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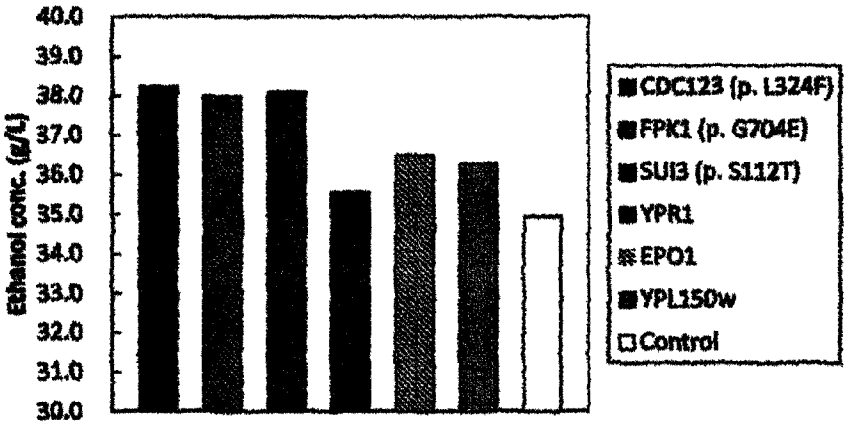
(54) Titre : GENES MUTES IMPLIQUES DANS L'AMELIORATION DE LA PRODUCTIVITE D'ETHANOL PAR FERMENTATION D'ETHANOL ET PROCEDE DE PRODUCTION D'ETHANOL LES UTILISANT

(54) Title: MUTANT GENE ASSOCIATED WITH IMPROVEMENT IN ETHANOL PRODUCTIVITY VIA ETHANOL FERMENTATION AND METHOD FOR PRODUCING ETHANOL USING THE SAME



(57) Abrégé/Abstract:
In order to improve the ethanol fermentation capability of a yeast having xylose metabolizing capability, provided are mutated genes coding for mutated proteins having consensus sequences obtained by replacing the 30-th amino acid of SEQ ID NO: 1, the 43-rd amino acid of SEQ ID NO: 4, and the 31- st amino acid of SEQ ID NO: 7 with other amino acids.

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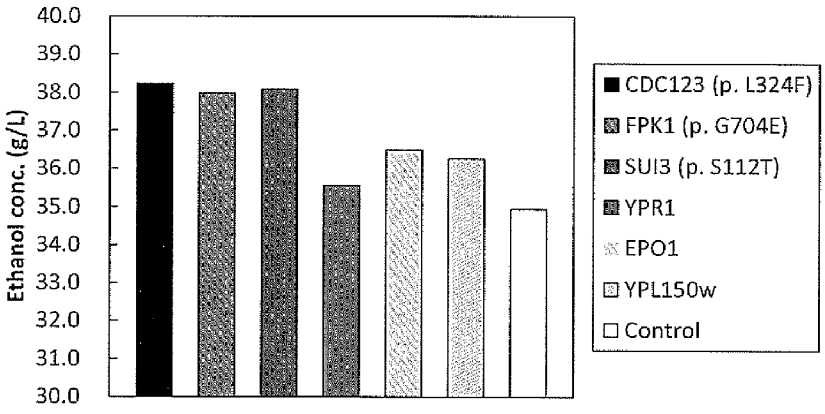
(54) **Title:** MUTATED GENES INVOLVED WITH IMPROVEMENT IN ETHANOL PRODUCTIVITY BY ETHANOL FERMENTATION AND METHOD FOR PRODUCING ETHANOL USING SAME

(54) 発明の名称： エタノール発酵によるエタノール生産性の向上に関与する変異遺伝子及びこれを用いたエタノールの製造方法



(57) **Abstract:** In order to improve the ethanol fermentation capability of a yeast having xylose metabolizing capability, provided are mutated genes coding for mutated proteins having consensus sequences obtained by replacing the 30-th amino acid of SEQ ID NO: 1, the 43-rd amino acid of SEQ ID NO: 4, and the 31-st amino acid of SEQ ID NO: 7 with other amino acids.

(57) 要約：キシロース代謝能を有する酵母おけるエタノール発酵能を向上させる。配列番号１の３０番目、配列番号４の４３番目、配列番号７の３１番目が他のアミノ酸に置換されたコンセンサス配列を有する変異タンパク質コードする変異遺伝子である。

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SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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DESCRIPTION

MUTANT GENE ASSOCIATED WITH IMPROVEMENT IN ETHANOL PRODUCTIVITY VIA ETHANOL FERMENTATION AND METHOD FOR PRODUCING ETHANOL USING THE SAME

[Technical Field]

[0001]

The present invention relates to a mutant gene associated with improvement in ethanol productivity in ethanol fermentation performed with a yeast strain or so on having xylose-metabolizing ability and a method for producing ethanol using the same.

[Background Art]

[0002]

A cellulosic biomass is an effective starting material for a useful alcohol, such as ethanol, or an organic acid. In order to increase the amount of ethanol produced with the use of a cellulosic biomass, yeast strains capable of utilizing xylose, which is pentose, as a substrate have been developed. For example, Patent Document 1 discloses a recombinant yeast strain resulting from integration of a xylose reductase (XR) gene and a xylitol dehydrogenase (XDH) gene derived from *Pichia stipitis* into its chromosome. Also, a report has been made concerning a xylose-assimilating yeast strain into which a xylose isomerase (XI) gene derived from the intestinal protozoa of *Reticulitermes speratus* has been introduced (Patent Document 2).

[0003]

An attempt for improving ethanol productivity of a xylose-assimilating yeast strain or a general yeast strain that produces ethanol from glucose via fermentation has been reported. For example, Patent Document 3 reports that the alcohol-producing capacity of *sake* yeast strain is improved upon introduction of a particular mutation into the PDR3 gene. Also, Patent Document 4 discloses a recombinant *Klebsiella oxytoca*, which is prepared by introducing alcohol dehydrogenase and pyruvate decarboxylase into a host and deleting a gene associated with production of butanediol or 2,3-butanediol therefrom. With the use of

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the recombinant disclosed in Patent Document 4, ethanol can be produced with high efficiency by converting a sugar-containing substrate in a medium mainly into ethanol and decreasing conversion thereof into butanediol or 2,3-butanediol.

[0004]

Patent Document 5 discloses that a recombinant yeast strain comprising a xylose metabolism-associated expression cassette introduced thereinto is subjected to acclimatization to improve the xylose fermentation ability of the recombinant yeast strain. In addition, Patent Document 6 discloses a xylose isomerase gene derived from the intestinal protozoa of *Reticulitermes speratus* or the intestinal protozoa of *Mastotermes darwiniensis*. The xylose isomerase gene disclosed in Patent Document 6 effectively functions in yeast and it is capable of improving xylose-metabolizing ability of yeast.

[Prior Art Documents]

[Patent Documents]

[0005]

[Patent Document 1] JP 2009-195220 A

[Patent Document 2] JP 2011-147445 A

[Patent Document 3] JP 2002-238582 A

[Patent Document 4] JP 2009-500035 A

[Patent Document 5] JP 2009-195220 A

[Patent Document 6] JP 2009-195220 A

[Summary of the Invention]

[Objects to Be Attained by the Invention]

[0006]

Yeast strains having xylose-metabolizing ability were insufficient in terms of ethanol productivity from xylose in a medium. Under the circumstances described above, accordingly, the present invention is intended to improve ethanol fermentation ability of a yeast strain having xylose-metabolizing ability.

[Means for Attaining the Objects]

[0007]

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The present inventors have conducted concentrated studies in order to attain the above objects. As a result, they succeeded in obtaining a yeast strain with improved fermentation performance when subjecting a yeast strain with xylose-metabolizing ability to long-term continuous culture and identifying a plurality of mutations associated with excellent ethanol fermentation ability of the yeast strain via thorough analysis of the obtained strain. This has led to the completion of the present invention.

[0008]

The present invention includes the following.

[0009]

(1) A mutant gene encoding the mutant CDC123 protein comprising a consensus sequence comprising a substitution of an amino acid residue in the 30th position from the N terminus with another amino acid residue in SEQ ID NO: 1.

[0010]

(2) The mutant gene according to (1), wherein the amino acid residue in the 30th position is any of leucine, valine, and isoleucine and the another amino acid residue is cysteine.

[0011]

(3) The mutant gene according to (1), wherein the mutant CDC123 protein comprises:

(a) the amino acid sequence as shown in SEQ ID NO: 3; or

(b) an amino acid sequence having 70% or higher identity to the amino acid sequence of SEQ ID NO: 3 in which an amino acid residue corresponding to the 324th position from the N terminus of the amino acid sequence of SEQ ID NO: 3 is cysteine.

[0012]

(4) A mutant gene encoding the SUI3 protein comprising a consensus sequence comprising a substitution of an amino acid residue in the 52nd position from the N terminus with another amino acid residue in SEQ ID NO: 4.

[0013]

(5) The mutant gene according to (4), wherein the amino acid residue in the 52nd position is serine or asparagine and the another amino acid residue is threonine.

[0014]

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(6) The mutant gene according to (4), wherein the mutant SUI3 protein comprises:

(a) the amino acid sequence as shown in SEQ ID NO: 6; or

(b) an amino acid sequence having 70% or higher identity to the amino acid sequence of SEQ ID NO: 6 in which an amino acid residue corresponding to the 112th position from the N terminus of the amino acid sequence of SEQ ID NO: 6 is threonine.

[0015]

(7) A mutant gene encoding the mutant FPK1 protein comprising a consensus sequence comprising a substitution of an amino acid residue in the 31st position from the N terminus with another amino acid residue in SEQ ID NO: 7.

[0016]

(8) The mutant gene according to (7), wherein the amino acid residue in the 31st position is glycine and the another amino acid residue is glutamic acid.

[0017]

(9) The mutant gene according to (7), wherein the mutant FPK1 protein comprises:

(a) the amino acid sequence as shown in SEQ ID NO: 9; or

(b) an amino acid sequence having 70% or higher identity to the amino acid sequence of SEQ ID NO: 9 in which an amino acid residue corresponding to the 704th position from the N terminus of the amino acid sequence of SEQ ID NO: 9 is glutamic acid.

[0018]

(10) A mutant yeast strain having xylose-metabolizing ability, which comprises the mutant gene according to any of (1) to (9).

[0019]

(11) A method for producing ethanol comprising a step of culturing the mutant yeast strain according to (10) in a xylose-containing medium and performing ethanol fermentation.

[0020]

(12) The method for producing ethanol according to (11), wherein the medium contains cellulose and the ethanol fermentation proceeds simultaneously at least with the cellulose saccharification.

[0021]

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This description includes part or all of the content as disclosed in the description and/or drawings of Japanese Patent Application No. 2017-214102, which is a priority document of the present application.

[Effects of the Invention]

[0022]

The mutant gene according to the present invention is capable of imparting a yeast strain having xylose-metabolizing ability with excellent ethanol fermentation ability. Specifically, the mutant yeast strain according to the present invention can exhibit excellent ethanol fermentation ability. Accordingly, the mutant gene according to the present invention, a mutant yeast strain comprising such mutant gene, and a method for producing ethanol using the same can provide excellent ethanol productivity.

[Brief Description of the Drawings]

[0023]

Fig. 1 shows a characteristic diagram demonstrating the results of evaluation of the ethanol fermentation ability of 6 types of mutant yeast strains each having xylose-metabolizing ability resulting from independent introduction of 6 types of mutant genes identified in the examples.

[Embodiments of the Invention]

[0024]

Hereafter, the present invention is described in greater detail with reference to the drawing and the examples.

[0025]

The mutant gene according to the present invention was identified in a mutant strain having excellent ethanol fermentation ability, which was obtained by long-term continuous culture of a recombinant yeast strain having xylose-metabolizing ability. As described in the examples below, specific 3 different mutant genes were identified. These 3 mutant genes are occasionally referred to as mutant genes 1 to 3 for convenience.

<Mutant gene 1 >

The mutant gene 1 encodes the mutant CDC123 protein comprising a substitution of a particular amino acid residue of the cell division cycle (CDC) 123 protein with another

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amino acid residue. CDC123 is an eIF2 translation initiation factor complex-associated factor (also referred to as an aggregation factor, architectural factor, or assembly factor) and it has a function of regulating initiation of translation. A systematic name of the gene encoding CDC123 is YLR215C.

