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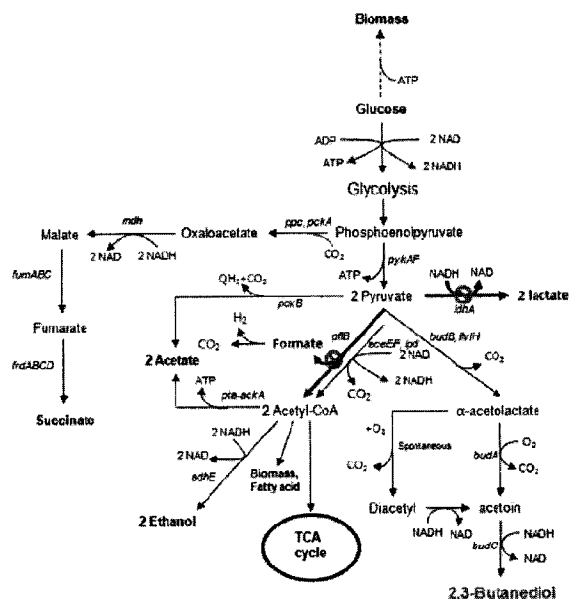
(72) **Inventeurs/Inventors:**
PARK, JONG-MYOUNG, KR;
SONG, HYO-HAK, KR;
YANG, TAEK-HO, KR

(73) **Propriétaire/Owner:**
GS CALTEX CORPORATION, KR

(74) **Agent:** BENOIT & COTE INC.

(54) **Titre : MICRO-ORGANISME RECOMBINE AYANT UNE PRODUCTIVITE ACCRUE DE 2,3-BUTANEDIOL, ET PROCEDE DE PRODUCTION DE 2,3-BUTANEDIOL L'UTILISANT**

(54) **Title: RECOMBINANT MICROORGANISM HAVING ENHANCED 2,3-BUTANEDIOL PRODUCING ABILITY AND METHOD FOR PRODUCING 2,3-BUTANEDIOL USING THE SAME**



(57) **Abrégé/Abstract:**

Disclosed herein is a recombinant microorganism having enhanced 2,3-butanediol producing ability, wherein a pathway for converting pyruvate to acetyl-CoA, a pathway for converting pyruvate to formic acid, or a pathway for converting pyruvate to lactate is inhibited in a microorganism having acetyl-CoA and lactate biosynthetic pathways.

【ABSTRACT】

Disclosed herein is a recombinant microorganism having enhanced 2,3-butanediol producing ability, wherein a pathway for converting pyruvate to acetyl-CoA, a pathway for converting
5 pyruvate to formic acid, or a pathway for converting pyruvate to lactate is inhibited in a microorganism having acetyl-CoA and lactate biosynthetic pathways.

【DESCRIPTION】**【Invention Title】**

RECOMBINANT MICROORGANISM HAVING ENHANCED 2,3-BUTANEDIOL
PRODUCING ABILITY AND METHOD FOR PRODUCING 2,3-BUTANEDIOL USING
5 THE SAME

【Technical Field】

[1] The present invention relates to a recombinant
microorganism having enhanced 2,3-butanediol producing ability,
10 and a method for producing 2,3-butanediol using the same.

【Background Art】

[2] 2,3-butanediol ($\text{CH}_3\text{CHOHCHOHCH}_3$) which is one of alcohols
having four carbons and two hydroxyl groups (-OH) is capable of
being converted to a chemical catalyst with 1,3-Butadiene which
15 is a raw material used in a manufacturing process of synthetic
rubber and methyl ethyl ketone (MEK) used as a fuel additive
and a solvent (Ji et al., Biotechnol. Adv., 29: 351, 2011). In
addition, 2,3-butanediol is applicable as an octane booster by
being mixed with gasoline, which is a significantly important
20 intermediate in fuel industries (Celinska et al., Biotechnol.
Adv., 27: 715, 2009).

[3]

[4] 2,3-butanediol is capable of being produced by a chemical
synthesis process and a microbial fermentation process.
25 However, since production cost for 2,3-butanediol through the

above-described processes is significantly high, industrial-scale production of 2,3-butanediol is not achieved. Meanwhile, in accordance with a rapid increase in price of fossil raw materials and reinforcement of regulation on international environmental pollution, together with the recent and fast development of a technology of producing 2,3-butanediol through a microorganism fermentation process, importance of an interest in production of bio-based 2,3-butanediol through microorganism fermentation and research and development thereof have been increased.

