culturing the transformant according to (6) or the transformed yeast according to any one of (7) to (9).

[0020]

(11) A transformant in which a polynucleotide encoding D-lactate dehydrogenase originated from the family Limulidae or DNA construct in which the polynucleotide and a promoter capable of expressing the polynucleotide are linked is introduced.

[0021]

(12) A transformed yeast in which a polynucleotide encoding D-lactate dehydrogenase originated from the family~~genus~~ *Limulidae*~~*Limulus*~~ or DNA construct in which the polynucleotide and a promoter capable of expressing the polynucleotide are linked is introduced.

[0022]

(13) The transformed yeast according to (12), wherein at least one of pyruvate decarboxylase 1 gene (PDC1 gene), suppression-of-exponential-defect 1 gene (SED1 gene) and glyceraldehyde-3-phosphate dehydrogenase 3 gene (TDH3 gene) of the above-described transformed yeast is substituted with the above-described polynucleotide encoding D-lactate dehydrogenase originated from the family Limulidae or DNA construct in which the polynucleotide and a promoter capable of expressing the polynucleotide are linked is introduced.

[0023]

(14) A method of producing D-lactic acid, which comprises the step of culturing the transformant according to (11) or the transformed yeast according to (12) or (13).

EFFECTS OF THE INVENTION

[0024]

According to the present invention, a polypeptide having a D-lactate

Limulidae and genus *Limulus*.

[0030]

Examples of the homolog of the polypeptide having the amino acid sequence shown in SEQ ID NO:1 or 2 include a polypeptide having the same amino acid sequence as shown in SEQ ID NO:1 or 2 except that one or several, preferably 1 to 10, more preferably 1 to 5, still more preferably 1 or 2 amino acids are substituted, deleted, inserted and/or added, which polypeptide also has a D-LDH activity.

[0031]

Further, a homolog of the polypeptide shown in SEQ ID NO:13 or 24 may also be a polypeptidehaving an amino acid sequence which has a sequence identity of not less than 80%, preferably 90%, more preferably not less than 95% to the amino acid sequence shown in SEQ ID NO:1 or 2, which polypeptide also has a D-LDH activity. Here, the sequence identity of an amino acid sequence can be easily checked using the BLAST, which is a software widely used in the art. Anyone can use the BLAST through the NCBI (National Center for Biotechnology Information) homepage, and the identity can be easily checked by using default parameters.

[0032]

In addition, the homolog of the polypeptide having the amino acid sequence shown in SEQ ID NO:1 or 2 may also be a polypeptide originated from a living organism belonging to the family Limulidae, preferably a living organism belonging to the genus *Limulus* or *Tachypleus*, more preferably *Limulus polyphemus* belonging to the genus *Limulus*, or *Tachypleus tridentatus*, *Tachypleus gigas* or *Tachypleus rotundicauda* belonging to the genus *Tachypleus*, which polypeptide has a D-LDH activity.

[0033]

The polypeptide having the amino acid sequence shown in SEQ ID NO:1 or 2 can be extracted from *Limulus polyphemus* by a known method or prepared using a

known peptide synthesis method. Further, it can be also prepared by gene recombination technique using a polynucleotide encoding the amino acid sequence shown in SEQ ID NO:1 or 2. The polypeptide having the amino acid sequence shown in SEQ ID NO:1 or 2 can be also extracted from a living organism belonging to the genus *Tachypleus* by a known method or prepared using a known peptide synthesis method. Further, it can be also prepared by gene recombination technique using a polynucleotide encoding the amino acid sequence of the polypeptide.

[0034]

The homolog of the polypeptide having the amino acid sequence shown in SEQ ID NO:1 or 2 may have an improved D-LDH activity and/or an improved thermal stability as compared to the polypeptide having the amino acid sequence shown in SEQ ID NO:1 or 2. Here, the term "an improved D-LDH activity" refers to an improvement in the substrate affinity and molecular activity (Kcat) for pyruvic acid and NADH, as well as a shift in the optimum pH for the enzyme activity of the polypeptide closer to a pH suitable for growth of cells expressing the polypeptide. Such polypeptide may be artificially designed based on the polypeptide having the amino acid sequence shown in SEQ ID NO:1 or 2, or may be separated from nature. Further, a mutation may be randomly introduced to the D-lactate dehydrogenase gene by an evolutionary molecular engineering method to screen a preferred polypeptide therefrom.

