

<170> PatentIn version 3.5

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<211> 529
<212> PRT
<213> Acremonium cellulolyticus

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Gly Ser Leu Leu Ala Thr Ala Gly Ala Gln Gln Ile Gly Thr Tyr Thr
            20              25              30

Ala Glu Thr His Pro Ser Leu Ser Trp Ser Thr Cys Lys Ser Gly Gly
        35              40              45

Ser Cys Thr Thr Asn Ser Gly Ala Ile Thr Leu Asp Ala Asn Trp Arg
    50              55              60

Trp Val His Gly Val Asn Thr Ser Thr Asn Cys Tyr Thr Gly Asn Thr
65              70              75              80

Trp Asn Ser Ala Ile Cys Asp Thr Asp Ala Ser Cys Ala Gln Asp Cys
            85              90              95

Ala Leu Asp Gly Ala Asp Tyr Ser Gly Thr Tyr Gly Ile Thr Thr Ser
            100             105             110

Gly Asn Ser Leu Arg Leu Asn Phe Val Thr Gly Ser Asn Val Gly Ser
        115             120             125

Arg Thr Tyr Leu Met Ala Asp Asn Thr His Tyr Gln Ile Phe Asp Leu
    130             135             140

Leu Asn Gln Glu Phe Thr Phe Thr Val Asp Val Ser His Leu Pro Cys
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9

| | 145 | | | | 150 | | | | 155 | | | | 160 |

Gly Leu Asn Gly Ala Leu Tyr Phe Val Thr Met Asp Ala Asp Gly Gly
                    165   170   175

Val Ser Lys Tyr Pro Asn Asn Lys Ala Gly Ala Gln Tyr Gly Val Gly
              180   185   190

Tyr Cys Asp Ser Gln Cys Pro Arg Asp Leu Lys Phe Ile Ala Gly Gln
              195   200   205

Ala Asn Val Glu Gly Trp Thr Pro Ser Ser Asn Asn Ala Asn Thr Gly
    210   215   220

Ile Gly Asn His Gly Ala Cys Cys Ala Glu Leu Asp Ile Trp Glu Ala
225   230   235   240

Asn Ser Ile Ser Glu Ala Leu Thr Pro His Pro Cys Asp Thr Pro Gly
              245   250   255

Leu Ser Val Cys Thr Thr Asp Ala Cys Gly Gly Thr Tyr Ser Ser Asp
              260   265   270

Arg Tyr Ala Gly Thr Cys Asp Pro Asp Gly Cys Asp Phe Asn Pro Tyr
          275   280   285

Arg Leu Gly Val Thr Asp Phe Tyr Gly Ser Gly Lys Thr Val Asp Thr
          290   295   300

Thr Lys Pro Phe Thr Val Val Thr Gln Phe Val Thr Asn Asp Gly Thr
305   310   315   320

Ser Thr Gly Ser Leu Ser Glu Ile Arg Arg Tyr Tyr Val Gln Asn Gly
              325   330   335

Val Val Ile Pro Gln Pro Ser Ser Lys Ile Ser Gly Ile Ser Gly Asn
              340   345   350

Val Ile Asn Ser Asp Tyr Cys Ala Ala Glu Ile Ser Thr Phe Gly Gly
          355   360   365

Thr Ala Ser Phe Ser Lys His Gly Gly Leu Thr Asn Met Ala Ala Gly
          370   375   380

Met Glu Ala Gly Met Val Leu Val Met Ser Leu Trp Asp Asp Tyr Ala
385   390   395   400

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        Val Asn Met Leu Trp Leu Asp Ser Thr Tyr Pro Thr Asn Ala Thr Gly
                        405             410             415

        Thr Pro Gly Ala Ala Arg Gly Thr Cys Ala Thr Thr Ser Gly Asp Pro
                        420             425             430

        Lys Thr Val Glu Ala Gln Ser Gly Ser Ser Tyr Val Thr Phe Ser Asp
                        435             440             445

        Ile Arg Val Gly Pro Phe Asn Ser Thr Phe Ser Gly Gly Ser Ser Thr
            450             455             460

        Gly Gly Ser Thr Thr Thr Thr Ala Ser Arg Thr Thr Thr Thr Ser Ala
        465             470             475             480

        Ser Ser Thr Ser Thr Ser Ser Thr Ser Thr Gly Thr Gly Val Ala Gly
                        485             490             495

        His Trp Gly Gln Cys Gly Gly Gln Gly Trp Thr Gly Pro Thr Thr Cys
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        Val Ser Gly Thr Thr Cys Thr Val Val Asn Pro Tyr Tyr Ser Gln Cys
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        Leu
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                        20              25              30

        Ala Glu Thr His Pro Ser Leu Ser Trp Ser Thr Cys Lys Ser Gly Gly
                        35              40              45

