



(51) International Patent Classification:

C12N 9/04 (2006.01) C12P 7/06 (2006.01)
C12N 9/92 (2006.01)

(21) International Application Number:

PCT/JP2014/003069

(22) International Filing Date:

9 June 2014 (09.06.2014)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

2013-124755 13 June 2013 (13.06.2013) JP

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: RECOMBINANT YEAST AND METHOD FOR PRODUCING ETHANOL USING THE SAME

(57) Abstract: The invention is intended to improve xylose assimilation ability and ethanol fermentation ability in a xylose-assimilating yeast into which a xylose isomerase gene has been introduced. The amount of NADH produced by the recombinant yeast into which the xylose isomerase gene had been introduced as a result of the enzymatic reaction of acetohydroxy acid reductoisomerase is lowered.



Description

Title of Invention: RECOMBINANT YEAST AND METHOD FOR PRODUCING ETHANOL USING THE SAME

Technical Field

[0001] The present invention relates to a recombinant yeast having xylose-metabolizing ability and a method for producing ethanol using the same.

Background Art

[0002] Major saccharides contained in lignocellulose are glucose that constitutes cellulose and xylose that constitutes hemicellulose. By chemically or enzymatically degrading lignocellulose, a saccharified composition mainly composed of such a monosaccharide can be obtained. Production of useful materials from lignocellulose at the industrial level necessitates microorganisms that are capable of effective utilization of saccharides contained in such saccharified compositions and fermentation of such useful substances with high yield and high productivity.

[0003] In general, yeasts with high ethanol fermentation ability, such as *Saccharomyces cerevisiae*, are capable of utilizing glucose, mannose, or galactose, although such yeasts are not capable of utilizing xylose. In order to perform fermentation using lignocellulose as a starting material with high efficiency, accordingly, it is necessary that such yeasts be modified to be capable of using xylose.

[0004] For example, production of recombinant *S. cerevisiae* capable of xylose utilization has been attempted (Patent Literature 1 and 2; Non-Patent Literature 1). Patent Literature 1 and Non-Patent Literature 1 each report improvement in ethanol yield and xylose utilization of a yeast to which xylose assimilability has been imparted through introduction of genes encoding xylose reductase (XR) and xylitol dehydrogenase (XDH) from different microorganisms. Such literature reports that the phosphoketolase (PK) pathway is potentiated and NADH is consumed by acetaldehyde dehydrogenase, in order to consume excessive NADH generated by introduction of a xylose assimilation pathway caused by XR and XDH in the subsequent reaction.

[0005] Patent Literature 2 reports the use of xylose isomerase (XI), which is an isomerase that converts xylose into xylulose. When XI is used, excessive NADH is not generated. That is, the glycolytic pentose phosphate pathway (PPP) is used without any processing, and the phosphoketolase pathway is not potentiated in the technique according to Patent Literature 2.

[0006] While NADH may be excessively consumed as a result of introduction of the acetaldehyde dehydrogenase gene into a xylose-assimilating yeast into which the XI gene had been introduced, the improvement of xylose assimilation as a result of introduction

of such gene has also been reported (Patent Literature 3).

Citation List

Patent Literature

- [0007] PTL 1: WO 2003/078643
PTL 2: JP 2005-514951 A
PTL 3: JP 2010-239925 A

Non Patent Literature

- [0008] NPL 1: Sonderegger M, Schumperli M, Sauer U. 2004, Metabolic engineering of a phosphoketolase pathway for pentose catabolism in *Saccharomyces cerevisiae*, Appl. Environ. Microbiol., 70 (5): 2892-2897

Summary of Invention

Technical Problem

- [0009] However, the xylose-assimilating yeast into which the XI gene had been introduced was not sufficient in terms of ethanol fermentation ability; that is, such strain was not sufficient in terms of ethanol production efficiency. Under the above circumstances, it is an object of the present invention to provide a xylose-assimilating yeast that is particularly excellent in terms of xylose assimilation ability and ethanol fermentation ability. It is another object of the present invention to provide a method for producing ethanol that results in an excellent ethanol yield with the use of such yeast.

Solution to Problem

- [0010] The present inventors have conducted concentrated studies in order to attain the above objects. As a result, they discovered that xylose assimilation ability and ethanol yield could be improved by decreasing the amount of NADH produced by an enzymatic reaction involving acetohydroxy acid reductoisomerase in a yeast having xylose metabolizing ability. This has led to the completion of the present invention.

The present invention is as follows.

(1) A recombinant yeast into which the xylose isomerase gene has been introduced, wherein a production of NADH as a result of the enzymatic reaction of acetohydroxy acid reductoisomerase is lowered.

(2) The recombinant yeast according to (1), wherein activity of endogenous acetohydroxy acid reductoisomerase is lowered.

(3) The recombinant yeast according to (1), wherein the expression level of the endogenous gene encoding acetohydroxy acid reductoisomerase is lowered.

(4) The recombinant yeast according to (3), wherein the endogenous gene is disrupted.

(5) The recombinant yeast according to (4), wherein the endogenous genes are hetero-disrupted.

(6) The recombinant yeast according to (1) into which a gene encoding mutant acetohydroxy acid reductoisomerase with lowered NAD^+ dependence and enhanced NADP^+ dependence is introduced.

(7) The recombinant yeast according to (3), wherein the endogenous gene encoding acetohydroxy acid reductoisomerase encodes the protein (a) or (b) below:

(a) the protein comprising the amino acid sequence as shown in SEQ ID NO: 2; or

(b) the protein comprising an amino acid sequence having 70% or higher identity with the amino acid sequence as shown in SEQ ID NO: 2 and having enzymatic activity of converting 2-acetolactate and NAD^+ into 2,3-dihydroxy-isovalerate and NADH , respectively.

(8) The recombinant yeast according to (6), wherein the gene encoding mutant acetohydroxy acid reductoisomerase encodes the protein (a) or (b) below:

(a) the protein comprising the amino acid sequence as shown in SEQ ID NO: 4 or 6; or

(b) the protein comprising an amino acid sequence having 70% or higher identity with the amino acid sequence as shown in SEQ ID NO: 4 or 6 and having enzymatic activity of converting 2-acetolactate and NADP^+ into 2,3-dihydroxy-isovalerate and NADPH , respectively.

(9) The recombinant yeast according to (1), wherein the xylose isomerase gene encodes the protein (a) or (b) below:

(a) the protein comprising the amino acid sequence as shown in SEQ ID NO: 8; or

(b) the protein comprising an amino acid sequence having 70% or higher identity with the amino acid sequence as shown in SEQ ID NO: 8 and having enzymatic activity of converting xylose into xylulose.

(10) A method for producing ethanol comprising a step of culturing the recombinant yeast according to any of (1) to (9) in a xylose-containing medium to perform ethanol fermentation.

Advantageous Effects of Invention

[0011] The recombinant yeast of the present invention is excellent in terms of the ability to assimilate xylose in a medium and efficiency for producing ethanol from xylose. With the use of the recombinant yeast of the present invention, accordingly, the ethanol yield in a xylose-containing medium can be remarkably improved.

[0012] According to the method for producing ethanol of the present invention, efficiency for ethanol fermentation using xylose in a medium as a saccharide source can be maintained at a high level, and an excellent ethanol yield can be achieved.