[0026]

The mutant CDC123 protein comprises a substitution mutation of a particular amino acid residue on the C-terminal side of the D123 domain. A region having such mutation is conserved among many CDC123 proteins derived from different organism species. SEQ ID NO: 1 shows the conserved region within the D123 domain comprising such mutation (i.e., a consensus sequence). The mutant CDC123 protein is defined to comprise an amino acid sequence comprising a substitution of an amino acid residue in the 30th position from the N terminus in SEQ ID NO: 1 with another amino acid residue.

[0027]

The conserved region comprising the amino acid sequence as shown in SEQ ID NO: 1 is defined to be a region of 34 amino acid residues from the N terminus (the left end) in the amino acid sequences shown in the Table 1 below (lines 2 to 13). An amino acid residue indicated as Xaa at position 1 in the amino acid sequence as shown in SEQ ID NO: 1 is glutamic acid, arginine, glutamine, lycine, or aspartic acid. An amino acid residue indicated as Xaa at position 5 in the amino acid sequence as shown in SEQ ID NO: 1 is leucine or phenylalanine. An amino acid residue indicated as Xaa at position 7 in the amino acid sequence as shown in SEQ ID NO: 1 is leucine or isoleucine. An amino acid residue indicated as Xaa at position 8 in the amino acid sequence as shown in SEQ ID NO: 1 is valine, isoleucine, or leucine. An amino acid residue indicated as Xaa at position 9 in the amino acid sequence as shown in SEQ ID NO: 1 is threonine, lycine, proline, or leucine. An amino acid residue indicated as Xaa at position 10 in the amino acid sequence as shown in SEQ ID NO: 1 is arginine, serine, or glutamic acid. An amino acid residue indicated as Xaa at position 11 in the amino acid sequence as shown in SEQ ID NO: 1 is histidine, asparagine, or threonine. An amino acid residue indicated as Xaa at position 13 in the amino acid sequence as shown in SEQ ID NO: 1 is threonine, isoleucine, or valine. An amino acid residue indicated as Xaa at position 14 in the amino acid sequence as shown in SEQ ID NO: 1 is glycine, cysteine, or

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alanine. An amino acid residue indicated as Xaa at position 17 in the amino acid sequence as shown in SEQ ID NO: 1 is alanine or valine. An amino acid residue indicated as Xaa at position 18 in the amino acid sequence as shown in SEQ ID NO: 1 is serine, threonine, histidine, or cysteine. An amino acid residue indicated as Xaa at position 23 in the amino acid sequence as shown in SEQ ID NO: 1 is glutamic acid or glutamine. An amino acid residue indicated as Xaa at position 25 in the amino acid sequence as shown in SEQ ID NO: 1 is histidine or glutamine. An amino acid residue indicated as Xaa at position 28 in the amino acid sequence as shown in SEQ ID NO: 1 is glutamine, lycine, arginine, isoleucine, or threonine. An amino acid residue indicated as Xaa at position 30 in the amino acid sequence as shown in SEQ ID NO: 1 is leucine, valine, or isoleucine. An amino acid residue indicated as Xaa at position 31 in the amino acid sequence as shown in SEQ ID NO: 1 is leucine, valine, or isoleucine. An amino acid residue indicated as Xaa at position 32 in the amino acid sequence as shown in SEQ ID NO: 1 is glutamic acid or aspartic acid. An amino acid residue indicated as Xaa at position 34 in the amino acid sequence as shown in SEQ ID NO: 1 is serine, alanine, or threonine.

[0028]

The "another amino acid residue" after substitution of the amino acid in the 30th position from the N terminus of the sequence shown in SEQ ID NO: 1 is different from the amino acid in the wild-type CDC123 protein. In the wild-type CDC123 proteins, the amino acid in the 30th position is not particularly limited, and it is often leucine, valine, or isoleucine. When the amino acid in the 30th position in a certain wild-type CDC123 protein is leucine, for example, the mutant CDC123 protein comprises an amino acid sequence in which leucine in the 30th position has been substituted with an amino acid residue other than leucine. In such a case, another amino acid residue other than leucine is not particularly limited, and it is preferably an amino acid other than valine and isoleucine. In the mutant CDC123 protein, the amino acid after substitution mutation is more preferably cysteine.

[0029]

As a method of substitution of the amino acid in the 30th position from the N terminus of the sequence shown in SEQ ID NO: 1 with another amino acid residue, a conventional genetic engineering technique can be adequately employed. Specifically, a

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nucleotide sequence of a wild-type gene encoding a target protein into which a mutation is to be introduced is identified, and a mutation can be introduced to encode a protein after the substitution with the use of, for example, a site-directed mutagenesis kit. The gene into which the mutation has been introduced can be recovered in accordance with a conventional technique. For example, the gene can be integrated into an expression vector and recovered in that state. A mutation can be introduced into a gene by a conventional technique, such as the Kunkel method or the Gapped duplex method, or a method in accordance therewith. For example, a mutation can be introduced with the use of a mutagenesis kit that adopts a site-directed mutagenesis technique (e.g., Mutan-K and Mutan-G, Takara Bio Inc.) or an LA PCR *in vitro* Mutagenesis series kit (Takara Bio Inc.).

[0030]

In the CDC123 protein derived from *Saccharomyces cerevisiae*, more specifically, the amino acid in the 30th position is leucine. SEQ ID NO: 2 and SEQ ID NO: 3 show the nucleotide sequence encoding the mutant CDC123 protein derived from *Saccharomyces cerevisiae* comprising a substitution of leucine in the 30th position with cysteine and the amino acid sequence of the mutant CDC123 protein, respectively. In the amino acid sequence of the mutant CDC123 protein as shown in SEQ ID NO: 3, the amino acid in the 30th position in SEQ ID NO: 1 corresponds to the amino acid in the 324th position from the N terminus. Specifically, cysteine in the 324th position in the amino acid sequence as shown in SEQ ID NO: 3 is leucine in the wild-type protein.

[0031]

The mutant CDC123 protein is not limited to the protein comprising the amino acid sequence as shown in SEQ ID NO: 3. For example, it may be a protein comprising an amino acid sequence having 70% or higher identity to the amino acid sequence as shown in SEQ ID NO: 3, provided that cysteine in the 324th position is maintained. As described above, the degree of sequence identity may be 70% or higher, preferably 80% or higher, more preferably 85% or higher, further preferably 90% or higher, and most preferably 95% or higher. The degree of sequence identity can be determined using the BLASTN or BLASTX Program equipped with the BLAST algorithm (at default settings). The degree of sequence identity is determined by subjecting a pair of amino acid sequences to pairwise alignment analysis,

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identifying completely identical amino acid residues, and calculating the percentage of all the amino acid residues subjected to comparison accounted for by such amino acid residues.

[0032]

The mutant CDC123 protein is not limited to the protein comprising the amino acid sequence as shown in SEQ ID NO: 3. As long as cysteine in the 324th position is maintained, a protein may comprise an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 3 by substitution, deletion, insertion, or addition of 1 or a plurality of amino acids and preferably 1 or several amino acids. The term "several" used herein refers to, for example, 2 to 40, preferably 2 to 30, more preferably 2 to 20, further preferably 2 to 10, and most preferably 2 to 5.

[0033]

In addition, the mutant CDC123 protein is not limited to the protein encoded by the nucleotide sequence as shown in SEQ ID NO: 2. For example, it may be a protein encoded by a polynucleotide hybridizing under stringent conditions to the full-length sequence or a partial sequence of a complementary strand of DNA comprising the nucleotide sequence as shown in SEQ ID NO: 2, provided that the protein maintaining cysteine in the 324th position is encoded. Under "stringent conditions," so-called specific hybrids are formed, but non-specific hybrids are not formed. For example, such conditions can be adequately determined with reference to Molecular Cloning: A Laboratory Manual (Third Edition). Specifically, the degree of stringency can be determined in accordance with the temperature and the salt concentration of a solution used for Southern hybridization and the temperature and the salt concentration of a solution used for the step of washing in Southern hybridization. Under stringent conditions, more specifically, the sodium concentration is 25 to 500 mM and preferably 25 to 300 mM, and temperature is 42°C to 68°C and preferably 42°C to 65°C. Further specifically, hybridization is carried out in the presence of 5× SSC (83 mM NaCl, 83 mM sodium citrate) at 42°C.

[0034]

In the case of the mutant CDC123 protein comprising an amino acid sequence other than the amino acid sequence as shown in SEQ ID NO: 3 or the mutant CDC123 protein encoded by a nucleotide sequence other than the nucleotide sequence as shown in SEQ ID

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NO: 2, as described above, the position of the cysteine residue after the mutation may not be the 324th position.

[0035]

The mutant CDC123 protein is not limited to one derived from *Saccharomyces cerevisiae*. The origin thereof is not limited, provided that the CDC123 protein has the conserved region comprising the amino acid sequence as shown in SEQ ID NO: 1 in which the amino acid in the 30th position from the N terminus has been substituted with another amino acid residue. For example, wild-type CDC123 proteins derived from various organism species can be identified from the databases storing amino acid sequences and nucleotide sequences on the basis of the amino acid sequence of the CDC123 protein derived from *Saccharomyces cerevisiae* or the nucleotide sequence encoding such CDC123 protein. From among the wild-type CDC123 proteins identified in the manner described above, those having the conserved region comprising the amino acid sequence as shown in SEQ ID NO: 1 may be subjected to substitution of the amino acid in the 30th position, so that the mutant CDC123 protein and the mutant gene 1 encoding such mutant CDC123 protein can be obtained.

[0036]

For example, the databases may be searched on the basis of the amino acid sequence of the CDC123 protein derived from *Saccharomyces cerevisiae*. Thus, wild-type CDC123 proteins each having the conserved region comprising the amino acid sequence of SEQ ID NO: 1 can be identified as shown in Table 1 (lines 2 to 13). Table 1 also shows the amino acid sequences comprising such conserved region.

[0037]

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[Table 1]

Gene name	Sequence	SEQ ID NO:
Mutant CDC123 protein	EDYELRLVTRHNTGRFASKEHSENHVPQD**C**VEASLNPEAIRELTQKWKELLSQQAKE-ESSDSESET	10
Cdc123p [*Saccharomyces cerevisiae* YJM1381]	EDYELRLVTRHNTGRFASKEHSENHVPQDLVEASLNPEAIRELTQKWKELLSQQAKE-ESSDSESET	11
CDC123-like protein [*Saccharomyces kudriavzevii* IFO 1802]	EDYELRLVTSHNTCRFASKEHSENHVPQDLVEASLNPEAIRELTQKWKELLSQQTQE-ESSDSEDGT	12
CDC123-like protein [*Saccharomyces eubayanus*]	EDYELRLVTRHNTGRFASKEHSENHVPQDLVEAGLDPEAIRELTQKWRELLNQQTQE-ESSGSEDEA	13
Cell division cycle protein 123 [*Candida glabrata*]	RDYELRLITENNIGRFASKEHSQNHVPKDVVDASLDPERIRELSQKWSELLLQQE-----KESSDEE	14
Hypothetical protein KNAG_0B02750 [*Kazachstania naganishii* CBS 8797]	QDYELRLVKENNTARFASKEHSENHVPKDIVDASLDPNAIRDLAQKWKELLSQQQAEDSSSGSEEEA	15
Hypothetical protein NDAI_0A02840 [*Naumovozyma dairenensis* CBS 421]	KDYELRLVKENNVGRFVSKEHSENQVPKDLIDAALDPQAIKELTEKWKELLSRQEKD------EENK	16
Hypothetical protein TBLA_0F02200 [*Tetrapisispora blattae* CBS 6284]	RDYELRLVKRNNVARFASKEHSENQVPKDVVDASLDPNVIKELASKWKELLSQQEAD-TDSDSDSAE	17
Hypothetical protein NCAS_0E03830 [*Naumovozyma castellii* CBS 4309]	KDYELRLLTENNTGRFASKEHSENQVPRDLVDASLNPDAIRELTQKWKDLLSRQNGSGSDTESESES	18
Hypothetical protein TDEL_0C02280 [*Torulaspora delbrueckii*]	EDYELRIVPENNVARFATKEHSENHVPKDVLEASLNPEAIKELSEKWQELLRCQELE-DDSDNE---	19
Hypothetical protein KAFR_0L01360 [*Kazachstania africana* CBS 2517]	KDYELRLVLENNTARFASKEHSENQVPRDVVDATTDPNAIRELIGKWKELLEQQE---EDTDSD---	20
Hypothetical protein TPHA_0A03030 [*Tetrapisispora phaffii* CBS 4417]	DDYELRLLTETNVGRFAHKEHSENQVPIDIVEASLNPDAIKELADKWSELLKKQDDY--DSDSHDN-	21
Hypothetical protein Kpol_1019p20 [*Vanderwaltozyma polyspora* DSM 70294]	EDYEFRLIKENNVGRFACKEHSENQVPTDIVEASLNPEAIRELTQKWKELLSKQSME-EDSSSDSNE	22

[0038]

Specifically, the mutant CDC123 protein may comprise an amino acid sequence comprising, for example, a substitution of an amino acid in SEQ ID NOs: 11 to 22 corresponding to the amino acid in the 30th position from the N terminus of the sequence in SEQ ID NO: 1 with another amino acid residue and preferably with cysteine.