[5]

[6] A method for producing the bio-based 2,3-butanediol is to convert renewable biomass to 2,3-butanediol through fermentation of microorganism having 2,3-butanediol producing ability. 2,3-butanediol is produced by various kinds of microorganisms such as *Klebsiella* species, *Enterobacter* species, *Bacillus* species, *Serratia* species, and the like (Maddox IS, *Biotechnol.*, 6: 269, 1996). In particular, *Klebsiella pneumoniae* (*K. pneumoniae*), *Klebsiella oxytoca* (*K. oxytoca*), and *Paenibacillus polymyxa* produce a relatively large amount of 2,3-butanediol. Particularly, *Klebsiella pneumoniae* (*K. pneumoniae*) and *Klebsiella oxytoca* (*K. oxytoca*) have advantages in that culturing is easily performed, a growth rate is rapid, and 2,3-butanediol is capable of being produced from various kinds of biomasses including lignocellulosic-derived

pentose (Ji et al., *Biotechnol. Adv.*, 29: 351, 2011; Chandel et al., *Sustainable Biotechnol.*, 63, 2010; Jansen et al., *Biotechnol. Bioeng.*, 26: 362, 1984; Jansen et al., *Adv. Biochem. Eng.*, 27: 85, 1983).

5 [7]

[8] Research on production of the bio-based 2,3-butanediol through the microorganism fermentation process has been conducted according to two fields divided into a field of optimization (temperature, pH, dissolved oxygen, and the like)
 10 of a fermentation process and a field of microorganism development (microorganism discovery, understanding of physiological characteristics, mutation, genetic engineering, and the like). In the field of optimization of the fermentation process, various conditions such as temperature,
 15 pH, concentration of dissolved oxygen, and the like for effectively producing 2,3-butanediol were identified (Ji et al., *Bioresour. Technol.*, 100: 3410, 2009; Nakashimada et al., *J. Biosci. Bioeng.*, 90: 661, 2000; Nakashimada et al., *Biotechnol. Lett.*, 20: 1133, 1998). However, production of
 20 2,3-butanediol through the microorganism fermentation process under the above-described conditions still has difficulty in being directly applied to commercial processes due to low productivity and yield. In addition, the production has a disadvantage in that various by-products such as organic acids
 25 including lactic acid, alcohols including ethanol, and the

like, also occur, together with 2,3-butanediol in the fermentation process. The occurrence of by-products reduces yield of 2,3-butanediol for biomass, and requires enormous cost for separation and purification in a process of recovering 2,3-
5 butanediol from a culture fluid.

[9]

[10] Accordingly, research on the development of microorganisms associated with the production of 2,3-butanediol has mainly progressed by reducing the by-products. As a representative
10 method, Ji et al., achieved success by partially inhibiting occurrence of organic acids which are by-products, by exposing UV to a wild-type *Klebsiella oxytoca* strain as one of the physical chemical mutagenesis methods (Ji et al., *Biotechnol. Lett.*, 30: 731, 2008). In addition, there is an attempt to
15 improve the production of 2,3-butanediol by applying an ion injection (ion beam) method to a *Klebsiella pneumoniae* strain to increase a consumption speed of biomass (Ma et al., *Appl. Microbiol. Biotechnol.*, 82: 49, 2009). However, the above-developed strains are still insufficient to be directly applied
20 to commercial processes in view of productivity, final concentration, and yield of 2,3-butanediol.

[11]

[12] Therefore, the present inventors studied a recombinant microorganism not only having high productivity, high
25 concentration, and high yield of 2,3-butanediol, but also

having high selectivity of 2,3-butanediol, which generates less by-products, and found that a recombinant microorganism from which specific genes are deleted has a small amount of by-products while simultaneously having high selectivity and high productivity of 2,3-butanediol, and accordingly, completed the present invention.

【Disclosure】

【Technical Problem】

10 **[13]** An object of the present invention is to provide a recombinant microorganism having enhanced 2,3-butanediol producing ability, and a method for producing 2,3-butanediol using the same.

【Technical Solution】

15 **[14]** In order to achieve the object of the present invention,
[15] according to an exemplary embodiment of the present invention, there is provided a recombinant microorganism having enhanced 2,3-butanediol producing ability,

[16] wherein a pathway for converting pyruvate to acetyl-CoA, a
20 pathway for converting pyruvate to formic acid, or a pathway for converting pyruvate to lactate is inhibited in a microorganism having acetyl-CoA and lactate biosynthetic pathways.

[17]

[18] According to another exemplary embodiment of the present invention,

[19] there is provided a method for producing 2,3-butanediol, the method including:

5 **[20]** culturing the recombinant microorganism as described above; and recovering 2,3-butanediol from the cultured recombinant microorganism.

[21]

10 **【Advantageous Effects】**

[22] The recombinant microorganism according to the present invention may produce 2,3-butanediol with high selectivity and concentration.

15 **【Description of Drawings】**

[23] FIG. 1 is a diagram illustrating a pathway in which 2,3-butanediol is synthesized in a Klebsiella strain.