Brief Description of Drawings

[0013] [fig.1] Fig. 1 is a characteristic diagram showing part of the pathway of valine/leucine biosynthesis.

[fig.2]Fig. 2 is a structural diagram schematically showing pUC-GRE3U-P_TDH1-XI-T_CYC1-P_TDH3-XKS1-T_HIS3-LoxP-G418-LoxP-GRE3D.

[fig.3]Fig. 3 is a structural diagram schematically showing pUC-ADH2part-T_CYC1-P_TDH3-M_DLD2-ilvC-mutant T_ACT1-TRP1-3U_ADH2.

[fig.4]Fig. 4 is a structural diagram schematically showing pUC-ILV5U-TRP1-ILV5D.

[fig.5]Fig. 5 is a structural diagram schematically showing pUC-ADH2part-T_CYC1-TRP1-ADH2D.

Description of Embodiments

[0014] Hereafter, the present invention is described in greater detail with reference to the drawings and the examples.

<Recombinant yeast>

The recombinant yeast of the present invention is obtained by introducing the xylose isomerase gene, in which a production of NADH (i.e., the reduced nicotinamide adenine dinucleotide) as a result of the enzymatic reaction of acetohydroxy acid reductoisomerase is lowered.

[0015] The recombinant yeast into which the xylose isomerase gene has been introduced is a recombinant yeast in which the xylose isomerase gene functions. When the xylose isomerase gene functions, the xylose isomerase gene that has been introduced is transcribed and translated, so as to express the xylose isomerase having enzymatic activity.

[0016] The term "the recombinant yeast into which the xylose isomerase gene has been introduced" refers to any of the following: a recombinant yeast that has acquired xylose-metabolizing ability as a result of introduction of a xylose isomerase gene into a yeast that does not inherently have xylose-metabolizing ability; a recombinant yeast that has acquired xylose-metabolizing ability as a result of introduction of a xylose isomerase gene and another xylose metabolism-associated gene into a yeast that does not inherently have xylose-metabolizing ability; and a recombinant yeast that has enhanced xylose-metabolizing ability as a result of introduction of a xylose isomerase gene into a yeast that inherently has xylose-metabolizing ability.

[0017] The recombinant yeast of the present invention is capable of assimilating xylose contained in a medium to produce 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 saccharification enzyme. The latter case is the so-called "simultaneous saccharification and fermentation system."

In the recombinant yeast of the present invention, the amount of NADH produced as

a result of the enzymatic reaction of acetohydroxy acid reductoisomerase is lowered. As shown in Fig. 1, acetohydroxy acid reductoisomerase is an enzyme having activity of converting 2-acetolactate and NAD^+ into 2,3-dihydroxy-isovalerate and NADH , respectively, in the pathway of valine/leucine biosynthesis (i.e., the enzyme indicated as "ILV5" in Fig. 1).

[0018] In order to lower the amount of NADH produced as a result of the enzymatic reaction of acetohydroxy acid reductoisomerase, for example, activity of acetohydroxy acid reductoisomerase inherent in the recombinant yeast may be lowered, or the expression level of acetohydroxy acid reductoisomerase gene inherent in the recombinant yeast may be lowered. Alternatively, a mutant acetohydroxy acid reductoisomerase gene having lowered NAD^+ dependence and enhanced NADP^+ dependence may be introduced. That is, expression of mutant acetohydroxy acid reductoisomerase encoded by the mutant acetohydroxy acid reductoisomerase gene leads to relatively-lowering in activity of endogenous acetohydroxy acid reductoisomerase. Thus, the amount of NADH produced by endogenous acetohydroxy acid reductoisomerase is decreased, and the amount of NADPH (reduced nicotinamide adenine dinucleotide phosphate) produced by mutant acetohydroxy acid reductoisomerase is increased as a consequence. In Fig. 1, mutant acetohydroxy acid reductoisomerase is indicated as "IilvC" (NADP -dependent).

[0019] In order to lower the activity level of acetohydroxy acid reductoisomerase inherent in the recombinant yeast, for example, a substance that inhibits activity of acetohydroxy acid reductoisomerase or an antibody that neutralizes activity of acetohydroxy acid reductoisomerase may be allowed to coexist with acetohydroxy acid reductoisomerase. In order to lower the expression level of acetohydroxy acid reductoisomerase gene endogenous to the recombinant yeast, for example, a promoter of the endogenous gene may be modified, or such gene may be deleted or disrupted. Examples of techniques for suppressing gene expression include the transposon technique, the transgene technique, the post-transcriptional gene silencing technique, the RNAi technique, the nonsense mediated decay (NMD) technique, the ribozyme technique, the anti-sense technique, the miRNA (micro-RNA) technique, and the siRNA (small interfering RNA) technique. It is particularly preferable that acetohydroxy acid reductoisomerase gene endogenous to the recombinant yeast be deleted or disrupted and that one of the alleles be deleted or disrupted.

[0020] The term "endogenous acetohydroxy acid reductoisomerase gene" refers to a gene that inherently exists in the recombinant yeast of the present invention and encodes a protein having enzymatic activity of converting 2-acetolactate and NAD^+ into 2,3-dihydroxy-isovalerate and NADH , respectively. Thus, the nucleotide sequence constituting endogenous acetohydroxy acid reductoisomerase gene is not particularly

limited.

- [0021] For example, SEQ ID NOs: 1 and 2 show the nucleotide sequence of acetohydroxy acid reductoisomerase gene inherent in *Saccharomyces cerevisiae* and the amino acid sequence of acetohydroxy acid reductoisomerase encoded by such gene, respectively.
- [0022] The acetohydroxy acid reductoisomerase gene is not limited to the gene identified by SEQ ID NOs: 1 and 2. It may be a paralogous gene or a homologous gene in the narrow sense having different nucleotide and amino acid sequences.
- [0023] The acetohydroxy acid reductoisomerase gene is not limited to the gene identified by SEQ ID NOs: 1 and 2. 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 with the amino acid sequence as shown in SEQ ID NO: 2 and encoding a protein having enzymatic activity of converting 2-acetolactate and NAD⁺ into 2,3-dihydroxy-isovalerate and NADH, respectively. 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.
- [0024] Further, acetohydroxy acid reductoisomerase gene is not limited to the gene identified by SEQ ID NOs: 1 and 2. For example, it may be a gene comprising an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 2 by substitution, deletion, insertion, or addition of one or several amino acids and encoding a protein having enzymatic activity of converting 2-acetolactate and NAD⁺ into 2,3-dihydroxy-isovalerate and NADH, respectively. 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.
- [0025] Furthermore, acetohydroxy acid reductoisomerase gene is not limited to the gene identified by SEQ ID NOs: 1 and 2. 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: 1 and encoding a protein having enzymatic activity of converting 2-acetolactate

and NAD⁺ into 2,3-dihydroxy-isovalerate and NADH, respectively. Under "stringent conditions," so-called specific hybrids are formed, but non-specific hybrids are not formed. Such conditions can be adequately determined with reference to, for example, *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 degrees C to 68 degrees C and preferably 42 degrees C to 65 degrees C, for example. Further specifically, the sodium concentration is 5x SSC (83 mM NaCl, 83 mM sodium citrate), and the temperature is 42 degrees C.