<Mutant gene 2>

The mutant gene 2 encodes a mutant SUI3 protein comprising a substitution of a particular amino acid with another amino acid in the β subunit of the translation initiator eIF2. The SUI3 protein is associated with the mechanism of eIF2 that detects an initiation codon as the β subunit of the translation initiator eIF2. The systematic name of the gene encoding the SUI3 protein is YPL237W.

[0039]

The mutant SUI3 protein has a substitution mutation of a particular amino acid residue in the vicinity of the N terminus of a functional domain as a transcription initiator. A region comprising such mutation is conserved among many SUI3 proteins derived from different organism species. SEQ ID NO: 4 represents a conserved region comprising such mutation (a consensus sequence). The mutant SUI3 protein can be defined to comprise an amino acid sequence comprising a substitution of the amino acid in the 43rd position from the N terminus in SEQ ID NO: 4 with another amino acid residue.

[0040]

The conserved region comprising the amino acid sequence as shown in SEQ ID NO: 4 is defined to be a region of 48 amino acid residues from the N terminus (the left end) in the amino acid sequences shown in the Table 2 below (lines 2 to 13). An amino acid residue indicated as Xaa at position 2 in the amino acid sequence as shown in SEQ ID NO: 4 is aspartic acid or glutamic acid. An amino acid residue indicated as Xaa at position 3 in the amino acid sequence as shown in SEQ ID NO: 4 is isoleucine, valine, leucine, or alanine. An amino acid residue indicated as Xaa at position 4 in the amino acid sequence as shown in SEQ ID NO: 4 is alanine, threonine, or serine. An amino acid residue indicated as Xaa at position 5 in the amino acid sequence as shown in SEQ ID NO: 4 is glutamic acid or aspartic acid. An amino acid residue indicated as Xaa at position 6 in the amino acid sequence as

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shown in SEQ ID NO: 4 is alanine or valine. An amino acid residue indicated as Xaa at position 7 in the amino acid sequence as shown in SEQ ID NO: 4 is leucine or phenylalanine. An amino acid residue indicated as Xaa at position 9 in the amino acid sequence as shown in SEQ ID NO: 4 is glutamic acid or leucine. An amino acid residue indicated as Xaa at position 11 in the amino acid sequence as shown in SEQ ID NO: 4 is serine, threonine, or lycine. An amino acid residue indicated as Xaa at position 19 in the amino acid sequence as shown in SEQ ID NO: 4 is threonine, serine, or alanine. An amino acid residue indicated as Xaa at position 20 in the amino acid sequence as shown in SEQ ID NO: 4 is lycine, alanine, or proline. An amino acid residue indicated as Xaa at position 21 in the amino acid sequence as shown in SEQ ID NO: 4 is aspartic acid, histidine, glutamic acid, or valine. An amino acid residue indicated as Xaa at position 22 in the amino acid sequence as shown in SEQ ID NO: 4 is serine, valine, threonine, or alanine. An amino acid residue indicated as Xaa at position 23 in the amino acid sequence as shown in SEQ ID NO: 4 is serine, alanine, threonine, aspartic acid, glutamic acid, or asparagine. An amino acid residue indicated as Xaa at position 24 in the amino acid sequence as shown in SEQ ID NO: 4 is valine or leucine. An amino acid residue indicated as Xaa at position 26 in the amino acid sequence as shown in SEQ ID NO: 4 is alanine, aspartic acid, or glutamic acid. An amino acid residue indicated as Xaa at position 29 in the amino acid sequence as shown in SEQ ID NO: 4 is lycine or glutamic acid. An amino acid residue indicated as Xaa 30 in the amino acid sequence as shown in SEQ ID NO: 4 is glutamine or glutamic acid. An amino acid residue indicated as Xaa at position 33 in the amino acid sequence as shown in SEQ ID NO: 4 is lycine, arginine, or serine. An amino acid residue indicated as Xaa at position 36 in the amino acid sequence as shown in SEQ ID NO: 4 is leucine or valine. An amino acid residue indicated as Xaa at position 37 in the amino acid sequence as shown in SEQ ID NO: 4 is aspartic acid, asparagine, or lycine. An amino acid residue indicated as Xaa at position 38 in the amino acid sequence as shown in SEQ ID NO: 4 is asparagine, serine, or valine. An amino acid residue indicated as Xaa at position 39 in the amino acid sequence as shown in SEQ ID NO: 4 is valine, isoleucine, aspartic acid, or alanine. An amino acid residue indicated as Xaa at position 40 in the amino acid sequence as shown in SEQ ID NO: 4 is aspartic acid, glutamic acid, threonine, serine, glycine, or valine. An amino acid residue indicated as Xaa at position 41 in the amino acid sequence as shown in SEQ ID

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NO: 4 is alanine, glycine, serine, glutamic acid, threonine, alanine, aspartic acid, or valine. An amino acid residue indicated as Xaa at position 42 in the amino acid sequence as shown in SEQ ID NO: 4 is glutamic acid, asparagine, or aspartic acid. An amino acid residue indicated as Xaa at position 43 in the amino acid sequence as shown in SEQ ID NO: 4 is serine or asparagine. An amino acid residue indicated as Xaa at position 44 in the amino acid sequence as shown in SEQ ID NO: 4 is lycine, serine, glutamic acid, or asparagine. An amino acid residue indicated as Xaa at position 45 in the amino acid sequence as shown in SEQ ID NO: 4 is glutamic acid, lycine, or aspartic acid. An amino acid residue indicated as Xaa at position 46 in the amino acid sequence as shown in SEQ ID NO: 4 is glycine, alanine, threonine, aspartic acid, serine, or glutamic acid. An amino acid residue indicated as Xaa at position 47 in the amino acid sequence as shown in SEQ ID NO: 4 is threonine or serine. An amino acid residue indicated as Xaa at position 48 in the amino acid sequence as shown in SEQ ID NO: 4 is proline or threonine.

[0041]

The "another amino acid residue" after substitution of the amino acid in the 43rd position from the N terminus of the sequence shown in SEQ ID NO: 4 is different from the amino acid in the wild-type SUI3 protein. In the wild-type SUI3 proteins, the amino acid in the 43rd position is not particularly limited, and it is often serine or asparagine. When the amino acid in the 43rd position in a certain wild-type SUI3 protein is serine, for example, the mutant SUI3 protein comprises an amino acid sequence in which serine in the 43rd position has been substituted with an amino acid residue other than serine. In such a case, another amino acid residue other than serine is not particularly limited, and it is preferably an amino acid other than asparagine. In the mutant SUI3 protein, the amino acid after substitution mutation is more preferably threonine.

[0042]

As a method of substitution of the amino acid in the 43rd position from the N terminus of the sequence shown in SEQ ID NO: 4 with another amino acid residue, a conventional genetic engineering technique can be adequately employed. Specifically, a nucleotide sequence of a wild-type gene encoding a target protein into which a mutation is to be introduced is identified, and a mutation can be introduced to encode a protein after the

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substitution with the use of, for example, a site-directed mutagenesis kit. The gene into which the mutation has been introduced can be recovered in accordance with a conventional technique. For example, the gene can be integrated into an expression vector and recovered in that state. A mutation can be introduced into a gene by a conventional technique, such as the Kunkel method or the Gapped duplex method, or a method in accordance therewith. For example, a mutation can be introduced with the use of a mutagenesis kit that adopts a site-directed mutagenesis technique (e.g., Mutan-K and Mutan-G, Takara Bio Inc.) or an LA PCR *in vitro* Mutagenesis series kit (Takara Bio Inc.).

[0043]

In the SUI3 protein derived from *Saccharomyces cerevisiae*, more specifically, the amino acid in the 43rd position is serine. SEQ ID NO: 5 and SEQ ID NO: 6 show the nucleotide sequence encoding the mutant SUI3 protein derived from *Saccharomyces cerevisiae* comprising substitution of serine in the 43rd position with threonine and the amino acid sequence of the mutant SUI3 protein, respectively. In the amino acid sequence of the mutant SUI3 protein as shown in SEQ ID NO: 6, the amino acid in the 43rd position in SEQ ID NO: 4 corresponds to the amino acid in the 112th position from the N terminus. Specifically, threonine in the 112th position in the amino acid sequence as shown in SEQ ID NO: 6 is serine in the wild-type protein.

[0044]

The mutant SUI3 protein is not limited to the protein comprising the amino acid sequence as shown in SEQ ID NO: 6. For example, it may be a protein comprising an amino acid sequence having 70% or higher identity to the amino acid sequence as shown in SEQ ID NO: 6, provided that threonine in the 112th position is maintained. As described above, the degree of sequence identity may be 70% or higher, preferably 80% or higher, more preferably 85% or higher, further preferably 90% or higher, and most preferably 95% or higher. The degree of sequence identity can be determined using the BLASTN or BLASTX Program equipped with the BLAST algorithm (at default settings). The degree of sequence identity is determined by subjecting a pair of amino acid sequences to pairwise alignment analysis, identifying completely identical amino acid residues, and calculating the percentage of all the

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amino acid residues subjected to comparison accounted for by such amino acid residues.

[0045]

The mutant SUI3 protein is not limited to the protein comprising the amino acid sequence as shown in SEQ ID NO: 6. For example, it may be a protein comprising an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 6 by substitution, deletion, insertion, or addition of 1 or a plurality of and preferably 1 or several amino acids, provided that threonine in the 112th position is maintained. The term "several" used herein refers to, for example, 2 to 30, preferably 2 to 20, more preferably 2 to 10, and most preferably 2 to 5.

[0046]

In addition, the mutant SUI3 protein is not limited to the protein encoded by the nucleotide sequence as shown in SEQ ID NO: 5. As long as a protein maintaining threonine in the 112th position is encoded, for example, it may be a protein encoded by a polynucleotide hybridizing under stringent conditions to the full-length sequence or a partial sequence of a complementary strand of DNA comprising the nucleotide sequence as shown in SEQ ID NO: 5. Under "stringent conditions," so-called specific hybrids are formed, but non-specific hybrids are not formed. For example, such conditions can be adequately determined with reference to Molecular Cloning: A Laboratory Manual (Third Edition). Specifically, the degree of stringency can be determined in accordance with the temperature and the salt concentration of a solution used for Southern hybridization and the temperature and the salt concentration of a solution used for the step of washing in Southern hybridization. Under stringent conditions, more specifically, the sodium concentration is 25 to 500 mM and preferably 25 to 300 mM, and temperature is 42°C to 68°C and preferably 42°C to 65°C. Further specifically, hybridization is carried out in the presence of 5× SSC (83 mM NaCl, 83 mM sodium citrate) at 42°C.

[0047]

In the case of the mutant SUI3 protein comprising an amino acid sequence other than the amino acid sequence as shown in SEQ ID NO: 6 or the mutant SUI3 protein encoded by a nucleotide sequence other than the nucleotide sequence as shown in SEQ ID NO: 5, as

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described above, the position of the threonine residue after the mutation may not be the 112th position.