        Ser Cys Thr Thr Asn Ser Gly Ala Ile Thr Leu Asp Ala Ala Trp Arg
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Trp Asn Ser Ala Ile Cys Asp Thr Asp Ala Ser Cys Ala Gln Asp Cys
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Ala Leu Asp Gly Ala Asp Tyr Ser Gly Thr Tyr Gly Ile Thr Thr Ser
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Gly Asn Ser Leu Arg Leu Asn Phe Val Thr Gly Ser Asn Val Gly Ser
            115             120             125

Arg Thr Tyr Leu Met Ala Asp Asn Thr His Tyr Gln Ile Phe Asp Leu
    130             135             140

Leu Asn Gln Glu Phe Thr Phe Thr Val Asp Val Ser His Leu Pro Cys
145             150             155             160

Gly Leu Asn Gly Ala Leu Tyr Phe Val Thr Met Asp Ala Asp Gly Gly
            165             170             175

Val Ser Lys Tyr Pro Asn Asn Lys Ala Gly Ala Gln Tyr Gly Val Gly
            180             185             190

Tyr Cys Asp Ser Gln Cys Pro Arg Asp Leu Lys Phe Ile Ala Gly Gln
        195             200             205

Ala Asn Val Glu Gly Trp Thr Pro Ser Ser Asn Asn Ala Asn Thr Gly
    210             215             220

Ile Gly Asn His Gly Ala Cys Cys Ala Glu Leu Asp Ile Trp Glu Ala
225             230             235             240

Asn Ser Ile Ser Glu Ala Leu Thr Pro His Pro Cys Asp Thr Pro Gly
            245             250             255

Leu Ser Val Cys Thr Thr Asp Ala Cys Gly Gly Thr Tyr Ser Ser Asp
            260             265             270

Arg Tyr Ala Gly Thr Cys Asp Pro Asp Gly Cys Asp Phe Asn Pro Tyr
    275             280             285

Arg Leu Gly Val Thr Asp Phe Tyr Gly Ser Gly Lys Thr Val Asp Thr
    290             295             300

Thr Lys Pro Phe Thr Val Val Thr Gln Phe Val Thr Asn Asp Gly Thr
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Ser Thr Gly Ser Leu Ser Glu Ile Arg Arg Tyr Tyr Val Gln Asn Gly
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12

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Val Ile Asn Ser Asp Tyr Cys Ala Ala Glu Ile Ser Thr Phe Gly Gly
            355             360             365

Thr Ala Ser Phe Ser Lys His Gly Gly Leu Thr Asn Met Ala Ala Gly
    370             375             380

Met Glu Ala Gly Met Val Leu Val Met Ser Leu Trp Asp Asp Tyr Ala
385             390             395             400

Val Asn Met Leu Trp Leu Asp Ser Thr Tyr Pro Thr Asn Ala Thr Gly
            405             410             415

Thr Pro Gly Ala Ala Arg Gly Thr Cys Ala Thr Thr Ser Gly Asp Pro
            420             425             430

Lys Thr Val Glu Ala Gln Ser Gly Ser Ser Tyr Val Thr Phe Ser Asp
            435             440             445

Ile Arg Val Gly Pro Phe Asn Ser Thr Phe Ser Gly Gly Ser Ser Thr
    450             455             460

Gly Gly Ser Thr Thr Thr Thr Ala Ser Arg Thr Thr Thr Thr Ser Ala
465             470             475             480

Ser Ser Thr Ser Thr Ser Ser Thr Ser Thr Gly Thr Gly Val Ala Gly
            485             490             495

His Trp Gly Gln Cys Gly Gly Gln Gly Trp Thr Gly Pro Thr Thr Cys
            500             505             510

Val Ser Gly Thr Thr Cys Thr Val Val Asn Pro Tyr Tyr Ser Gln Cys
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Leu
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Claims

1. A method for producing cellobiohydrolase with higher activity, comprising:

    substituting, with alanine, asparagine that is the amino acid at position 62 from the N-terminal of cellobiohydrolase shown in SEQ ID NO: 1, or
    asparagine at position corresponding to position 62 of SEQ ID NO: 1 in an amino acid sequence of cellobiohydrolase classified as GH7 of a glycoside hydrolase family.

2. Cellobiohydrolase with higher activity, comprising:

    a mutation by substitution, with alanine, of asparagine that is the amino acid at position 62 of SEQ ID NO: 1 from the N-terminal of cellobiohydrolase shown in SEQ ID NO: 1, or
    asparagine at the position corresponding to position 62 of SEQ ID NO: 1 in an amino acid sequence of cellobiohydrolase classified as GH7 of a glycoside hydrolase family.