[0026] As described above, whether or not a gene comprising a nucleotide sequence that differs from the sequence shown in SEQ ID NO: 1 or a gene encoding an amino acid sequence that differs from the sequence shown in SEQ ID NO: 2 would function as acetohydroxy acid reductoisomerase gene may be determined by, for example, preparing an expression vector comprising the gene of interest incorporated into an adequate site between a promoter and a terminator, transforming an *E. coli* host using such expression vector, and assaying acetohydroxy acid reductoisomerase activity of the protein expressed. The term "acetohydroxy acid reductoisomerase activity" refers to activity of converting 2-acetolactate and NAD⁺ into 2,3-dihydroxy-isovalerate and NADH, respectively. Thus, acetohydroxy acid reductoisomerase activity can be evaluated by preparing a solution containing 2-acetolactate and NAD⁺ as substrates, allowing the target protein to react at an adequate temperature, and measuring the amount of 2-acetolactate and NAD⁺ that had decreased and/or the amount of 2,3-dihydroxy-isovalerate and NADH that had been produced.

[0027] The mutant acetohydroxy acid reductoisomerase gene with lowered NAD⁺ dependence and enhanced NADP⁺ dependence is not particularly limited. For example, such gene may encode a mutant enzyme prepared by modifying amino acid residues constituting the NADPH-binding site in wild-type acetohydroxy acid reductoisomerase, so as to lower NAD⁺ dependence and enhance NADP⁺ dependence. The gene disclosed in, for example, US Patent No. 8,097,440 or *Arch. Biochem. Biophys.*, 338, pp. 83-89, 1997 can be adequately used as such mutant acetohydroxy acid reductoisomerase gene.

[0028] Specifically, the mutations R68D, K69L, K75V, and R76D may be introduced into the *E. coli*-derived acetohydroxy acid reductoisomerase, so as to lower NAD⁺ dependence and enhance NADP⁺ dependence. Also, the mutations A71S, R76D, S78D, Q110V, D146G, and G185R may be introduced into the *E. coli*-derived acetohydroxy

acid reductoisomerase, so as to lower NAD⁺ dependence and enhance NADP⁺ dependence.

[0029] More specifically, the nucleotide sequence of the *E. coli*-derived mutant acetohydroxy acid reductoisomerase gene resulting from introduction of the mutations R68D, K69L, K75V, and R76D is shown in SEQ ID NO: 3, and the amino acid sequence of mutant acetohydroxy acid reductoisomerase encoded by such gene is shown in SEQ ID NO: 4. Also, the nucleotide sequence of the *E. coli*-derived mutant acetohydroxy acid reductoisomerase gene resulting from introduction of the mutations A71S, R76D, S78D, Q110V, D146G, and G185R is shown in SEQ ID NO: 5, and the amino acid sequence of mutant acetohydroxy acid reductoisomerase encoded by such gene is shown in SEQ ID NO: 6.

[0030] The mutant acetohydroxy acid reductoisomerase gene is not limited to the gene identified by SEQ ID NOs: 3 to 6. 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 with the amino acid sequence as shown in SEQ ID NO: 4 or 6 and encoding a protein having enzymatic activity of converting 2-acetolactate and NADP⁺ into 2,3-dihydroxy-isovalerate and NADPH, respectively. An amino acid sequence having a given level of sequence similarity or identity with the amino acid sequence as shown in SEQ ID NO: 4 is required to conserve the mutations R68D, K69L, K75V, and R76D. Also, an amino acid sequence having a given level of sequence similarity or identity with the amino acid sequence as shown in SEQ ID NO: 6 is required to conserve the mutations A71S, R76D, S78D, Q110V, D146G, and G185R. The degree of sequence similarity or identity can be determined using the BLASTN or BLASTX Program equipped with the BLAST algorithm (at default settings).

[0031] 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.

[0032] Further, the mutant acetohydroxy acid reductoisomerase gene is not limited to the gene identified by SEQ ID NOs: 3 to 6. For example, it may be a gene comprising an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 4

or 6 by substitution, deletion, insertion, or addition of one or several amino acids and encoding a protein having enzymatic activity of converting 2-acetolactate and NADP⁺ into 2,3-dihydroxy-isovalerate and NADPH, respectively. 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. An amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 4 by substitution, deletion, insertion, or addition of a given number of amino acids is required to conserve the mutations R68D, K69L, K75V, and R76D. Also, an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 6 by substitution, deletion, insertion, or addition of a given number of amino acids is required to conserve the mutations A71S, R76D, S78D, Q110V, D146G, and G185R.

[0033] Furthermore, the mutant acetohydroxy acid reductoisomerase gene is not limited to the gene identified by SEQ ID NOs: 3 to 6. 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: 3 or 5 and encoding a protein having enzymatic activity of converting 2-acetolactate and NADP⁺ into 2,3-dihydroxy-isovalerate and NADPH, respectively. Under "stringent conditions," so-called specific hybrids are formed, but non-specific hybrids are not formed. Such conditions can be adequately determined with reference to, for example, 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.

[0034] Under stringent conditions, more specifically, the sodium concentration is 25 to 500 mM and preferably 25 to 300 mM, and the temperature is 42 degrees C to 68 degrees C and preferably 42 degrees C to 65 degrees C, for example. Further specifically, the sodium concentration is 5x SSC (83 mM NaCl, 83 mM sodium citrate), and the temperature is 42 degrees C. 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: 3 is required to encode an amino acid sequence that has conserved the mutations R68D, K69L, K75V, and R76D. Also, 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: 6 is required to encode an amino acid sequence that has conserved the mutations A71S, R76D, S78D, Q110V, D146G, and G185R.

[0035] As described above, whether or not a gene comprising a nucleotide sequence that

differs from the sequence shown in SEQ ID NO: 3 or 5 or a gene encoding an amino acid sequence that differs from the sequence shown in SEQ ID NO: 4 or 6 would function as the mutant acetohydroxy acid reductoisomerase gene may be determined by, for example, preparing an expression vector comprising the gene of interest incorporated into an adequate site between a promoter and a terminator, transforming an *E. coli* host using such expression vector, and assaying mutant acetohydroxy acid reductoisomerase activity of the protein expressed. The term "mutant acetohydroxy acid reductoisomerase activity" refers to activity of converting 2-acetolactate and NADP⁺ into 2,3-dihydroxy-isovalerate and NADPH, respectively. Thus, mutant acetohydroxy acid reductoisomerase activity can be evaluated by preparing a solution containing 2-acetolactate and NADP⁺ as substrates, allowing the target protein to react at an adequate temperature, and measuring the amount of 2-acetolactate and NADP⁺ that had decreased and/or the amount of 2,3-dihydroxy-isovalerate and NADPH that had been produced.

[0036] As described above, the recombinant yeast of the present invention comprises a xylose isomerase gene that has been introduced therein. 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 termites 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 another bacterial strain (i.e., *Clostridium phytofermentans*), and a gene derived from the *Streptomyces murinus* cluster.