[0035]

The present invention is characterized by including a polynucleotide having the nucleotide sequence shown in SEQ ID NO:3 or 4 or a homolog thereof, which polynucleotide encodes a ~~polynucleotide~~polypeptide having a D-LDH activity. Here, the origin of the "polynucleotide" does not matter and may be a cDNA, genomic DNA, synthetic DNA, mRNA, synthetic RNA, replicon RNA; however, it is preferably a DNA or RNA, more preferably DNA. Also, the "polynucleotide" may be either

The DNA construct for homologous recombination prepared in the above-described manner was transformed into *Saccharomyces cerevisiae* SW092-2D strain (hereinafter, referred to as "SW092-2D strain"), which is a *Saccharomyces cerevisiae* NBRC10505 strain (hereinafter, referred to as "NBRC10505 strain") in which the lysine auxotrophy is reverted.

[0093]

The SW092-2D strain was prepared by the following method. Using the genomic DNA of the *Saccharomyces cerevisiae* BY4741 strain manufactured by Funakoshi Corporation as template and the oligonucleotides (SEQ ID NOs:29 and 30) as primer set, PCR was performed to amplify a PCR fragment of about 2 kb corresponding to the former half of the LYS2 gene. After separating the PCR fragment by 1% agarose gel electrophoresis and purifying it in accordance with a conventional method, the PCR fragment was transformed into the NBRC10505 strain to cancel the amber mutation in the LYS2 gene. By culturing the yeast strain in a lysine-free medium, a transformant in which the lysine synthesis capacity was reverted (NBRC10505(LYS2) strain) was selected. In order to confirm that the thus obtained transformant is a yeast in which the amber mutation in the LYS2 gene was canceled, the following steps were carried out. First, diploid cells were obtained by mating the thus obtained transformant with the *Saccharomyces cerevisiae* LO~~0~~GY7 strain having the wild-type LYS2 gene and the diploid cells were cultured in an ascus formation medium to allow ascus formation. The resulting asci were anatomized using a micromanipulator to obtain respective haploid cells (tetrad) and the auxotrophy was examined for each of the haploid cells. All of the thus obtained haploid cells were confirmed to have lysine synthesis capacity. The thus obtained NBRC10505(LYS2) strain and the NBRC10506 strain were mated and the resulting asci were anatomized using a micromanipulator to obtain the SW092-2D strain (genotype: MATa ura3 leu2 trp1 his3 ade2 LYS2).

[0094]

The SW092-2D strain was transformed with the above-described DNA construct for homologous recombination and cultured in a tryptophan-free medium to select transformed yeasts. The transformed yeasts obtained in this manner are hereinafter referred to as "SW092-2D (Δpdc1::Lp.D-LDH1-TRP1) strain" and "SW092-2D (Δpdc1::Lp.D-LDH2~~DLH2~~-TRP1) strain".

[0095]

(Example 6) D-lactic acid productivity test 2

Using the SW092-2D (Δpdc1::Lp.D-LDH1-TRP1) strain, SW092-2D (Δpdc1::Lp.D-LDH2-TRP1) strain and a mini-jar fermentor (manufactured by B.E. Marubishi Co., Ltd., 5 L), their fermentation was evaluated.

[0096]

The SC3 medium shown in Table 1 was added to a test tube in an amount of 10 mL and a small amount of each strain was inoculated thereto and cultured overnight at 30°C (pre-preculture). Then, to 45 mL of SC3 medium placed in an Erlenmeyer flask, 5 mL of the thus obtained pre-preculture medium was added and cultured at 30°C for another 8 hours (preculture). The entire amount of the thus obtained preculture medium was inoculated to 1 L of SC3 medium placed in the mini-jar fermentor and cultured for 30 hours. The culturing conditions are shown below:

pH: pH5

Aeration: 100 mL/min

Stirring: 120 rpm

Neutralizer: 1N sodium hydroxide.

[0097]

The amount of the culture medium at the end of the culture period and the D-lactic acid and glucose concentrations in the culture medium were measured by

into the PDC1 gene locus was prepared. Here, as the yeast used for the introduction of the gene into the chromosome, a NBRC10506 strain in which the adenine auxotrophy is reverted (SU013-1D strain) was used. The SU013-1D strain was prepared by the following method. Using the plasmid pRS422 as template and the oligonucleotides (SEQ ID NOs:34 and 35) as primer set, PCR was performed to amplify a PCR fragment of about 2 kb corresponding to the ADE2 gene. After separating the PCR fragment by 1% agarose gel electrophoresis and purifying it in accordance with a conventional method, the PCR fragment was transformed to cancel the mutation in the ADE2 gene. By culturing the yeast strain in an adenine-free medium, a transformant in which the adenine synthesis capacity was reverted was selected.