[0048]

The mutant SUI3 protein is not limited to one derived from *Saccharomyces cerevisiae*. The origin thereof is not limited, provided that the SUI3 protein has the conserved region comprising the amino acid sequence as shown in SEQ ID NO: 4 in which the amino acid in the 43rd position from the N terminus has been substituted with another amino acid residue. For example, wild-type SUI3 proteins derived from various organism species can be identified from the databases storing amino acid sequences and nucleotide sequences on the basis of the amino acid sequence of the SUI3 protein derived from *Saccharomyces cerevisiae* or the nucleotide sequence encoding such SUI3 protein. From among the wild-type SUI3 proteins identified in the manner described above, those having the conserved region comprising the amino acid sequence as shown in SEQ ID NO: 4 may be subjected to substitution of the amino acid in the 43rd position, so that the mutant SUI3 protein and the mutant gene 2 encoding such mutant SUI3 protein can be obtained.

[0049]

For example, the databases may be searched on the basis of the amino acid sequence of the SUI3 protein derived from *Saccharomyces cerevisiae*. Thus, wild-type SUI3 proteins each having the conserved region comprising the amino acid sequence of SEQ ID NO: 4 can be identified as shown in Table 2 (lines 2 to 13). Table 2 also shows the amino acid sequences comprising such conserved region.

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[Table 2]

Gene name	Sequence	SEQ ID NO:
Mutant SUI3 protein	KE-PTDDIAEALGELSLKKKKKK-TKDSSVDAFEKELAKAGLDNVD-AE--TKEGTP--S--ANS-SIQQEVGLPYSELL	23
Sui3p [*Saccharomyces cerevisiae* YJM450]	KE-PTDDIAEAFGELSLKKKKKK-TKDSSVDAFEKELAKAGLDNVD-AE--SKEGTP--S--ANS-SIQQEVGLPYSELL	24
SUI3-like protein [*Saccharomyces eubayanus*]	TE-PTDDIAEALGELSLKKKKKK-TKDSSVDAFEKELAKAGLDNVD-AE--SKEATP--A--ASA-SIQQEVGLPYPELL	25
sui3p [*Saccharomyces arboricola* H-6]	KG-PTDDIAEALGELSLKKKKKK-TKDSSVDAFEKELAKAGLDSVE-GE--SKEATP--V--ASS-SIQQEVGLPYPELL	26
Hypothetical protein KAFR_0F01140 [*Kazachstania africana* CBS 2517]	DN-TTDDITEALGELSLKKKKKK-TKDVALDDFEKELAKAG---VT-SE--SKETTP--Q--NIS-VVQQEAGLPYDKLL	27
Hypothetical protein KNAG_0D00820 [*Kazachstania naganishii* CBS 8797]	DG-ELDDVSEALGELTLKKKKKK-SKDSTLDDFEKELARAG---IN-EE--SSKDST--P--TGE-IGNDEVGLPYADLL	28
Hypothetical protein NCAS_0G01200 [*Naumovozyma castellii* CBS 4309]	NN-SVDELSDVLGDLTIKKKKKK-AAHVDVDAFEKELAKAG---VS-TE--SKEATP--SGDNES-SIQNSIGLPYPELL	29
Hypothetical protein TDEL_0A06770 [*Torulaspora delbrueckii*]	SD-SVDDISEALGELKLKKKKKK-AKDTDLDDFEQQLAKAGVNVDE-AN--NKEATP--T--VDS-ALQQEVGLAYPELL	30
Hypothetical protein NDAI_0F01330 [*Naumovozyma dairenensis* CBS 421]	NNTSVDDLSDVLGDLTLKKKKKK-SKEATTDDFEKELAKAG---VS-T---SKDGTPISEGNSESETLQKEVGLPYPQLL	31
Probable Eukaryotic translation initiation factor 2 subunit beta [*Zygosaccharomyces bailii* ISA1307]	SG-SVDEISEALGELKLKKKKK--SKETEVDDFEQQLAKAGVKVAG-GN--SKESTP--V--AES-SIQQDVGLTYQDLL	32
Hypothetical protein TBLA_0A02260 [*Tetrapisispora blattae* CBS 6284]	NG-EIDEASEALGELSLKKKKKKkTKEANLDEFEKELAKAG---VVVDE--NKEETP--S--NES-TLQEDIGLPYQDLL	33
Hypothetical protein ZYGR_0AN00550 [*Zygosaccharomyces rouxii*]	SE-SVDEISEALGELKLKKKKK--SKEAEVDDFEKQLASAGVNVDG-GN--SQESTP--A--LES-SLQQDVGLSYPGLL	34
Hypothetical protein TPHA_0H01900 [*Tetrapisispora phaffii* CBS 4417]	D---VDDITEALGDLKLKKKKKK-APVADVDEFEQELAKAG---VV-VDETSNEATP--G--HES-SLQQDVGLPYDKLL	35

[0051]

Specifically, the mutant SUI3 protein may comprise an amino acid sequence comprising, for example, a substitution of an amino acid in SEQ ID NOs: 24 to 35 corresponding to the amino acid in the 43rd position from the N terminus of SEQ ID NO: 4 with another amino acid residue and preferably with threonine.

<Mutant gene 3>

The mutant gene 3 encodes a mutant FPK1 protein comprising a substitution of a particular amino acid with another amino acid in a serine/threonine protein kinase. The FPK1 protein phosphorylates a member of the aminophospholipid translocase family and regulates translocation and membrane asymmetry of a phospholipid. The FPK1 protein phosphorylates and inhibits an upstream inhibitory kinase Ypk1p. The systematic name of a gene encoding the FPK1 protein is YNR047W.

[0052]

The mutant FPK1 protein comprises a substitution mutation of particular amino acid residues in the vicinity of the ATP-binding site and an active site within the catalytic domain of a serine/threonine protein kinase. A region comprising such mutation is conserved among many FPK1 proteins derived from different organism species. SEQ ID NO: 7 represents a conserved region within the catalytic domain comprising such mutation (a consensus sequence). The mutant FPK1 protein can be defined to comprise an amino acid sequence a substitution of the amino acid in the 31st position from the N terminus in SEQ ID NO: 7 with another amino acid residue.

[0053]

The conserved region comprising the amino acid sequence as shown in SEQ ID NO: 7 is defined to be a region of 80 amino acid residues from the N terminus (the left end) in the amino acid sequences shown in the Table 3 below (lines 2 to 13). An amino acid residue indicated as Xaa at position 41 in the amino acid sequence as shown in SEQ ID NO: 7 is proline or isoleucine. An amino acid residue indicated as Xaa at position 46 in the amino acid sequence as shown in SEQ ID NO: 7 is glycine or alanine. An amino acid residue indicated as Xaa at position 47 in the amino acid sequence as shown in SEQ ID NO: 7 is aspartic acid, glutamic acid, or serine. An amino acid residue indicated as Xaa at position 48 in the amino

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acid sequence as shown in SEQ ID NO: 7 is asparagine, glutamic acid, or serine. An amino acid residue indicated as Xaa at position 49 in the amino acid sequence as shown in SEQ ID NO: 7 is threonine or serine. An amino acid residue indicated as Xaa at position 51 in the amino acid sequence as shown in SEQ ID NO: 7 is glutamic acid, glutamine, arginine, or leucine. An amino acid residue indicated as Xaa at position 54 in the amino acid sequence as shown in SEQ ID NO: 7 is threonine, serine, or cysteine. An amino acid residue indicated as Xaa at position 56 in the amino acid sequence as shown in SEQ ID NO: 7 is isoleucine or valine. An amino acid residue indicated as Xaa at position 59 in the amino acid sequence as shown in SEQ ID NO: 7 is asparagine, lycine, or serine. An amino acid residue indicated as Xaa at position 60 in the amino acid sequence as shown in SEQ ID NO: 7 is glutamic acid or aspartic acid. An amino acid residue indicated as Xaa 6 at position 2 in the amino acid sequence as shown in SEQ ID NO: 7 is serine, threonine, isoleucine, or asparagine. An amino acid residue indicated as Xaa at position 68 in the amino acid sequence as shown in SEQ ID NO: 7 is glutamic acid or aspartic acid. An amino acid residue indicated as Xaa at position 69 in the amino acid sequence as shown in SEQ ID NO: 7 is isoleucine or valine. An amino acid residue indicated as Xaa at position 70 in the amino acid sequence as shown in SEQ ID NO: 7 is serine or glycine. An amino acid residue indicated as Xaa at position 72 in the amino acid sequence as shown in SEQ ID NO: 7 is threonine, asparagine, alanine, or serine. An amino acid residue indicated as Xaa at position 78 in the amino acid sequence as shown in SEQ ID NO: 7 is lycine or arginine. An amino acid residue indicated as Xaa at position 79 in the amino acid sequence as shown in SEQ ID NO: 7 is lycine or arginine.

[0054]

The "another amino acid residue" after substitution of the amino acid in the 31st position from the N terminus of SEQ ID NO: 7 is different from the amino acid in the wild-type FPK1 protein. In the wild-type FPK1 proteins, the amino acid in the 31st position is not particularly limited, and it is often glycine. When the amino acid in the 31st position in a certain wild-type FPK1 protein is glycine, for example, the mutant FPK1 protein comprises an amino acid sequence in which glycine in the 31st position has been substituted with an amino acid residue other than glycine. In such a case, another amino acid residue other than glycine is not particularly limited, and it is preferably glutamic acid.

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[0055]

As a method of substitution of the amino acid in the 31st position from the N terminus of the sequence shown in SEQ ID NO: 7 with another amino acid residue, a conventional genetic engineering technique can be adequately employed. Specifically, a nucleotide sequence of a wild-type gene encoding a target protein into which a mutation is to be introduced is identified, and a mutation can be introduced to encode a protein after the substitution with the use of, for example, a site-directed mutagenesis kit. The gene into which the mutation has been introduced can be recovered in accordance with a conventional technique. For example, the gene can be integrated into an expression vector and recovered in that state. A mutation can be introduced into a gene by a conventional technique, such as the Kunkel method or the Gapped duplex method, or a method in accordance therewith. For example, a mutation can be introduced with the use of a mutagenesis kit that adopts a site-directed mutagenesis technique (e.g., Mutan-K and Mutan-G, Takara Bio Inc.) or an LA PCR *in vitro* Mutagenesis series kit (Takara Bio Inc.).

[0056]

In the FPK1 protein derived from *Saccharomyces cerevisiae*, more specifically, the amino acid in the 31st position is glycine. SEQ ID NO: 8 and SEQ ID NO: 9 show the nucleotide sequence encoding the mutant FPK1 protein derived from *Saccharomyces cerevisiae* comprising a substitution of glycine in the 31st position with glutamic acid and the amino acid sequence of the mutant FPK1 protein, respectively. In the amino acid sequence of the mutant FPK1 protein as shown in SEQ ID NO: 9, the amino acid in the 31st position in SEQ ID NO: 7 corresponds to the amino acid in the 704th position from the N terminus. Specifically, glutamic acid in the 704th position in the amino acid sequence as shown in SEQ ID NO: 9 is glycine in the wild-type protein.

[0057]

The mutant FPK1 protein is not limited to the protein comprising the amino acid sequence as shown in SEQ ID NO: 9. For example, it may be a protein comprising an amino acid sequence having 70% or higher identity to the amino acid sequence as shown in SEQ ID NO: 9, provided that glutamic acid in the 704th position is maintained. As described above, the degree of sequence identity may be 70% or higher, preferably 80% or higher, more

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preferably 85% or higher, further preferably 90% or higher, and most preferably 95% or higher. The degree of sequence identity can be determined using the BLASTN or BLASTX Program equipped with the BLAST algorithm (at default settings). The degree of sequence identity is determined by subjecting a pair of amino acid sequences to pairwise alignment analysis, identifying completely identical amino acid residues, and calculating the percentage of all the amino acid residues subjected to comparison accounted for by such amino acid residues.