[0037] Specifically, use of a xylose isomerase gene derived from the intestinal protozoa of *Reticulitermes speratus* as the xylose isomerase gene is preferable. 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 are shown in SEQ ID NOs: 7 and 8, respectively.

The xylose isomerase gene is not limited to the gene identified by SEQ ID NOs: 7 and 8. It may be a paralogous gene or a homologous gene in the narrow sense having different nucleotide and amino acid sequences.

[0038] The xylose isomerase gene is not limited to the gene identified by SEQ ID NOs: 7 and 8. 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 with the amino acid sequence as shown in SEQ ID NO: 8 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.

- [0039] Further, the xylose isomerase gene is not limited to the gene identified by SEQ ID NOs: 7 and 8. For example, it may be a gene comprising an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 8 by substitution, deletion, insertion, or addition of one or several amino acids and encoding a 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.
- [0040] Furthermore, the xylose isomerase gene is not limited to the gene identified by SEQ ID NOs: 7 and 8. 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: 7 and encoding a protein having xylose isomerase activity. Under "stringent conditions," so-called specific hybrids are formed, but non-specific hybrids are not formed. Such conditions can be adequately determined with reference to, for example, 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 degrees C to 68 degrees C and preferably 42 degrees C to 65 degrees C, for example. Further specifically, the sodium concentration is 5x SSC (83 mM NaCl, 83 mM sodium citrate), and the temperature is 42 degrees C.
- [0041] As described above, whether or not a gene comprising a nucleotide sequence that differs from the sequence shown in SEQ ID NO: 7 or a gene encoding an amino acid sequence that differs from the sequence shown in SEQ ID NO: 8 would function as the

xylose isomerase gene may be determined by, for example, preparing an expression vector comprising the gene of interest incorporated into an adequate site between a promoter and a terminator, transforming an *E. coli* host using such expression vector, and assaying xylose isomerase activity of the protein expressed. The term "xylose isomerase activity" refers to activity of isomerizing xylose into xylulose. Thus, xylose isomerase activity can be evaluated by preparing a solution containing xylose as a substrate, allowing the target protein to react at an adequate temperature, and measuring the amount of xylose that had decreased and/or the amount of xylulose that had been produced.

[0042] As described above, another xylose metabolism-associated gene may be introduced into the recombinant yeast of the present invention, in addition to the xylose isomerase gene. The xylose metabolism-associated gene other than the xylose isomerase gene may be the xylose reductase gene encoding xylose reductase that converts xylose into xylitol, the xylitol dehydrogenase gene encoding xylitol dehydrogenase that converts xylitol into xylulose, or the xylulokinase gene encoding the xylulokinase that phosphorylates xylulose to produce xylulose 5-phosphate. Xylulose 5-phosphate produced by xylulokinase is metabolized by the pentose phosphate pathway.

[0043] More specific 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*, xylitol dehydrogenase genes derived from *Candida tropicalis* and *Candida prapsilosis*, and a xylulokinase gene derived from *Pichia stipitis* can be used.

[0044] It is particularly preferable that the recombinant yeast of the present invention comprise a xylulokinase gene that has been introduced therinto, in addition to the xylose isomerase gene. Xylulokinase is involved in a reaction that generates xylulose 5-phosphate with the use of xylulose generated by xylose isomerase as a substrate. With the introduction of the xylulokinase gene, accordingly, metabolic activity of the xylose-metabolizing pathway in which the xylose isomerase is involved can be increased.

[0045] When *Saccharomyces cerevisiae* serves as a host of the recombinant yeast of the present invention, expression of the xylulokinase gene inherent in *Saccharomyces cerevisiae* may be enhanced, so that metabolic activity of the xylose-metabolizing pathway in which the xylose isomerase is involved can be increased. The nucleotide sequence of the xylulokinase gene inherent in *Saccharomyces cerevisiae* and the amino acid sequence of acetohydroxy acid reductoisomerase encoded by such gene are shown

in SEQ ID NOs: 9 and 10, respectively.

[0046] The xylulokinase gene is not limited to the gene identified by SEQ ID NOs: 9 and 10. It may be a paralogous gene or a homologous gene in the narrow sense having different nucleotide and amino acid sequences.

[0047] The xylulokinase gene is not limited to the gene identified by SEQ ID NOs: 9 and 10. 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 with the amino acid sequence as shown in SEQ ID NO: 10 and encoding a protein having xylulokinase 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.

[0048] Further, the xylulokinase gene is not limited to the gene identified by SEQ ID NOs: 9 and 10. For example, it may be a gene comprising an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 10 by substitution, deletion, insertion, or addition of one or several amino acids and encoding a protein having xylulokinase 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.

[0049] Furthermore, the xylulokinase gene is not limited to the gene identified by SEQ ID NOs: 9 and 10. 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: 9 and encoding a protein having xylulokinase activity. Under "stringent conditions," so-called specific hybrids are formed, but non-specific hybrids are not formed. Such conditions can be adequately determined with reference to, for example, 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 degrees C to 68 degrees C and preferably 42 degrees C to 65 degrees C, for example. Further specifically, the sodium concentration is 5x SSC (83 mM NaCl, 83 mM sodium citrate), and the temperature is 42 degrees C.

[0050] As described above, whether or not a gene comprising a nucleotide sequence that differs from the sequence shown in SEQ ID NO: 9 or a gene encoding an amino acid sequence that differs from the sequence shown in SEQ ID NO: 10 would function as the xylulokinase gene may be determined by, for example, preparing an expression vector comprising the gene of interest incorporated into an adequate site between a promoter and a terminator, transforming an E. coli host using such expression vector, and assaying xylulokinase activity of the protein expressed. The term "xylulokinase activity" refers to activity of converting xylulose into xylulose 5-phosphate. Thus, xylulokinase activity can be evaluated by preparing a solution containing xylulose and ATP as substrates, allowing the target protein to react at an adequate temperature, and measuring the amount of xylulose and ATP that had decreased and/or the amount of xylulose 5-phosphate that had been produced.

<Preparation of recombinant yeast>

The recombinant yeast of the present invention can be prepared by introducing the xylose isomerase gene into a host yeast and by modifying the host to produce a decreased amount of NADH as a result of the enzymatic reaction of acetohydroxy acid reductoisomerase. A host yeast is not particularly limited. A yeast that does not have xylose-metabolizing ability or a yeast that inherently has xylose-metabolizing ability may be used. Examples of host yeasts 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 preferable. Examples of yeasts that inherently have xylose-metabolizing ability include, but are not particularly limited to, *Pichia stipitis*, *Candida tropicalis*, and *Candida prapsilosis*.

[0051] Experimental yeast strains used from the viewpoint of experimental convenience or industrial (practical) strains used from the viewpoint of practical usefulness may also be used. Examples of industrial strains include yeast strains used for the production of wine, sake, and Shochu. Use of a host yeast strain having homothallic properties is preferable. According to the technique disclosed in JP 2009-34036 A, the multiple copies of a gene can be easily introduced into the genome with the use of a yeast having homothallic properties. The term "yeast having homothallic properties" is the same as the term "homothallic yeast." Yeasts having homothallic properties are not particularly limited, and any yeasts can be used. An example of a yeast having homothallic properties is, but is not limited to, the *Saccharomyces cerevisiae* OC-2 strain (NBRC2260). Examples of other yeasts 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 exhibiting heterothallic phenotypes in an expressible manner, and the resulting strain can be used as a yeast having homothallic properties. That is, the term "yeast having homothallic properties" used herein also refers to a yeast into which the HO gene has been introduced in an expressible manner.