3. The cellobiohydrolase with higher activity according to claim 2, wherein
    the cellobiohydrolase has a sequence shown in SEQ ID NO: 2.

Patentansprüche

1. Verfahren zur Herstellung einer Zellobiohydrolase mit höherer Aktivität, wobei das Verfahren die Schritte umfasst:

    Substitution

    - von Asparagin, das die Aminosäure an Position 62 ausgehend vom N-Terminus der in SEQ ID NO: 1 gezeigten Zellobiohydrolase ist, oder
    - von Asparagin an der Position, die zur Position 62 der SEQ ID NO: 1 in einer als GH7 klassifizierten Zellobiohydrolase einer Glykosid-Hydrolase Familie korrespondiert,

    mit Alanin.

2. Zellobiohydrolase mit höherer Aktivität, umfassend:

    eine Mutation, erzeugt durch eine Substitution

    - von Asparagin, das die Aminosäure an Position 62 ausgehend vom N-Terminus der in SEQ ID NO: 1 gezeigten Cellobiohydrolase ist, oder
    - von Asparagin an der Position die zur Position 62 der SEQ ID NO: 1 in einer als GH7 klassifizierten Cellobiohydrolase einer Glykosid-Hydrolase Familie korrespondiert,

    mit Alanin.

3. Die Zellobiohydrolase mit höherer Aktivität gemäß Anspruch 2, wobei die Zellobiohydrolase die in SEQ ID NO: 2 gezeigte Sequenz hat.

Revendications

1. Procédé de production d'une cellobiohydrolase ayant une activité plus élevée, comprenant :

la substitution, par une alanine, d'une asparagine qui est un acide aminé en position 62 de la terminaison N d'une cellobiohydrolase présentée dans SEQ. ID. n° 1, ou
une asparagine en une position correspondant à la position 62 de SEQ. ID. n° 1 dans une séquence d'acide aminé d'une cellobiohydrolase classifiée comme GH7 d'une famille de glycoside hydrolase.

2. Cellobiohydrolase ayant une activité plus élevée, comprenant :

une mutation par substitution, par une alanine, d'une asparagine qui est l'acide aminé en position 62 de SEQ. ID. n° 1 de la terminaison N d'une cellobiohydrolase présentée dans SEQ. ID. n° 1, ou
une asparagine à la position correspondant à la position 62 de SEQ. ID. n° 1 dans une séquence d'acides aminés d'une cellobiohydrolase classifiée comme GH7 d'une famille de glycoside hydrolase.