[0058]

The mutant FPK1 protein is not limited to the protein comprising the amino acid sequence as shown in SEQ ID NO: 9. For example, it may be a protein comprising an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 9 by substitution, deletion, insertion, or addition of 1 or a plurality of and preferably 1 or several amino acids, provided that glutamic acid in the 704th position is maintained. The term "several" used herein refers to, for example, 2 to 90, preferably 2 to 80, more preferably 2 to 70, more preferably 2 to 60, more preferably 2 to 50, more preferably 2 to 40, more preferably 2 to 30, more preferably 2 to 20, more preferably 2 to 10, and most preferably 2 to 5.

[0059]

Furthermore, the mutant FPK1 protein is not limited to the protein encoded by the nucleotide sequence as shown in SEQ ID NO: 8. For example, it may be a protein encoded by a polynucleotide hybridizing under stringent conditions to the full-length sequence or a partial sequence of a complementary strand of DNA comprising the nucleotide sequence as shown in SEQ ID NO: 8, provided that the protein maintaining the glutamic acid in the 704th position is encoded. Under "stringent conditions," so-called specific hybrids are formed, but non-specific hybrids are not formed. For example, such conditions can be adequately determined with reference to Molecular Cloning: A Laboratory Manual (Third Edition). Specifically, the degree of stringency can be determined in accordance with the temperature and the salt concentration of a solution used for Southern hybridization and the temperature and the salt concentration of a solution used for the step of washing in Southern hybridization. Under stringent conditions, more specifically, the sodium concentration is 25 to 500 mM and preferably 25 to 300 mM, and temperature is 42°C to 68°C and preferably 42°C to 65°C.

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Further specifically, hybridization is carried out in the presence of 5× SSC (83 mM NaCl, 83 mM sodium citrate) at 42°C.

[0060]

In the case of the mutant SUI3 protein comprising an amino acid sequence other than the amino acid sequence as shown in SEQ ID NO: 9 or the mutant SUI3 protein encoded by a nucleotide sequence other than the nucleotide sequence as shown in SEQ ID NO: 8, as described above, the position of the threonine residue after the mutation may not be the 704th position.

[0061]

The mutant FPK1 protein is not limited to one derived from *Saccharomyces cerevisiae*. The origin thereof is not limited, provided that the FPK1 protein has the conserved region comprising the amino acid sequence as shown in SEQ ID NO: 7 in which the amino acid in the 31st position from the N terminus has been substituted with another amino acid residue. For example, wild-type FPK1 proteins derived from various organism species can be identified from the databases storing amino acid sequences and nucleotide sequences on the basis of the amino acid sequence of the FPK1 protein derived from *Saccharomyces cerevisiae* or the nucleotide sequence encoding such FPK1 protein. From among the wild-type FPK1 proteins identified in the manner described above, those having the conserved region comprising the amino acid sequence as shown in SEQ ID NO: 7 may be subjected to substitution of the amino acid in the 31st position, so that the mutant FPK1 protein and the mutant gene 3 encoding such mutant FPK1 protein can be obtained.

[0062]

For example, the databases may be searched on the basis of the amino acid sequence of the FPK1 protein derived from *Saccharomyces cerevisiae*. Thus, wild-type FPK1 proteins each having the conserved region comprising the amino acid sequence of SEQ ID NO: 7 can be identified as shown in Table 3 (lines 2 to 13). Table 3 also shows the amino acid sequences comprising such conserved region.

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[Table 3]

Gene name	Sequence	SEQ ID NO:
Mutant FPK1 protein	TNSFVGTEEYIAPEVIRGNGHTAAVDWWTLEILIYEMLFGFTPFKGDNTNETFTNILKNEVSFPNNNEISRTCKDLIKKL	36
Fpk1p [*Saccharomyces cerevisiae* YJM1078]	TNSFVGTEEYIAPEVIRGNGHTAAVDWWTLGILIYEMLFGFTPFKGDNTNETFTNILKNEVSFPNNNEISRTCKDLIKKL	37
Flippase kinase 1 [*Candida glabrata*]	TNSFVGTEEYIAPEVIRGNGHTAAVDWWTLGILIYEMLFGFTPFKGENTNETFSNILKKDVTFPNNNEVSRNCKDLIKKL	38
Hypothetical protein TDEL_0A07860 [*Torulaspora delbrueckii*]	TNSFVGTEEYIAPEVIRGNGHTAAVDWWTLGILTYEMLFGFTPFKGDNTNETFCNILKSEVTFPNNNEISRACKDLIKKL	39
Hypothetical protein NCAS_0A05570 [*Naumovozyma castellii* CBS 4309]	TNSFVGTEEYIAPEVIRGNGHTAAVDWWTLGILIYEMLFGFTPFKGSSSNETFSNILKNDVSFPNNNDISRNCKDLIKKL	40
LAFE_0C00628g1_1 [*Lachancea fermentati*]	TNSFVGTEEYIAPEVIRGNGHTAAVDWWTLGILIYEMLFGFTPFKGDNTNQTFSNILKNDVIFPNNNEISRTCKDLIKRL	41
LALA0S02e06326g1_1 [*Lachancea lanzarotensis*]	TNSFVGTEEYLAPEVIRGNGHTAAVDWWTLGILIYEMLFGFTPFKGDNTNRTFSNVLKNDVTFPNNNEISRSCKDLIRRL	42
LAQU0S09e04104g1_1 [*Lachancea quebecensis*]	TNSFVGTEEYIAPEVIRGNGHTAAVDWWTLGILIYEMLFGFTPFKADTTNKTFSNVLKNEVTFPNNNEISRNCKDLIKKL	43
LADA_0F15170g1_1 [*Lachancea dasiensis* CBS 10888]	TNSFVGTEEYIAPEVIRGNGHTAAVDWWTLGILIYEMLFGFTPFKGDNTNKTFSNVLKNDVNFPNNNEVSRSCKDLIRKL	44
KLTH0A07458p [*Lachancea thermotolerans* CBS 6340]	TNSFVGTEEYIAPEVIRGNGHTAAVDWWTLGILIYEMLFGFTPFKADTTNKTFSNVLKNEVTFPNNNEVSRNCKDLIKKL	45
LANO_0A00738g1_1 [*Lachancea nothofagi* CBS 11611]	TNSFVGTEEYIAPEVIRGNGHTAAVDWWTLGILIYEMLFGFTPFKGDNTNKTFSNVLKNEVSFPNNNEVSRSCKDLIRKL	46
Hypothetical protein Kpol_1028p16 [*Vanderwaltozyma polyspora* DSM 70294]	TNSFVGTEEYIAPEVIRGNGHTAAVDWWTLGILIYEMLFGFTPFKGDNTNETFCNVLKNDVNFPNNNEISRTCKDLIKKL	47
Hypothetical protein ZYGR_0AK07530 [*Zygosaccharomyces rouxii*]	TNSFVGTEEYIAPEVIRGNGHTAAVDWWTLGILIYEMLFGITPFKASNTNETFCNILKNEVTFPNNNDIGRSCKDLIKKL	48

[0064]

Specifically, the mutant FPK1 protein may comprise an amino acid sequence comprising a, for example, a substitution of an amino acid in SEQ ID NOs: 37 to 48 corresponding to the amino acid in the 31st position from the N terminus of the sequence shown in SEQ ID NO: 7 with another amino acid residue and preferably with threonine.

<Mutant yeast strain>

The mutant yeast strain according to the present invention comprises the mutant gene described above and has xylose-metabolizing ability. The mutant yeast strain comprising the mutant gene described above can be produced by, for example, a method of introducing the mutation as described above so as to modify the wild-type gene endogenous in the genome. Specifically, the mutant yeast strain comprising the mutant gene according to the present invention can be produced by the technique of site-directed mutagenesis as described above.

[0065]

A mutant yeast strain of interest can also be produced via homologous recombination between the mutant gene prepared in advance and the wild-type gene in the genome. Alternatively, the mutant yeast strain comprising the mutant gene according to the present invention may be produced by deleting the wild-type gene from the genome and introducing the mutant gene thereinto in an expressible manner. Alternatively, the mutant yeast strain comprising the mutant gene according to the present invention may be produced by introducing the mutant gene so as to overexpress the same in the genome while refraining from deleting the wild-type gene from the genome. Also, a mutant yeast strain comprising the mutant gene described above can be produced by mutagen treatment.

[0066]

Mutagen treatment may be carried out with the use of chemical mutagenic agents typified by EMS (ethylmethane sulfonate), 5-bromouracil, 2-aminopurine, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine, and other carcinogenic compounds, via treatment with radiation typified by X rays, alpha rays, beta rays, gamma rays, and ion beams, or via ultraviolet treatment.

[0067]

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The term "yeast strain having xylose-metabolizing ability" used herein refers to: a yeast strain that has acquired the xylose-assimilating ability as a result of introduction of a xylose metabolism-associated enzyme gene into a yeast strain that does not inherently has xylose-metabolizing ability (synonymous with "assimilating ability"); and a yeast strain that inherently comprises a xylose metabolism-associated enzyme gene and has the xylose-metabolizing ability.

[0068]

Examples of yeast strains having xylose-metabolizing ability include: a yeast strain that has been provided with xylose-assimilating ability as a result of introduction of a xylose isomerase gene into a yeast strain that does not inherently has xylose-metabolizing ability; and a yeast strain that has been provided with xylose-metabolizing ability as a result of introduction of another xylose metabolism-associated gene.

[0069]

The mutant yeast strain according to the present invention may have an ability of metabolizing xylose (i.e., the xylose-metabolizing ability), specifically, it can assimilate xylose contained in a medium to generate ethanol. Xylose contained in a medium may be obtained by saccharification of xylan or hemicellulose comprising xylose as a constituent sugar. Alternatively, it may be supplied to a medium as a result of saccharification of xylan or hemicellulose contained in a medium by a saccharifying enzyme. The latter case refers to the so-called simultaneous saccharification and fermentation process.

[0070]

The xylose isomerase gene (the XI gene) is not particularly limited, and a gene originating from any organism species may be used. For example, a plurality of the xylose isomerase genes derived from the intestinal protozoa of *Reticulitermes speratus* disclosed in JP 2011-147445 A can be used without particular limitation. Examples of the xylose isomerase genes that can be used include a gene derived from the anaerobic fungus *Piromyces sp.* strain E2 (JP 2005-514951 A), a gene derived from the anaerobic fungus *Cyllamyces aberensis*, a gene derived from a bacterial strain (i.e., *Bacteroides thetaiotaomicron*), a gene derived from a bacterial strain (i.e., *Clostridium phytofermentans*), and a gene derived from the *Streptomyces murinus* cluster.

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[0071]

Specifically, a xylose isomerase gene derived from the intestinal protozoa of *Reticulitermes speratus* may preferably be used. SEQ ID NO: 49 and SEQ ID NO: 50 show the nucleotide sequence of the coding region of the xylose isomerase gene derived from the intestinal protozoa of *Reticulitermes speratus* and the amino acid sequence of a protein encoded by such gene, respectively.

[0072]

The xylose isomerase gene is not limited to the gene identified by SEQ ID NO: 49 and SEQ ID NO: 50. It may be a paralogous gene or a homologous gene in the narrow sense having different nucleotide and amino acid sequences.

[0073]

The xylose isomerase gene is not limited to the gene identified by SEQ ID NO: 49 and SEQ ID NO: 50. For example, it may be a gene comprising an amino acid sequence having 70% or higher, preferably 80% or higher, more preferably 90% or higher, and most preferably 95% or higher sequence similarity or identity to the amino acid sequence as shown in SEQ ID NO: 50 and encoding a protein having xylose isomerase activity. The degree of sequence similarity or identity can be determined using the BLASTN or BLASTX Program equipped with the BLAST algorithm (at default settings). The degree of sequence similarity is determined by subjecting a pair of amino acid sequences to pairwise alignment analysis, identifying completely identical amino acid residues and amino acid residues exhibiting physicochemically similar functions, determining the total number of such amino acid residues, and calculating the percentage of all the amino acid residues subjected to comparison accounted for by the total number of such amino acid residues. The degree of sequence identity is determined by subjecting a pair of amino acid sequences to pairwise alignment analysis, identifying completely identical amino acid residues, and calculating the percentage of all the amino acid residues subjected to comparison accounted for by such amino acid residues.