The *Saccharomyces cerevisiae* OC-2 strain is particularly preferable since it has heretofore been used for wine brewing, and the safety thereof has been verified. As described in the examples below, the *Saccharomyces cerevisiae* OC-2 strain is preferable in terms of its excellent promoter activity at high sugar concentration. In particular, the *Saccharomyces cerevisiae* OC-2 strain is preferable in terms of its excellent promoter activity for the pyruvate decarboxylase gene (PDC1) at high sugar concentrations.

- [0052] Promoters of the xylose isomerase gene or mutant acetohydroxy acid reductoisomerase 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.
- [0053] Specifically, such gene may be introduced into the yeast genome together with an expression-regulating promoter or another expression-regulated region. Such 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.
- [0054] The gene 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 recombinant yeast of the present invention, 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 at least xylose as a carbon source. Another carbon source, such as glucose, may be contained in a medium in advance.

[0055] Xylose contained in a medium used for ethanol fermentation can be derived from a biomass. In other words, a medium used for ethanol fermentation may be composed of a cellulosic biomass and a hemicellulase that generates xylose via saccharification of hemicellulose contained in the 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.

[0056] When producing ethanol with the use of the recombinant yeast of the present invention, the medium may further comprise cellulose and cellulase. In such a case, the medium would contain glucose generated by the action of cellulase to 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 cellulose contained in a cellulosic biomass.

[0057] 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, cellulase and xylose derived from hemicellulose contained in a cellulosic biomass.

[0058] As described above, the method for producing ethanol of 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 of the present invention, ethanol can be produced through ethanol fermentation using xylose as a saccharide source. According to the method for producing ethanol with the use of the recombinant yeast of 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 or solid matter via solid-solution separation. Thereafter, ethanol contained in a liquid layer is separated and purified by distillation, so that 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.

[0059] The method for producing ethanol of 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 process of ethanol fermentation carried out with the use of saccharide sources (i.e., xylose and glucose generated by saccharification). In the simultaneous saccharification and fermentation process, the step of saccharification of a cellulosic biomass is carried out simultaneously with the step of ethanol fermentation.

[0060] Methods of saccharification are not particularly limited, and, 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. A cellulase preparation is not particularly limited. For example, cellulase produced by *Trichoderma reesei* or *Acremonium cellulolyticus* can be used. A commercially available cellulase preparation may also be used.

[0061] In the simultaneous saccharification and fermentation process, a cellulase preparation and the above-described recombinant microorganism are added to a medium containing a cellulosic biomass (a biomass after pretreatment may be used), and the recombinant yeast is cultured at a given temperature. Culture may be carried out at any temperature without particular limitation, and the temperature may be 25 degrees C to 45 degrees C, and preferably 30 degrees C to 40 degrees C, from the viewpoint of efficiency for ethanol fermentation. The pH level of the culture solution is preferably 4 to 6. When conducting culture, stirring or shaking may be carried out. 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 (40 degrees C to 70 degrees C), temperature is lowered to a given level (30 degrees C to 40 degrees C), and a yeast is then added thereto.

Examples

[0062] 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.

Example 1

[0063] In the present example, a recombinant yeast that had acquired xylose assimilation ability as a result of introduction of a xylose isomerase gene was modified, so that the resulting recombinant yeast would produce a decreased amount of NADH as a result of the enzymatic reaction of acetohydroxy acid reductoisomerase. The xylose assimilation ability and the ethanol productivity of such recombinant yeast were then evaluated. The recombinant yeast prepared in the present example has improved xylose-metabolizing activity as a result of introduction of the xylulokinase gene.

<Preparation of vectors for gene introduction>

(1) Vector for XI and XKS1 gene introduction and GRE3 gene disruption

As a vector capable of introducing the xylose isomerase gene derived from the intestinal protozoa of *Reticulitermes speratus* and the xylulokinase gene derived from the yeast into the GRE3 locus of a yeast while disrupting the GRE3 gene, pUC-GRE3U-P_TDH1-XI-T_CYC1-P_TDH3-XKS1-T_HIS3-LoxP-G418-LoxP-GRE3D was prepared (Fig. 2).

[0064] This vector was constructed so as to comprise: the xylose isomerase gene derived from the intestinal protozoa of *Reticulitermes speratus* in which the TDH1 promoter and the CYC1 terminator of the *Saccharomyces cerevisiae* BY4742 strain are added to the 5' side and the 3' side, respectively (RsXI-C1; see JP 2011-147445 A); the xylulokinase gene of the *Saccharomyces cerevisiae* BY4742 strain in which the TDH3 promoter and the HIS3 terminator of the *Saccharomyces cerevisiae* BY4742 strain are added to the 5' side and the 3' side, respectively (XKS1); regions to be integrated into the yeast genome via homologous recombination, i.e., a gene sequence of approximately 700 bp upstream from the 5' end of GRE3 (GRE3U) and a DNA sequence of approximately 800 bp downstream from the 3' end of GRE3 (GRE3D); and a marker gene sequence containing the G418 gene (the G418 marker). The marker gene is flanked by LoxP sequences, so that the marker can be removed from the marker gene sequence.

[0065] Each DNA sequence can be amplified with the use of the primers shown in Table 1. In order to allow DNA fragments to bind to each other, each target DNA fragment was amplified using the primers each prepared by adding a DNA sequence to a primer shown in Table 1 so as to overlap with an adjacent DNA sequence by about 15 bp, and a DNA fragment was allowed to bind to an adjacent DNA fragment using the In-Fusion HD Cloning Kit (Takara Bio). Thus, the vector was prepared.

(2) Vector for ilvC (NADP-dependent) gene introduction

As a vector for introducing the *E. coli*-derived NADP-dependent acetohydroxy acid reductoisomerase gene into a yeast, pUC-

ADH2part-T_CYC1-P_TDH3-M_DLD2-ilvC-mutant T_ACT1-TRP1-3U_ADH2) was prepared (Fig. 3). This vector was constructed so as to comprise: a NADP-dependent mutant of the ilvC gene of acetohydroxy acid reductoisomerase derived from the *E. coli* K12 strain (the Genebank: 948286) in which the TDH3 promoter of the *Saccharomyces cerevisiae* BY4742 strain and a fragment of the DLD2 gene that is predicted to be a mitochondrial-targeting signal peptide (i.e., 135 nucleotides from the 5' end, M_DLD2) are added to the 5' side and the ACT1 terminator is added to the 3' side; regions to be integrated into the yeast genome via homologous recombination, i.e., a gene sequence of approximately 450 bp upstream from the 3' end (ADH2part) and a DNA sequence of approximately 700 bp downstream from the 3' end (ADH2D) of the ADH2 gene; the CYC1 terminator region as the ADH2 terminator; and a marker gene sequence containing the TRP1 gene (the TRP1 marker). In the present example, a gene encoding an amino acid sequence having the mutations R68D, K69L, K75V, and R76D (Arch. Biochem. Biophys., 338, 83-89, 1997), and comprising a nucleotide sequence in which codons had been altered in accordance with the codon usage frequency of the yeast was used. In the present example, the NADP-dependent ilvC gene was fully synthesized. In the present example, another NADP-dependent ilvC gene encoding an amino acid sequence having the mutations A71S, R76D, S78D, Q110V, D146G, and G185R (United States Patent 8097440), and comprising a nucleotide sequence in which codons had been altered in accordance with the codon usage frequency of the yeast was used. In the present example, also, this NADP-dependent ilvC gene was fully synthesized.