3. Cellobiohydrolase ayant une activité plus élevée selon la revendication 2, dans laquelle la cellobiohydrolase a une séquence présentée dans SEQ. ID. n° 2.
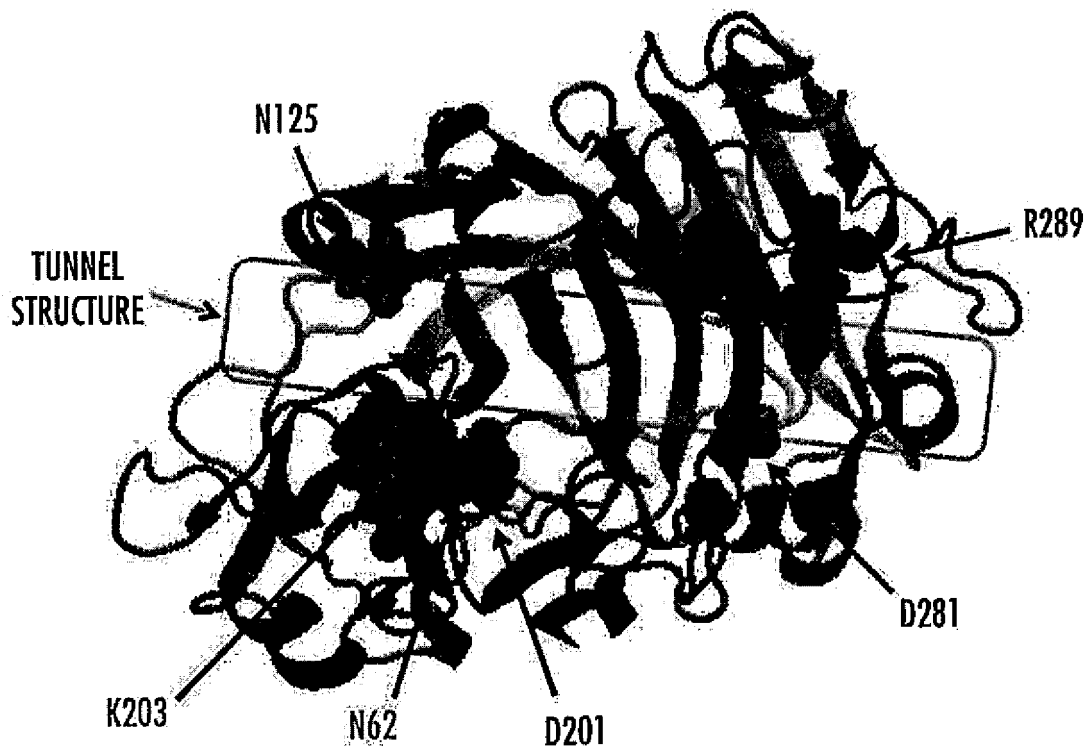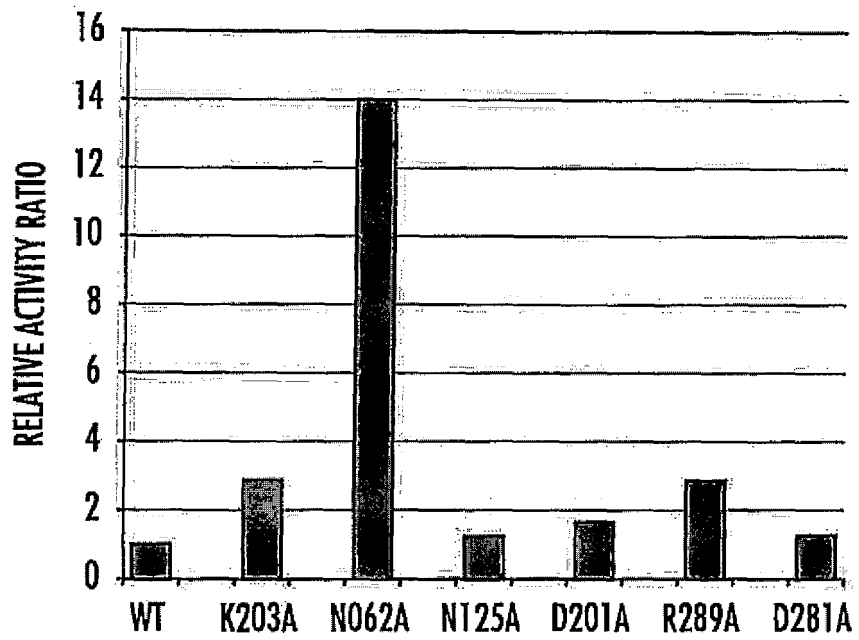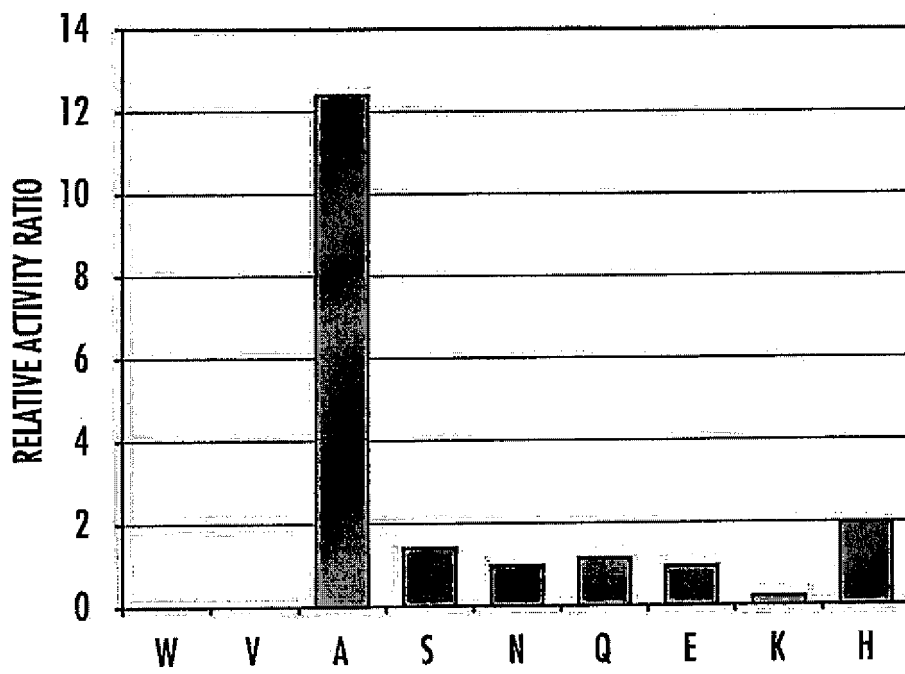
FIG.1

FIG.2

FIG.3

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- US 8790894 B **[0005]**
- JP 2010046034 A **[0005]**
- JP 2014246121 A **[0054]**

Non-patent literature cited in the description

- *Mol.Gen.Genet.,* 1989, vol. 218, 99-104 **[0036]**