[0074]

Further, the xylose isomerase gene is not limited to the gene identified by SEQ ID NO: 49 and SEQ ID NO: 50. For example, it may be a gene comprising an amino acid

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sequence derived from the amino acid sequence as shown in SEQ ID NO: 50 by substitution, deletion, insertion, or addition of one or several amino acids and encoding the protein having xylose isomerase activity. The term "several" used herein refers to, for example, 2 to 30, preferably 2 to 20, more preferably 2 to 10, and most preferably 2 to 5.

[0075]

Furthermore, the xylose isomerase gene is not limited to the gene identified by SEQ ID NO: 49 and SEQ ID NO: 50. For example, it may be a gene hybridizing under stringent conditions to the full-length sequence or a partial sequence of a complementary strand of DNA comprising the nucleotide sequence as shown in SEQ ID NO: 49 and encoding the protein having xylose isomerase activity. Under "stringent conditions," so-called specific hybrids are formed, but non-specific hybrids are not formed. For example, such conditions can be adequately determined with reference to Molecular Cloning: A Laboratory Manual (Third Edition). Specifically, the degree of stringency can be determined in accordance with the temperature and the salt concentration of a solution used for Southern hybridization and the temperature and the salt concentration of a solution used for the step of washing in Southern hybridization. Under stringent conditions, more specifically, the sodium concentration is 25 to 500 mM and preferably 25 to 300 mM, and the temperature is 42°C to 68°C and preferably 42°C to 65°C. Further specifically, hybridization is carried out in the presence of 5× SSC (83 mM NaCl, 83 mM sodium citrate) at 42°C.

[0076]

As described above, whether or not a gene comprising a nucleotide sequence that differs from the sequence as shown in SEQ ID NO: 49 or a gene encoding an amino acid sequence that differs from the sequence as shown in SEQ ID NO: 50 would function as a xylose isomerase gene may be determined by, for example, preparing an expression vector comprising the gene of interest integrated into an adequate site between a promoter and a terminator, transforming an *E. coli* host using such expression vector, and assaying the xylose isomerase activity of the protein expressed. The term "xylose isomerase activity" refers to activity of isomerizing xylose into xylulose. Accordingly, xylose isomerase activity can be evaluated by preparing a xylose-containing solution as a substrate, allowing the target protein to react at an adequate temperature, and measuring the amount of xylose that has decreased

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and/or the amount of xylulose that has been generated.

[0077]

In particular, a xylose isomerase gene preferably comprises an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 50 by introduction of a particular mutation into a particular amino acid residue and encodes a mutant xylose isomerase with improved xylose isomerase activity. A specific example of a gene encoding a mutant xylose isomerase is a gene encoding an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 50 by substitution of asparagine with cysteine in the 337th position. Xylose isomerase activity of such mutant xylose isomerase is superior to that of wild-type xylose isomerase. In addition, mutant xylose isomerase is not limited to the xylose isomerase resulting from substitution of asparagine with cysteine in the 337th position. It may be xylose isomerase resulting from substitution of asparagine with an amino acid residue other than cysteine in the 337th position, xylose isomerase resulting from substitution of an amino acid residue at a position different from the 337th position, in addition to substitution of asparagine in the 337th position, or xylose isomerase resulting from substitution of an amino acid residue other than cysteine in the 337th position.

[0078]

Meanwhile, examples of xylose metabolism-associated genes other than the xylose isomerase gene include a xylose reductase gene encoding a xylose reductase that converts xylose into xylitol, a xylitol dehydrogenase gene encoding a xylitol dehydrogenase that converts xylitol into xylulose, and a xylulokinase gene encoding a xylulokinase that phosphorylates xylulose to produce xylulose 5-phosphate. Xylulose 5-phosphate produced by a xylulokinase enters the pentose phosphate pathway, and it is then metabolized therein.

[0079]

Examples of xylose metabolism-associated genes include, but are not particularly limited to, a xylose reductase gene and a xylitol dehydrogenase gene derived from *Pichia stipitis* and a xylulokinase gene derived from *Saccharomyces cerevisiae* (see Eliasson A. et al., Appl. Environ. Microbiol., 66: 3381-3386; and Toivari M. N. et al., Metab. Eng., 3: 236-249). In addition, xylose reductase genes derived from *Candida tropicalis* and *Candida prapsilosis*,

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xylitol dehydrogenase genes derived from *Candida tropicalis* and *Candida prapsilosis*, and a xylulokinase gene derived from *Pichia stipitis* can be used.

[0080]

Examples of yeast strains that inherently have xylose-metabolizing ability include, but are not particularly limited to, *Pichia stipitis*, *Candida tropicalis*, and *Candida prapsilosis*.

[0081]

The mutant yeast strain according to the present invention may further comprise other gene(s) introduced thereinto, and such other gene(s) are not particularly limited. For example, a gene involved in the sugar metabolism of glucose may be introduced into such mutant yeast strain. For example, a mutant yeast strain can have β-glucosidase activity resulting from the introduction of the β-glucosidase gene.

[0082]

The term "β-glucosidase activity" used herein refers to the activity of catalyzing a hydrolysis reaction of a β-glycoside bond of a sugar. Specifically, β-glucosidase is capable of degrading a cellooligosaccharide, such as cellobiose, into glucose. The β-glucosidase gene can be introduced in the form of a cell-surface display gene. The term "cell-surface display gene" used herein refers to a gene that is modified to display a protein to be encoded by the gene on a cell surface. For example, a cell-surface display β-glucosidase gene results from fusion of a β-glucosidase gene with a cell-surface localized protein gene. A cell-surface localized protein is fixed and present on a yeast cell surface layer. Examples include agglutinative proteins, such as α- or a-agglutinin and FLO proteins. In general, a cell-surface localized protein comprises an N-terminal secretory signal sequence and a C-terminal GPI anchor attachment signal sequence. While a cell-surface localized protein shares properties with a secretory protein in terms of the presence of a secretory signal, its secretory signal differs in that the cell-surface localized protein is transported while fixed to a cell membrane through a GPI anchor. When a cell-surface localized protein passes through a cell membrane, a GPI anchor attachment signal sequence is selectively cut, it binds to a GPI anchor at a newly protruded C-terminal region, and it is then fixed to the cell membrane. Thereafter, the root of the GPI anchor is cut by phosphatidylinositol-dependent phospholipase C (PI-PLC). Subsequently, a protein separated from the cell membrane is integrated into a cell wall, fixed

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onto a cell surface layer, and then localized on a cell surface layer (see, for example, JP 2006-174767 A).

[0083]

The β-glucosidase gene is not particularly limited, and an example is a β-glucosidase gene derived from *Aspergillus aculeatus* (Murai et al., Appl. Environ. Microbiol., 64: 4857-4861). In addition, a β-glucosidase gene derived from *Aspergillus oryzae*, a β-glucosidase gene derived from *Clostridium cellulovorans*, and a β-glucosidase gene derived from *Saccharomycopsis fibligera* may be used.

[0084]

In addition to or other than the β-glucosidase gene, a gene encoding another cellulase-constituting enzyme may have been introduced into the mutant yeast strain according to the present invention. Examples of cellulase-constituting enzymes other than β-glucosidase include exo-cellobiohydrolases that liberate cellobiose from the terminus of crystalline cellulose (CBH1 and CBH2) and endo-glucanase (EG) that cannot degrade crystalline cellulose but cleaves a non-crystalline cellulose (amorphous cellulose) chain at random.

[0085]

A particular example of another gene to be introduced into a mutant yeast strain is a gene capable of promoting the use of xylose in a medium. A further specific example thereof is a gene encoding xylulokinase having activity of generating xylulose-5-phosphate using xylulose as a substrate. The metabolic flux of the pentose phosphate pathway can be improved through the introduction of the xylulokinase gene.

[0086]

Further, a gene encoding an enzyme selected from the group of enzymes constituting a non-oxidative process in the pentose phosphate pathway can be introduced into the mutant yeast strain according to the present invention. Examples of enzymes constituting a non-oxidative process in the pentose phosphate pathway include ribose-5-phosphate isomerase, ribulose-5-phosphate-3-epimerase, transketolase, and transaldolase. It is preferable that one or more genes encoding such enzymes be introduced, more preferable that two or more such

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genes be introduced in combination, further preferable that three or more genes be introduced in combination, and the most preferable that all of the genes above be introduced.

[0087]

More specifically, the xylulokinase (XK) gene of any origin can be used without particular limitation. A wide variety of microorganisms, such as bacterial and yeast strains, which assimilate xylulose, possess the XK gene. Information concerning XK genes can be obtained by searching the website of NCBI or other institutions, according to need. Preferable examples of such genes include the XK genes derived from yeast strains, lactic acid bacteria, *E. coli* bacteria, and plants. An example of an XK gene is XKS1, which is an XK gene derived from the *S. cerevisiae* S288C strain (GenBank: Z72979) (the nucleotide sequence and the amino acid sequence in the CDS coding region).

[0088]

More specifically, a transaldolase (TAL) gene, a transketolase (TKL) gene, a ribulose-5-phosphate epimerase (RPE) gene, and a ribose-5-phosphate ketoisomerase (RKI) gene of any origin can be used without particular limitation. A wide variety of organisms comprising the pentose phosphate pathway possess such genes. For example, a common yeast strain such as *S. cerevisiae* possesses such genes. Information concerning such genes can be obtained from the website of NCBI or other institutions, according to need. Genes belonging to the same genus as the host eukaryotic cells, such as eukaryotic or yeast cells, are preferable, and genes originating from the same species as the host eukaryotic cells are more preferable. A TAL1 gene, a TKL1 gene and a TKL2 gene, an RPE1 gene, and an RKI1 gene can be preferably used as the TAL gene, the TKL genes, the RPE gene, and the RKI gene, respectively. Examples of such genes include a TAL1 gene derived from the *S. cerevisiae* S288 strain (GenBank: U19102), a TKL1 gene derived from the *S. cerevisiae* S288 strain (GenBank: X73224), an RPE1 gene derived from the *S. cerevisiae* S288 strain (GenBank: X83571), and an RKI1 gene derived from the *S. cerevisiae* S288 strain (GenBank: Z75003).

[0089]

When the mutant genes or the xylose metabolism-associated gene are to be introduced into a yeast strain, such genes may be simultaneously introduced thereinto, or such genes may be successively introduced with the use of different expression vectors.

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[0090]

Examples of host yeast strains that can be used include, but are not particularly limited to, *Candida Shehatae*, *Pichia stipitis*, *Pachysolen tannophilus*, *Saccharomyces cerevisiae*, and *Schizosaccaromyces pombe*, with *Saccharomyces cerevisiae* being particularly preferable. Experimental yeast strains may also be used from the viewpoint of experimental convenience, or industrial (practical) strains may also be used from the viewpoint of practical usefulness. Examples of industrial strains include yeast strains used for the production of wine, *sake*, and *shochu*.

[0091]

Use of a host yeast strain having homothallic properties is preferable. According to the technique disclosed in JP 2009-34036 A, multiple copies of genes can be easily introduced into a genome with the use of a yeast strain having homothallic properties. The term "yeast strain having homothallic properties" has the same meaning as the term "homothallic yeast strain." Yeast strains having homothallic properties are not particularly limited, and any yeast strains can be used. An example of a yeast strain having homothallic properties is, but is not limited to, the *Saccharomyces cerevisiae* OC-2 train (NBRC2260). Examples of other yeast strains having homothallic properties include an alcohol-producing yeast (Taiken No. 396, NBRC0216) (reference: "*Alcohol kobo no shotokusei*" ("Various properties of alcohol-producing yeast"), Shuken Kaiho, No. 37, pp. 18-22, 1998.8), an ethanol-producing yeast isolated in Brazil and in Japan (reference: "*Brazil to Okinawa de bunri shita Saccharomyces cerevisiae yaseikabu no idengakuteki seishitsu*" ("Genetic properties of wild-type *Saccharomyces cerevisiae* isolated in Brazil and in Okinawa"), the Journal of the Japan Society for Bioscience, Biotechnology, and Agrochemistry, Vol. 65, No. 4, pp. 759-762, 1991.4), and 180 (reference: "*Alcohol Hakkoryoku no tsuyoi kobo no screening*" ("Screening of yeast having potent alcohol-fermenting ability"), the Journal of the Brewing Society of Japan, Vol. 82, No. 6, pp. 439-443, 1987.6). In addition, the HO gene may be introduced into a yeast strain exhibiting heterothallic phenotypes in an expressible manner, and the resulting strain can be used as a yeast strain having homothallic properties. That is, the term "yeast strain having homothallic properties" used herein also refers to a yeast strain into which the HO gene has been introduced in an expressible manner.