[0066] Each DNA sequence can be amplified with the use of the primers shown in Table 1. In order to allow DNA fragments to bind to each other, each target DNA fragment was amplified using the primers each prepared by adding a DNA sequence to a primer shown in Table 1 so as to overlap with an adjacent DNA sequence by about 15 bp, and a DNA fragment was allowed to bind to an adjacent DNA fragment using the In-Fusion HD Cloning Kit (Takara Bio). Thus, the vector was prepared.

[0067] (3) Vector for ILV5 gene disruption

A vector for disrupting the ILV5 gene, pUC-ILV5U-TRP1-ILV5D, was prepared (Fig. 4). This vector was constructed so as to comprise: a region to be integrated into the yeast genome via homologous recombination and a region for disruption of acetohydroxy acid reductoisomerase (ILV5) gene, i.e., a DNA sequence of approximately 850 bp upstream of the ILV5 gene (ILV5U) and a DNA sequence of approximately 800 bp downstream of the ILV5 gene (ILV5D); and a marker gene sequence containing TRP1 (the TRP1 marker).

[0068] Each DNA sequence can be amplified with the use of the primers shown in Table 1. In order to allow DNA fragments to bind to each other, each target DNA fragment was

amplified using the primers each prepared by adding a DNA sequence to a primer shown in Table 1 so as to overlap with an adjacent DNA sequence by about 15 bp, and a DNA fragment was allowed to bind to an adjacent DNA fragment using the In-Fusion HD Cloning Kit (Takara Bio). Thus, the vector was prepared.

(4) Control vector (marker gene only)

A control vector for introducing a marker gene only, pUC-ADH2part-T_CYC1-TRP1-ADH2D, was prepared (Fig. 5). This vector was constructed so as to comprise: regions to be integrated into the yeast genome via homologous recombination, i.e., a gene sequence of approximately 450 bp upstream from the 3' end (ADH2part) and a DNA sequence of approximately 700 bp downstream from the 3' end (ADH2D) of the ADH2 gene; the CYC1 terminator sequence as the ADH2 terminator; and a marker gene sequence containing TRP1 (the TRP1 marker).

[0069] Each DNA sequence can be amplified with the use of the primers shown in Table 1. In order to allow DNA fragments to bind to each other, each target DNA fragment was amplified using the primers each prepared by adding a DNA sequence to a primer shown in Table 1 so as to overlap with an adjacent DNA sequence by about 15 bp, and a DNA fragment was allowed to bind to an adjacent DNA fragment using the In-Fusion HD Cloning Kit (Takara Bio). Thus, the vector was prepared.

[0070]

[Table 1]

Amplified DNA fragment	Primer sequence	SEQ ID NO
pUC-GRE3U-P_TDH1-XI-T_CYC1-P_TDH3-XKS1-T_HIS3-LoxP-G418-LoxP-GRE3D		
TDH1 promoter	5'- GGAAATTTTTTAAAGCTTCCCTTTTACAGTGCTTC -3'	11
	5'- AAAAATTGAGACATTTTGTGTTGTGTGTAAATTTAGTGAAG -3'	12
RsXI-C1	5'- ATGTCTCAAATTTTAAAGGATATCCAG -3'	13
	5'- GGGGCCTGTCTTAAGTTATTGAAACAAAATTTGGTTAATAATACTTTC -3'	14
CYC1 terminator	5'- CTTAAGACAGGCCCTTTTCCTTTG -3'	15
	5'- TAACATTCAACGCTACTGCAGGAATTCGATATC -3'	16
TDH3 promoter	5'- TAGCGTTGAATGTTAGCGTCAACAAC -3'	17
	5'- TACTGAACACAACATTTTGTGTTTATGTGTGTTTATTTCG -3'	18
XKS1	5'- ATGTTGTGTTTCAGTAATTCAGAGACAG -3'	19
	5'- AAATAATCGGTGTCATTAGATGAGAGTCTTTTCCAGTTC -3'	20
HIS3 terminator	5'- TGACACCGATTATTTAAAGCTGCAG -3'	21
	5'- AGAGCGCGCCTCGTTC -3'	22
GRE3U	5'- TGGGAATATTACCGCTCGAAG -3'	23
	5'- CTTTAAAAAATTTCCAATTTTCCTTTACG -3'	24
GRE3D	5'- AACGAGGCGCGCTCTCCAGCCAGTAAAATCCATAC -3'	25
	5'- AAGGGGGAAGGTGTGGAATC -3'	26
pUC- ADH2part-T_CYC1-P_TDH3-M_DLD2-ilvC-mutant-T_ACT1-TRP1-3U_ADH2		
TDH3 promoter	5'- TAGCGTTGAATGTTAGCGTCAACAAC -3'	27
	5'- TTTGTTTGTGTTATGTGTGTTTATTCGAAACTAAGTTCTTGG -3'	28
M_DLD2	5'- ATGCTAAGAAACATTTTGGTGAGAAG -3'	29
	5'- AGTCAGTCTGGTTTGTATCTTG -3'	30
ilvC mutant	5'- ATGGCAACTACTTCAACACTTTGAATTTG -3'	31
	5'- TTAACCTGCAACAGCGATTC -3'	32
ADH2part	5'- CCAACTGTCCTCAGCTGAC -3'	33
	5'- CTTATTTAGAAGTGCAACAACGTATCTACC -3'	34
ADH2D	5'- GCGGATCTCTTATGTCTTTACGATTTATAGTTTTTC -3'	35
	5'- GAGGGTTGGGCATTATCATCAG -3'	36
pUC-ILV5U-TRP1-ILV5D		
CYC1 terminator	5'- CTTAAGACAGGCCCTTTTCCTTTG -3'	37
	5'- CTGCAGGAATTCGATATCAAGCTTATC -3'	38
TRP1 marker	5'- ACGACATTACTATATATATAATATAGGAAGCATTTAATAG -3'	39
	5'- GACATAAGAGATCCGCAGG -3'	40
ILV5U	5'- AAACGGCCAGTAGCCAAGAC -3'	41
	5'- ACTCCAAGCTGCCTTTGTGTGC -3'	42
ILV5D	5'- AGAGGAAAATAATATCAAGTGCTGGAAAC -3'	43
	5'- CCGGAGGCTTTCAATTGTTC -3'	44
pUC-ADH2part-T_CYC1-TRP1-ADH2D		
CYC1 terminator	5'- CTTAAGACAGGCCCTTTTCCTTTG -3'	45
	5'- ATATAGTAATGTCGTCTGCAGGAATTCGATATCAAG -3'	46
TRP1 marker	5'- ACGACATTACTATATATATAATATAGGAAGCATTTAATAG -3'	47
	5'- ACATAAGAGATCCGCAGGCAAGTGCACAAACAATAC -3'	48
ADH2part	5'- CCAACTGTCCTCAGCTGAC -3'	49
	5'- GGGGCCTGTCTTAAGCTTATTAGAAGTGTCAACAACG -3'	50
ADH2D	5'- GCGGATCTCTTATGTCTTTACGATTTATAGTTTTTC -3'	51
	5'- GAGGGTTGGGCATTATCATCAG -3'	52

<Preparation of yeasts comprising vectors introduced therinto>

The diploid yeasts auxotrophic for tryptophan, *Saccharomyces cerevisiae* OC2-T strain

(Saitoh, S. et al., J. Ferment. Bioeng., 1996, vol. 81, pp. 98-103), were designated as host strains. Yeasts were transformed using the Frozen-EZ Yeast Transformation II (ZYMO RESEARCH) in accordance with the protocols included therein.