[24] FIG. 2 is an agarose gel image obtained by PCR amplification and electrophoresis in order to confirm that ldhA
20 which is a gene of lactate dehydrogenase associated with the occurrence of lactic acid is removed.

[25] FIG. 3 is an agarose gel image obtained by PCR amplification and electrophoresis in order to confirm that pflB
which is a gene of pyruvate-formate lyase associated with
25 occurrence of formate is removed.

[26] FIG. 4 illustrates fermentation results of a *Klebsiella oxytoca* strain of Comparative Example 1.

[27] FIG. 5 illustrates fermentation results of a recombinant *Klebsiella oxytoca* strain of Comparative Example 2.

5 [28] FIG. 6 illustrates fermentation results of a recombinant *Klebsiella oxytoca* strain of Example 1.

[29] FIGS. 7 to 11 illustrate batch fermentation results of a recombinant *Klebsiella oxytoca* strain of Example 1 depending on an agitation speed of 150 rpm (FIG. 7), 250 rpm (FIG. 8), 350
10 rpm (FIG. 9), and 450 rpm (FIG. 10) (FIG. 11: concentrations of 2,3-butanediol per hour depending on agitation speed).

[30] FIG. 12 illustrates fed-batch fermentation results of a recombinant *Klebsiella oxytoca* strain of Example 1 performed by retaining an agitation speed at 450 rpm under aerobic
15 condition.

[31] FIG. 13 illustrates fed-batch fermentation results of a recombinant *Klebsiella oxytoca* strain of Example 1 performed by controlling an agitation speed.

20 **【DESCRIPTION OF THE EXEMPLARY EMBODIMENTS】**

[32] The present invention relates to:

[33] a recombinant microorganism having enhanced 2,3-butanediol producing ability,

[34] wherein a pathway for converting pyruvate to acetyl-CoA, a
25 pathway for converting pyruvate to formic acid, or a pathway

for converting pyruvate to lactate is inhibited in a microorganism having acetyl-CoA and lactate biosynthetic pathways.

[35]

5 **[36]**

[37] In addition, the present invention relates to:

[38] a method for producing 2,3-butanediol, the method including: culturing the recombinant microorganism of the present invention; and

10 **[39]** recovering 2,3-butanediol from the cultured recombinant microorganism.

[40]

[41] Hereinafter, the present invention is described in detail.

[42]

15 **[43]** Recombinant microorganism having enhanced producing ability of 2,3-butanediol

[44] The recombinant microorganism of the present invention is

[45] a recombinant microorganism having enhanced 2,3-butanediol producing ability,

20 **[46]** wherein the pathway for converting pyruvate to acetyl-CoA, the pathway for converting pyruvate to formic acid, or the pathway for converting pyruvate to lactate is inhibited in a microorganism having acetyl-CoA and lactate biosynthetic pathways.

25 **[47]**

[48]

[49] In addition, the recombinant microorganism of the present invention is a recombinant microorganism from which a gene encoding alcohol dehydrogenation enzyme (aldehyde/alcohol
5 dehydrogenase), that is, adhE, is not deleted.

[50]

[51] Preferably, as illustrated in FIG. 1, the recombinant microorganism of the present invention is a recombinant microorganism in which the pathway for converting pyruvate to
10 acetyl-CoA, the pathway for converting pyruvate to formic acid, or the pathway for converting pyruvate to lactate is inhibited while having acetyl-CoA and lactate biosynthetic pathways.

[52]

[53] In addition, the recombinant microorganism of the present
15 invention has high selectivity, yield, concentration, and productivity of 2,3-butanediol. That is, in the recombinant microorganism of the present invention, selectivity of 2,3-butanediol is 70% or more, preferably, 80% or more, and a yield of 2,3-butanediol is 0.35 g/g or more based on batch culture or
20 fed-batch culture. Further, due to the recombination, the recombinant microorganism of the present invention better inhibits a producing ability of formic acid, acetic acid, or ethanol as compared to a wild-type microorganism.

[54]

25 [55] Biosynthetic pathway of acetyl-CoA

[56] A biosynthetic pathway of acetyl-CoA of the present invention means a pathway in which acetyl-CoA is synthesized from a specific metabolite in a microorganism. The biosynthetic pathway of acetyl-CoA of the present invention may
5 be a pathway for synthesizing acetyl-CoA from pyruvate, and the like.

[57]

[58] Biosynthetic pathway of lactate

[59] A biosynthetic pathway of lactate of the present invention
10 means a pathway in which lactate is synthesized from a specific metabolite in a microorganism. The biosynthetic pathway of lactate of the present invention may be a pathway for synthesizing lactate from pyruvate, and the like.