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[0092]

Promoters of genes to be introduced are not particularly limited. For example, promoters of the glyceraldehyde-3-phosphate dehydrogenase gene (TDH3), the 3-phosphoglycerate kinase gene (PGK1), and the high-osmotic pressure response 7 gene (HOR7) can be used. The promoter of the pyruvate decarboxylase gene (PDC1) is particularly preferable in terms of its high capacity for expressing target genes in a downstream region at high levels.

[0093]

Specifically, such mutant gene may be introduced into the yeast genome together with an expression-regulated promoter or another expression-regulated region. Such mutant gene may be introduced into a host yeast genome in such a manner that expression thereof is regulated by a promoter or another expression-regulated region of a gene that is inherently present therein.

[0094]

The mutant genes can be introduced into the genome by any conventional technique known as a yeast transformation technique. Specific examples include, but are not limited to, electroporation (Meth. Enzym., 194, p. 182, 1990), the spheroplast technique (Proc. Natl. Acad. Sci., U.S.A., 75, p. 1929, 1978), and the lithium acetate method (J. Bacteriology, 153, p. 163, 1983; Proc. Natl. Acad. Sci., U.S.A., 75, p. 1929, 1978; Methods in yeast genetics, 2000 Edition: A Cold Spring Harbor Laboratory Course Manual).

<Production of ethanol>

When producing ethanol with the use of the mutant yeast strain described above, ethanol fermentation is carried out by culture in a medium containing at least xylose. Specifically, a medium in which ethanol fermentation is carried out contains, as a carbon source, at least metabolizable xylose. The medium may be supplemented with another carbon source, such as glucose, in advance.

[0095]

A xylose, that is contained in a medium to be used for ethanol fermentation can be derived from a biomass. In other words, a medium to be used for ethanol fermentation may comprise a cellulosic biomass and hemicellulase that generates xylose, through

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saccharification of hemicellulose contained in a cellulosic biomass. The cellulosic biomass may have been subjected to a conventional pretreatment technique. Examples of pretreatment techniques include, but are not particularly limited to, degradation of a lignin with a microorganism and grinding of a cellulosic biomass. For example, a ground cellulosic biomass may be subjected to pretreatment, such as soaking thereof in a dilute sulfuric acid solution, alkaline solution, or ionic solution, hydrothermal treatment, or fine grinding. Thus, the efficiency of biomass saccharification can be improved.

[0096]

When producing ethanol with the use of the mutant yeast strain described above, the medium may further comprise cellulose and cellulase. In such a case, the medium contains glucose generated by the action of cellulase imposed upon cellulose. When a medium used for ethanol fermentation contains cellulose, such cellulose can be derived from a biomass. In other words, a medium used for ethanol fermentation may comprise cellulase that is capable of saccharifying cellulase contained in a cellulosic biomass.

[0097]

A saccharified solution resulting from saccharification of a cellulosic biomass may be added to the medium used for ethanol fermentation. In such a case, the saccharified solution contains remaining cellulose or cellulase and xylose derived from hemicellulose contained in a cellulosic biomass.

[0098]

As described above, the method for producing ethanol according to the present invention comprises a step of ethanol fermentation involving the use of at least xylose, as a saccharide source. According to the method for producing ethanol with the use of the mutant yeast strain according to the present invention, ethanol fermentation is followed by recovery of ethanol from the medium. Ethanol may be recovered by any conventional means without particular limitation. After the completion of the process of ethanol fermentation mentioned above, for example, a liquid layer containing ethanol is separated from a solid layer containing the recombinant yeast strain or solid matter via solid-solution separation. Thereafter, ethanol contained in a liquid layer is separated and purified by distillation, so that

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highly purified ethanol can be recovered. The degree of ethanol purification can be adequately determined in accordance with the purpose of use of the ethanol.

[0099]

The method for producing ethanol according to the present invention may employ the so-called simultaneous saccharification and fermentation process in which the step of saccharification of cellulose contained in a medium with a cellulase proceeds simultaneously with the step of ethanol fermentation involving the use of saccharide sources; i.e., xylose and glucose generated by saccharification. With the simultaneous saccharification and fermentation process, the step of saccharification of a cellulosic biomass is carried out simultaneously with the process of ethanol fermentation.

[0100]

Methods of saccharification are not particularly limited. For example, an enzymatic method involving the use of a cellulase preparation, such as cellulase or hemicellulase, may be employed. A cellulase preparation contains a plurality of enzymes involved in degradation of a cellulose chain and a hemicellulose chain, and it exhibits a plurality of types of activity, such as endoglucanase activity, endoxylanase activity, cellobiohydrolase activity, glucosidase activity, and xylosidase activity. Cellulase preparations are not particularly limited, and examples include cellulases produced by *Trichoderma reesei* and *Acremonium cellulolyticus*. Commercially available cellulase preparations may also be used.

[0101]

In the simultaneous saccharification and fermentation process, a cellulase preparation and the recombinant microorganism are added to a medium containing a cellulosic biomass (a biomass after pretreatment may be used), and the recombinant yeast strain is cultured at a given temperature. Culture may be carried out at any temperature without particular limitation, and the temperature may be 25°C to 45°C and preferably 30°C to 40°C, from the viewpoint of ethanol fermentation efficiency. The pH level of the culture solution is preferably 4 to 6. Agitation or shake culture may be employed. Alternatively, the simultaneous saccharification and fermentation process may be carried out irregularly in such a manner that saccharification is first carried out at an optimal temperature for an enzyme

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(40°C to 70°C), temperature is lowered to a given level (30°C to 40°C), and a yeast strain is then added thereto.

[0102]

The method for producing ethanol according to the present invention involves the use of the mutant yeast strain comprising the mutant gene described above. In comparison with the use of a xylose-metabolizing enzyme that does not comprise a mutant gene, accordingly, ethanol of higher concentration can be produced. More specifically, the mutant yeast strain comprising the mutant gene described above has an ability of producing ethanol from xylose via fermentation that has been improved to a significant extent. With the use of such mutant yeast strain, accordingly, ethanol productivity can be improved.

EXAMPLES

[0103]

Hereafter, the present invention is described in greater detail with reference to the examples, although the technical scope of the present invention is not limited to these examples.

[Method for producing xylose-assimilating yeast strain]

In accordance with the method disclosed in JP 2014-193152 A, a yeast strain Uz736 having xylose-metabolizing ability was prepared. The detail is described below.

[0104]

At the outset, uracil auxotrophic strains (OC2-U) of the homothallic wine yeast *Saccharomyces cerevisiae* OC2 strain were obtained via UV-induced mutation. While disrupting the ribosomal RNA gene locus, the HIS3 gene locus, the LEU2 gene locus, the TRP1 gene locus, and the GRE3 gene locus of the OC2-U strain, a xylose isomerase (XI) gene derived from the intestinal protozoa of *Reticulitermes speratus*, a yeast-derived TAL1 gene, a yeast-derived TKL1 gene, a yeast-derived PRE1 gene, a yeast-derived RKI1 gene, and a yeast-derived XKS1 gene were introduced thereinto with the use of marker genes (i.e., hygromycin-resistant gene, HIS3 gene, LEU2 gene, URA3 gene, and TRP1 gene) to produce the OC700 strain. While disrupting the ADH2 gene, the *E. coli*-derived acetaldehyde dehydrogenase (mhpF) gene and a yeast-derived ADH1 gene were introduced into the ADH2 gene locus of the OC700 strain. Thus, the Uz736 strain was produced.

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[Method for breeding xylose-assimilating yeast strain]

Subsequently, the Uz736 strain was subjected to long-term culture to breed yeast strains with improved ethanol fermentation ability. At the outset, the Uz736 strain was subjected to long-term culture for 30 to 60 days in a liquid medium prepared by biomass saccharification. The cultured yeast was inoculated into a YPD agar medium (10 g/l dry yeast extract, 20 g/l bactopeptone, 20 g/l glucose, and 20 g/l agar) to obtain single colonies. The ethanol fermentation ability of the single colonies was evaluated to obtain a breeding yeast with improved ethanol fermentation ability.

[0105]

Specifically, the single colonies were inoculated into a YPD agar medium (10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose) and subjected to shake culture (80 rpm, amplitude 35 mm, 30°C) or stationary culture at 30°C for 24 hours. Thereafter, the single colonies were separately inoculated into various media for ethanol production of different components and then subjected to shake culture (80 rpm, amplitude 35 mm, 30°C) or stationary culture in an incubator at 31°C to perform the fermentation test. The inside of the reaction vessel was maintained in the anaerobic condition.

[0106]

Ethanol in a fermentation liquor was assayed using a biosensor (BF-5, Oji Scientific Instruments) or via HPLC (LC-10A, Shimadzu Corporation) under the conditions described below.

[Biosensor]

Temperature: 37°C

Flow rate: 0.8 ml/min

[HPLC]

Column: AminexHPX-87H

Mobile phase: 0.01 N H_2SO_4

Flow rate: 0.6 ml/min

Temperature: 30°C

Detector: Differential refractometer (RID-10A)

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Subsequently, yeast strains with the improved fermentation ability were cultured in a sporulation medium at 25°C for 5 days and then recovered. The recovered yeast strains were treated with a 1 ml of a reaction solution comprising 125 U of zymolyase in 50 mM phosphate buffer (pH 7.5) for 2 hours for cell wall lysis. Thereafter, Tween 80 was added to a concentration of 1% therein, the resultant was vigorously stirred to separate spores from each other, and the separated spores were inoculated in an agar medium to form single colonies. The yeast single colonies were repeatedly subjected to long-term culture to formation of single colonies. Thus, 4 types of breeding strains; i.e., Uz1015, Uz1229, Uz1230, and Uz1235, with the improved ethanol fermentation ability were obtained.

[Method of mutation analysis]

OC700 and Uz736 used in the example and Uz1015, Uz1229, Uz1230, and Uz1235 produced in the example were subjected to next-generation sequence analysis (Hiseq) (Takara Bio Inc.). The obtained sequence data were analyzed in terms of the sites of mutation using analytical software (NextGENe, SoftGenetics). The gene sequence data of *Saccharomyces cerevisiae* S288C were used for reference, and default settings of analytical parameters were employed. The obtained data of mutations were compared, and 6 types of gene mutations that were common between Uz1230 and Uz1235 with excellent ethanol fermentation ability and were not present in other 4 types of genes were identified.

[0107]

Specifically, a mutation causing substitution of leucine 324 with cysteine in the CDC123 gene (L324C), G704E in the FPK1 gene, S112T in the SUI3 gene, V195* in the YPR1 gene, G599D in the EPO1 gene, and G328E in the YPL150w gene were identified.

[Method of producing mutant yeast]

In the same manner as with the case of the Uz736 strain described above, a xylose isomerase (XI) gene derived from the intestinal protozoa of *Reticulitermes speratus* and a yeast-derived XKS1 gene were introduced into the laboratory yeast strain *Saccharomyces cerevisiae* BY4742 with the use of the marker genes (i.e., hygromycin-resistant gene and URA3d gene) while disrupting the ribosomal RNA gene locus and the GRE3 gene locus thereof. Thus, the Uz2443 strain having xylose-metabolizing ability was produced. Six types of plasmids necessary for introduction of mutations into the CDC123 gene, the FPK1 gene,

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the SUI3 gene, the YPR1 gene, the EPO1 gene, and the YPL150w gene of the Uz2443 strain were prepared.

[0108]

Specifically, the genome of the Uz1230 strain as a template was amplified via PCR, so that the resultant would comprise an upstream 500-bp region and a downstream 500-bp region of ORF of the relevant mutant gene.

[0109]

More specifically, the genome of the Uz2443 strain as a template was amplified with the use of the primers shown in Table 4. Thus, the CDC123 gene, the FPK1 gene, the SUI3 gene, the YPR1 gene, the EPO1 gene, and the YPL150w gene into which mutations had been introduced were amplified. The amplified fragments were cloned into vectors comprising hygromycin-resistant genes to prepare 6 types of vectors.