[0103]

In order to confirm that the thus obtained transformant is a yeast in which the mutation in the ADE2~~AED2~~ gene was canceled, the following steps were carried out. First, diploid cells were obtained by mating the thus obtained transformant with the *Saccharomyces cerevisiae* LQ~~0~~GY77 strain having the wild-type ADE2 gene and the diploid cells were cultured in an ascus formation medium to allow ascus formation. The resulting asci were anatomized using a micromanipulator to obtain respective haploid cells (tetrad) and the auxotrophy was examined for each of the haploid cells. All of the thus obtained haploid cells were confirmed to have adenine synthesis capacity. The thus obtained NBRC10506(ADE2) strain and the NBRC10505 strain were mated and the resulting asci were anatomized using a micromanipulator to obtain the SU013-1D strain (genotype: MATα ura3 leu2 trp1 his3 ADE2 lys2). The thus obtained transformed yeast is hereinafter referred to as "SU013-1D (Δpdc1::X. L-LDH-TRP1) strain".

[0104]

(Example 7, Comparative Example 2) D-lactic acid productivity test 3

transformed into the SU013-1D strain to cancel the mutation in the LEU2 gene. By culturing the yeast strain in a leucine-free medium, a transformant in which the leucine synthesis capacity was reverted was selected. In order to confirm that the thus obtained transformant is a yeast in which the mutation in the LEU2 gene was canceled, the following steps were carried out. First, diploid cells were obtained by mating the thus obtained transformant with the *Saccharomyces cerevisiae* LQ0GY77 strain having the wild-type LEU2 gene and the diploid cells were cultured in an ascus formation medium to allow ascus formation. The resulting asci were anatomized using a micromanipulator to obtain respective haploid cells (tetrad) and the auxotrophy was examined for each of the haploid cells. All of the thus obtained haploid cells were confirmed to have leucine synthesis capacity. The thus obtained SU013-1D(LEU2) strain and the NBRC10505 strain were mated and the resulting asci were anatomized using a micromanipulator to obtain the SW087-2C strain (genotype: MATα ura3 LEU2 trp1 his3 ADE2 lys2).

[0111]

The SW087-2C strain was transformed with the above-described DNA construct for homologous recombination and cultured in a uracil-free medium for selection to obtain a transformed yeast introduced with the Lp. D-LDH1 gene at the TDH3 gene locus. The thus obtained transformed yeast is hereinafter referred to as "SW087-2C (ΔTDH3::Lp. D-LDH-URA3) strain".

[0112]

[Introduction of the D-LDH gene into SED1 gene locus]

The introduction into the SED1 gene locus was carried out by modifying the method described in Example 2 of the international application PCT/JP 2008/072129. That is, using the pTRS205 in place of the pTRS102 as PCR template and the SEQ ID NO:39 in place of the SEQ ID NO:14 of the above-described international application publication as primer, a DNA construct for homologous recombination to

originated from the family Limulidae or a DNA construct in which said polynucleotide and a promoter capable of expressing said polynucleotide are linked is introduced.

12. A transformed yeast in which a polynucleotide encoding D-lactate dehydrogenase originated from the familygenus *Limulidae*~~*Limulus*~~ or a DNA construct in which said polynucleotide and a promoter capable of expressing said polynucleotide are linked is introduced.

13. The transformed yeast according to claim 12, wherein at least one of pyruvate decarboxylase 1 gene (PDC1 gene), suppression-of-exponential-defect 1 gene (SED1 gene) and glyceraldehyde-3-phosphate dehydrogenase 3 gene (TDH3 gene) of said transformed yeast is substituted with said polynucleotide encoding said D-lactate dehydrogenase originated from the family Limulidae or said DNA construct in which said polynucleotide and a promoter capable of expressing said polynucleotide are linked is introduced.

14. A method of producing D-lactic acid, which comprises the step of culturing the transformant according to claim 11 or the transformed yeast according to claim 12 or 13.