[0071] At the outset, a region to be subjected to homologous recombination of the vector, pUC-5U_GRE3-P_TDH1-XI-T_CYC1-P_TDH3-XKS1-T_HIS3-LoxP-G418-LoxP-3U_GRE3, was amplified by PCR, the resulting fragment was transformed into the OC2-T strains, the resultants were applied to a tryptophan-free SD agar medium, and the grown colonies were then subjected to purification. The purified strain was designated as the Uz979 strain. The resulting strain was allowed to sporulate in a sporulation medium (1% potassium phosphate, 0.1% yeast extract, 0.05% glucose, and 2% agar), so as to cause diploidization with the utilization of homothallic properties. Diploid strain containing the XI and XKS1 genes integrated into and the GRE3 gene disrupted in the GRE3 genetic loci of the chromosomes was obtained and designated as the Uz979 strain.

Subsequently, regions subjected to homologous recombination of the vector, pUC-ADH2part-T_CYC1-P_TDH3-M_DLD2-ilvC-mutant R68D K69L K75V R76D - T_ACT1-TRP1-3U_ADH2, pUC-ADH2part-T_CYC1-P_TDH3-M_DLD2-ilvC-mutant A71S R76D S78D Q110V D146G G185R -T_ACT1-TRP1-3U_ADH2, pUC-ILV5U-TRP1-ILV5D, or pUC-ADH2part-T_CYC1-TRP1-ADH2D, were amplified by PCR, the resulting fragments were transformed into the Uz979 strain, the resultants were applied to a tryptophan-free SD agar medium, and the grown colonies were subjected to purification. The purified strains were designated as the Uz999, Uz1000, Uz1089, and Uz1034 strains, respectively. Heterozygous recombination (in one copy) was observed in each strain.

<Fermentation test>

Two strains exhibiting high fermentation ability were selected from each of the Uz999, Uz1000, Uz1089, and Uz1034 strains obtained in the manner described above, and the selected strains were subjected to a fermentation test in flasks in the manner described below. At the outset, the test strains were introduced into 100-ml baffled flasks each containing 20 ml of YPD liquid medium containing glucose at 20 g/l (10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose), and culture was conducted at 30 degrees C and 120 rpm for 24 hours. The strains were collected and introduced into 20-ml flasks each containing 10 ml of the D5X65YPAc3 medium (5 g/l glucose, 65 g/l xylose, 10 g/l yeast extract, 20 g/l peptone, and 3 g/l acetic acid) (cell density: 0.3 g of dry cells/l). The fermentation test was carried out via shake culture (80 rpm; shake width: 35 mm; 30 degrees C). Each flask was stoppered with a rubber cap comprising a needle (inner diameter: 1.5 mm), and anaerobic conditions inside the flask were maintained by mounting a check valve at the tip of the needle.

[0072] Sampling was carried out 90 hours, 114 hours, and 138 hours after the initiation of fermentation, and the xylose concentration and the ethanol concentration in the fermentation liquid were assayed via HPLC (LC-10A, Shimadzu Seisakusho) under the conditions described below. From among the data obtained through three sampling instances, the data attained when the highest ethanol concentration was observed were employed as the results of the fermentation test (and such data represent the average of two strains).

[0073] [HPLC conditions]

Column: AminexHPX-87H

Mobile phase: 0.01N H₂SO₄

Flow rate: 0.6 ml/min

Temperature: 30 degrees C

Detector: differential refractometer (RID-10A)

<Results of fermentation test>

The results of the fermentation test are shown in Table 2.

[0074] [Table 2]

	Control Uz1034	ilvC mutant Uz999 R68D K69L K75V R76D	ilvC mutant Uz1000 A71S R76D S78D Q110V D146G G185R	ILV5 hetero-disrupted Uz1089
Ethanol concentration (g/l)	2.07	7.16	6.92	6.48
Xylose concentration (g/l)	57.2	35.3	37	40.1

As is apparent from Table 2, the Uz999 strain and the Uz1000 strain into which the NADP-dependent ilvC genes had been introduced and the Uz1089 strain comprising a hetero-disruption of the endogenous ILV5 gene showed remarkable improvement in the rate of xylose assimilation, compared with the control Uz1034 strain, and ethanol productivity was improved as a consequence. On the basis of the results demonstrated above, xylose assimilation ability and ethanol productivity were found to be remarkably improved in a recombinant yeast having xylose assimilation ability into which the xylose isomerase gene had been introduced by reducing the amount of NADH produced as a result of the enzymatic reaction of acetohydroxy acid reductoisomerase.

Claims

- [Claim 1] A recombinant yeast into which the xylose isomerase gene has been introduced, wherein a production of NADH as a result of the enzymatic reaction of acetohydroxy acid reductoisomerase is lowered.
- [Claim 2] The recombinant yeast according to claim 1, wherein activity of endogenous acetohydroxy acid reductoisomerase is lowered.
- [Claim 3] The recombinant yeast according to claim 1, wherein the expression level of the endogenous gene encoding acetohydroxy acid reductoisomerase is lowered.
- [Claim 4] The recombinant yeast according to claim 3, wherein the endogenous gene is disrupted.
- [Claim 5] The recombinant yeast according to claim 4, wherein the endogenous genes are hetero-disrupted.
- [Claim 6] The recombinant yeast according to claim 1 into which a gene encoding mutant acetohydroxy acid reductoisomerase with lowered NAD⁺ dependence and enhanced NADP⁺ dependence is introduced.
- [Claim 7] The recombinant yeast according to claim 3, wherein the endogenous gene encoding acetohydroxy acid reductoisomerase encodes the protein (a) or (b) below:
(a) the protein comprising the amino acid sequence as shown in SEQ ID NO: 2; or
(b) the protein comprising an amino acid sequence having 70% or higher identity with the amino acid sequence as shown in SEQ ID NO: 2 and having enzymatic activity of converting 2-acetolactate and NAD⁺ into 2,3-dihydroxy-isovalerate and NADH, respectively.
- [Claim 8] The recombinant yeast according to claim 6, wherein the gene encoding mutant acetohydroxy acid reductoisomerase encodes the protein (a) or (b) below:
(a) the protein comprising the amino acid sequence as shown in SEQ ID NO: 4 or 6; or
(b) the protein comprising an amino acid sequence having 70% or higher identity with the amino acid sequence as shown in SEQ ID NO: 4 or 6 and having enzymatic activity of converting 2-acetolactate and NADP⁺ into 2,3-dihydroxy-isovalerate and NADPH, respectively.
- [Claim 9] The recombinant yeast according to claim 1, wherein the xylose isomerase gene encodes the protein (a) or (b) below:
(a) the protein comprising the amino acid sequence as shown in SEQ