[0110]

[Table 4]

Primer name	Sequence	SEQ ID NO:
V_CDC123 INF	CTGACTTGAGCGTCGAAGATTACAAGCAAGTATTAGTAGCCTC	51
V_CDC123 INR	CTATACAGCGGAATTCCCATTTGAAATGGTTTGAAAATGAATT	52
V_FPK1 INF	CTGACTTGAGCGTCGCCATCTTCGATCCAGGAGCTCACCGATG	53
V_FPK1 INR	CTATACAGCGGAATTGCCGGTTTCCTGGATTTTTGAGCATTTTGC	54
V_SUI3 INF	CTGACTTGAGCGTCGGTGACTTGTTCAATTTCTGTACCCTTTG	55
V_SUI3 INR	CTATACAGCGGAATTGATATTTGGTCTTTGGGTTGTACGTTCT	56
V_YMR124w INF	CTGACTTGAGCGTCGTGCCCTCCTAATTTTTTTTTTTTTTAGT	57
V_YMR124w INR	CTATACAGCGGAATTATAATCCTAGGAATGTAAAACAAAGTAA	58
V_YPL150w INF	CTGACTTGAGCGTCGTGAGCACCCTTACTTAATAAAAGAGTTG	59
V_YPL150w INR	CTATACAGCGGAATTGACTTCCTTTCATCAAAAATGAAGGATC	60
V_YRP1 INF	CTGACTTGAGCGTCGGACTATTTTAATTACGTTGGTGTCATTG	61
V_YRP1 INR	CTATACAGCGGAATTAGATTCGTTTTCTTTTTCTCGTTGTTCA	62

[0111]

In order to knock out the CDC123 gene, the FPK1 gene, the SUI3 gene, the YPR1 gene, the EPO1 gene, and the YPL150w gene endogenous in the Uz2443 strain and separately introduce the mutant CDC123, FPK1, SUI3, YPR1, EPO1, and YPL150w genes into the Uz2443 strain, PCR was carried out with the use of the primers shown in Table 5 and the prepared vectors as templates. The 6 types of vectors were linearized. The linearized vectors were each transformed into the Uz2443 strain, and yeast strains grown on a hygromycin-

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containing selection medium were subjected to screening. As a result, 6 types of mutant yeast strains were obtained by introducing the relevant mutations into the Uz2443 strain.

[0112]

[Table 5]

Primer name	Sequence	SEQ ID NO:
CDC123d_F	ATACCAGTGACAAGAGAGCAGGTTGAACAC	63
CDC123_R	GTCTATAAAAGTTGTTTATTCTTGTGAGG	64
FPK1d_F	CGACCACGAGCAAGAACACGAACACGATTC	65
FPK1_R	CGCTCTTATTCATGTTCGTGATGGTGTCC	66
SUI3d_F	CTACACTAAAGAAGAAAAAGAAGACTAAAA	67
SUI3_R	GGTCGAATCCTAACTAAGCAGCTAAATCGG	68
YMR124wd_F	TAAGCAATAATCGCGATAATGTTAATGGTA	69
YMR124w_R	GTGATGGTTAGGTGAAGTTATGCTGCATG	70
YPL150wd_F	AATATAAAAAGCATTATAGGATCATCGTAC	71
YPL150w_R	GTTTTGTTCCAATTACGAAGATCCAACAGG	72
YPR1d_F	TACATTAAAACTAAATACTGGTGCCTCCAT	73
YPR1_R	GCAGAAGAATTCTTTTACGTAGCAGGCATG	74

[0113]

[Evaluation of mutant gene]

One platinum loopful each of 6 types of mutant yeast strains having the xylose-metabolizing ability each comprising a relevant mutant gene among the 6 types of the mutant genes and a yeast strain having xylose-metabolizing ability into which no mutation had been introduced was fractionated from an agar medium and subjected to shake culture in a triangular flask containing 8 ml of YPD medium (10 g/l dry yeast extract, 20 g/l bactopeptone, and 20 g/l glucose) at 32°C and 150 rpm for 24 hours. Thereafter, the initial PCVs of the yeast strains were adjusted to 0.12 and subjected to shake culture in 8 ml of the medium (80 g/l glucose, 100 g/l xylose, 0.3 g/l vanillin, 0.2 g/l syringaldehyde, 10 g/l acetic acid, 0.8 g/l furfural, and 10 g/l dry yeast extract) at 35°C and 80 rpm for 90 hours.

[0114]

After the completion of culture, ethanol concentration was analyzed via HPLC (column: AminexHPX-87H; mobile phase: 0.01 N H_2SO_4; flow rate: 0.6 ml/min; temperature: 30°C; detector: differential refractometer RID-10A). The results are shown in Fig. 1. As shown in Fig. 1, L324C in the CDC123 gene, G704E in the FPK1 gene, and S112T in the SUI3 gene among the 6 type of mutant genes were found to improve the ethanol fermentation ability. In contrast, it was found that other mutant genes identified in the example would not

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improve or would slightly improve the ethanol fermentation ability. Thus, L324C in the CDC123 gene, G704E in the FPK1 gene, and S112T in the SUI3 gene were found to be excellent mutations to achieve the improved ethanol fermentation ability.

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Claims

1. A mutant gene encoding a mutant CDC123 (Cell Division Cycle 123) protein comprising:

(a) an amino acid sequence as shown in SEQ ID NO: 3; or

(b) an amino acid sequence having 95% or higher identity to the amino acid sequence of SEQ ID NO: 3 in which an amino acid residue corresponding to the 324th position from the N terminus of the amino acid sequence of SEQ ID NO: 3 is cysteine.

2. A mutant yeast strain having xylose-metabolizing ability, which comprises the mutant gene according to claim 1.

3. A method for producing ethanol comprising a step of culturing the mutant yeast strain according to claim 2 in a xylose-containing medium and performing ethanol fermentation.

4. The method for producing ethanol according to claim 3, wherein the medium contains cellulose and the ethanol fermentation proceeds simultaneously at least with the cellulose saccharification.

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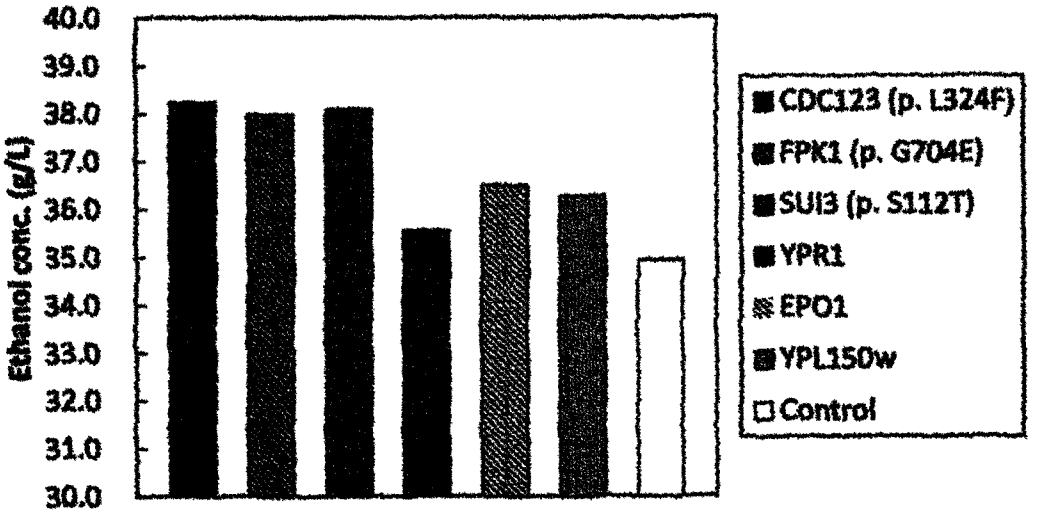
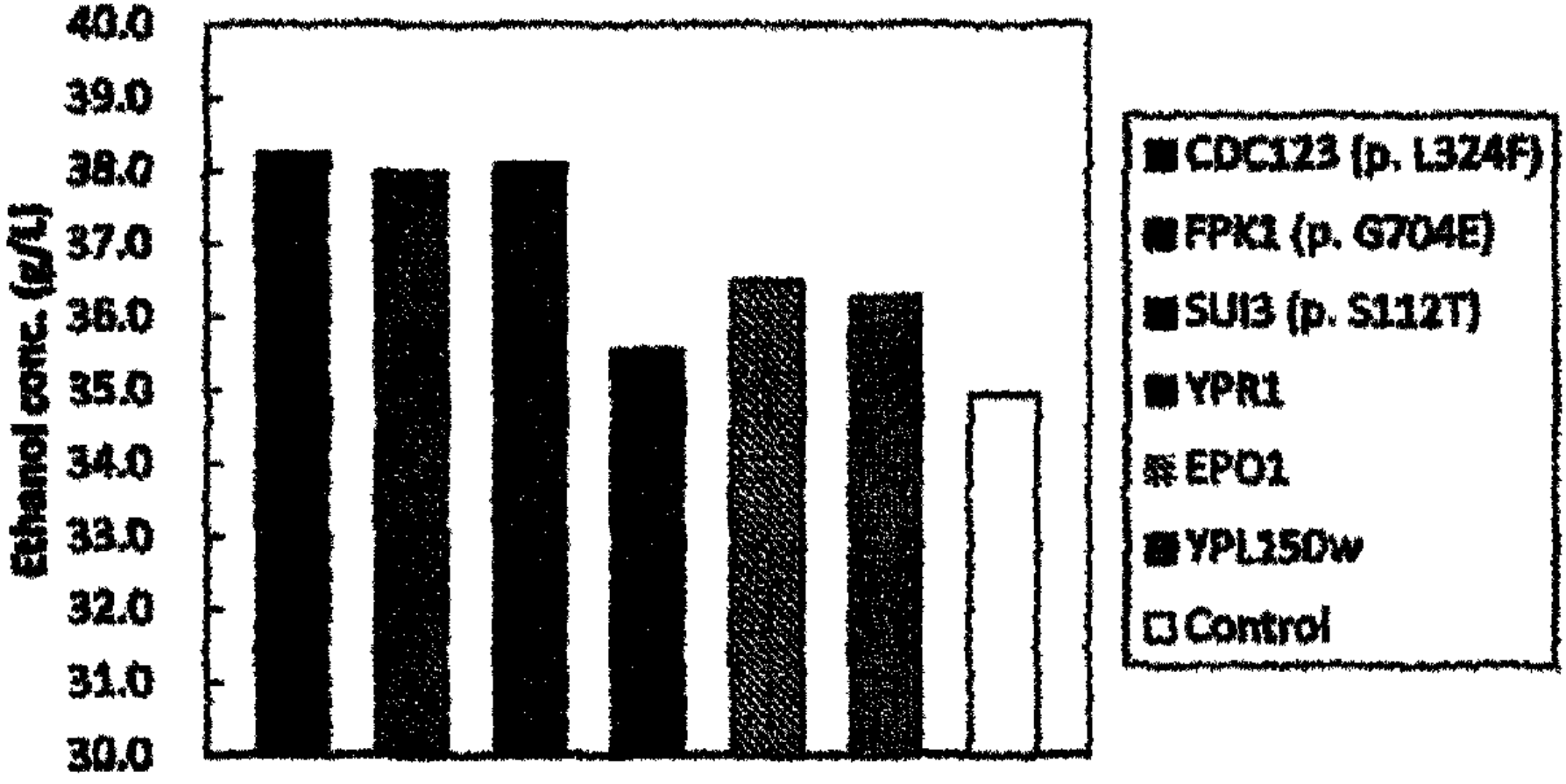
Fig. 1


40.0
39.0
38.0
37.0
36.0
35.0
34.0
33.0
32.0
31.0
30.0
Ethanol conc. (g/L)
CDC123 (p. L324F)
FPK1 (p. G704E)
SUI3 (p. S112T)
YPR1
EPO1
YPL150w
Control


40.0
39.0
38.0
37.0
36.0
35.0
34.0
33.0
32.0
31.0
30.0
Ethanol conc. (g/L)
CDC123 (p. L324F)
FPK1 (p. G704E)
SUI3 (p. S112T)
YPR1
EPO1
YPL150w
Control