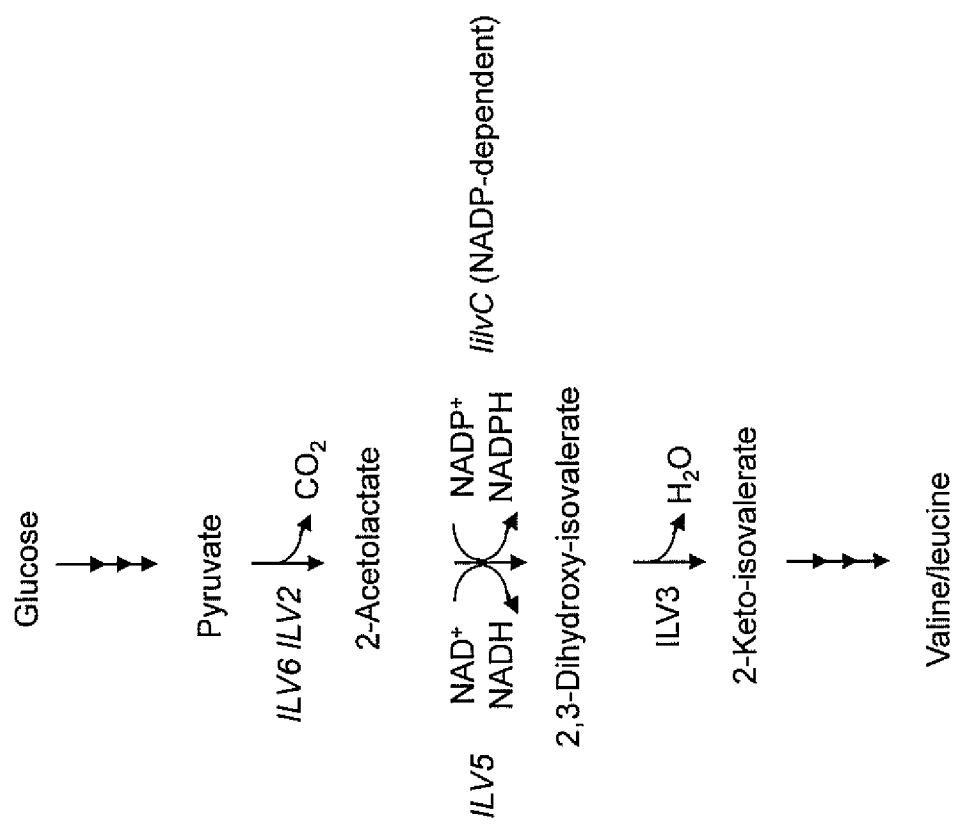
ID NO: 8; or

(b) the protein comprising an amino acid sequence having 70% or higher identity with the amino acid sequence as shown in SEQ ID NO: 8 and having enzymatic activity of converting xylose into xylulose.

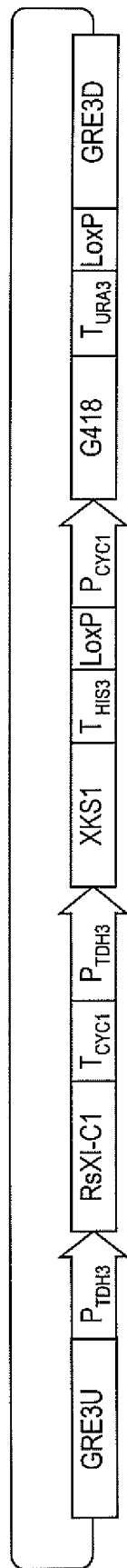
[Claim 10]

A method for producing ethanol comprising a step of culturing the recombinant yeast according to any one of claims 1 to 9 in a xylose-containing medium to perform ethanol fermentation.

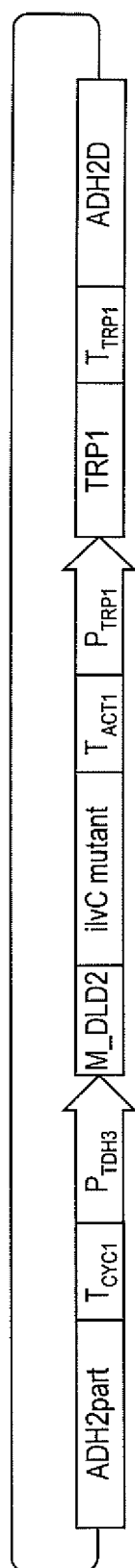
[Fig. 1]



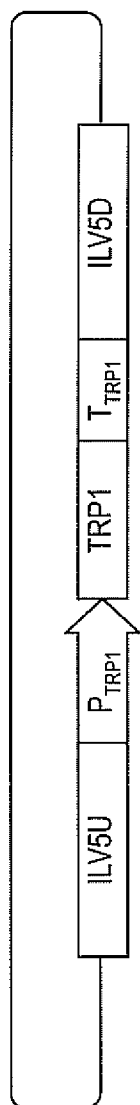
[Fig. 2]



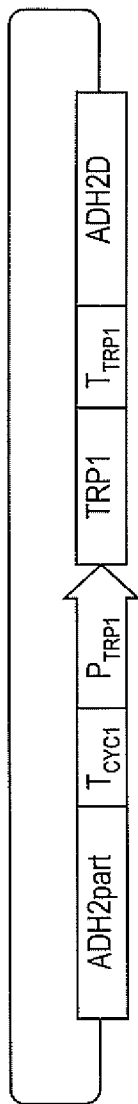
[Fig. 3]



[Fig. 4]



[Fig. 5]



INTERNATIONAL SEARCH REPORT

International application No

PCT/JP2014/003069

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N9/04 C12N9/92 C12P7/06
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, Sequence Search, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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A	WO 2010/151525 A1 (GEVO INC [US]; FELDMAN REID M RENNY [US]; GUNAWARDENA UVINI [US]; URAN) 29 December 2010 (2010-12-29) paragraphs [0153] - [0155], [0252] - [0286]; claims 5, 6, 16-19, 23 -----	1-10
A	WO 2013/048599 A2 (GEVO INC [US]; CALIFORNIA INST OF TECHN [US]; BUELTER THOMAS [US]; LIE) 4 April 2013 (2013-04-04) paragraphs [0156], [0157]; claims -----	1-10
A	WO 2011/078262 A1 (TOYOTA CHUO KENKYUSHO KK [JP]; RIKEN [JP]; KATAHIRA SATOSHI [JP]; TOKU) 30 June 2011 (2011-06-30) claims ----- -/-	1-10



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

29 September 2014

Date of mailing of the international search report

21/10/2014

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INTERNATIONAL SEARCH REPORT

International application No

PCT/JP2014/003069

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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Information on patent family members

International application No

PCT/JP2014/003069

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