[60]

15 [61] Microorganism having acetyl-CoA and lactate biosynthetic pathways

[62] The microorganism having the biosynthetic pathway of acetyl-CoA and the biosynthetic pathway of lactate of the present invention is not specifically limited as long as it has
20 the above-described biosynthetic pathways. In addition, the microorganism of the present invention may be a microorganism having the acetyl-CoA biosynthetic pathway and the lactate biosynthetic pathway in a wild-type or a recombinant microorganism having the acetyl-CoA biosynthetic pathway and
25 the lactate biosynthetic pathway by gene recombination. For

example, the microorganisms of the present invention may be included in Klebsiella genus, Bacillus genus, Serratia genus, or Enterobacter genus, preferably, Klebsiella oxytoca (K. oxytoca), Klebsiella pneumoniae (K. pneumoniae), and the like, 5 and the most preferably, Klebsiella oxytoca (K. oxytoca).

[63]

[64] Inhibition of pathway for converting pyruvate to acetyl-CoA

[65] Pyruvate-formate lyase controls conversion of pyruvate to 10 acetyl-CoA. A pathway for converting pyruvate to acetyl-CoA may be inhibited by inhibiting the pyruvate-formate lyase. The inhibition of the pyruvate-formate lyase may be achieved by inhibition of expression of the pyruvate-formate lyase, inhibition of enzyme activity of the pyruvate-formate lyase, 15 and the like. For example, the pyruvate-formate lyase may be inhibited by selecting appropriate methods by a person skilled in the art, the appropriate methods such as deletion of pflB which is a gene encoding the pyruvate-formate lyase, development of mutants in the gene (a mutant inhibiting 20 expression of a normal gene by mutating, substituting or deleting partial bases from the gene, or by introducing partial bases into the gene), regulation of gene expression in a transcription process or a translation process, and the like.

[66]

[67] Inhibition of pathway for converting pyruvate to formic acid

[68] Pyruvate-formate lyase controls conversion of pyruvate to formic acid. A pathway for converting pyruvate to formic acid
 5 may be inhibited by inhibiting the pyruvate-formate lyase. The inhibition of the pyruvate-formate lyase may be achieved by inhibition of expression of the pyruvate-formate lyase, inhibition of enzyme activity of the pyruvate-formate lyase, and the like. For example, the pyruvate-formate lyase may be
 10 inhibited by selecting appropriate methods by a person skilled in the art, the appropriate methods such as deletion of pflB which is a gene encoding the pyruvate-formate lyase, development of mutants in the gene (a mutant inhibiting expression of a normal gene by mutating, substituting or
 15 deleting partial bases from the gene, or by introducing partial bases into the gene), regulation of gene expression in a transcription process or a translation process, and the like.

[69]

[70] Inhibition of pathway for converting pyruvate to lactate

20 **[71]** Lactate dehydrogenase controls conversion of pyruvate to lactate A pathway for converting pyruvate to lactate may be inhibited by inhibiting the lactate dehydrogenase. The inhibition of the lactate dehydrogenase may be achieved by inhibition of expression of the lactate dehydrogenase,
 25 inhibition of enzyme activity of the lactate dehydrogenase, and

the like. For example, the lactate dehydrogenase may be inhibited by selecting appropriate methods by a person skilled in the art, the appropriate methods such as deletion of *ldha* which is a gene encoding the lactate dehydrogenase, development
5 of mutants in the gene (a mutant inhibiting expression of a normal gene by mutating, substituting or deleting partial bases from the gene, or by introducing partial bases into the gene), regulation of gene expression in a transcription process or a translation process, and the like.

10 **[72]**

[73] Alcohol dehydrogenation enzyme

[74] An alcohol dehydrogenation enzyme (aldehyde/alcohol dehydrogenase) controls a pathway for converting acetyl-CoA to ethanol. Accordingly, there is a case of promoting an increase
15 of production of 2,3-butanediol by deleting *adhE* which is a gene encoding alcohol dehydrogenation enzyme to inhibit occurrence of ethanol (Ji et al., Appl. Microbiol. Biotechnol., 85: 1751, 2010). However, in the recombinant microorganism of the present invention, when *adhE* is additionally deleted, a
20 production amount, selectivity, and productivity of 2,3-butanediol are remarkably decreased. Accordingly, *adhE* which is a gene encoding the alcohol dehydrogenation enzyme is not deleted in the present invention.

[75]

25 **[76]** Method for producing 2,3-butanediol

[77] A method for producing 2,3-butanediol of the present invention includes culturing the recombinant microorganism of the present invention; and recovering 2,3-butanediol from the cultured recombinant microorganism.

5 **[78]**

[79] The culturing is performed under aerobic condition, preferably, microaerobic condition. For example, the culturing is performed while supplying oxygen, that is, air during the culturing, and specifically, the supplying of the oxygen may be
10 performed by agitating. Preferably, the culturing is performed while agitating at an agitation speed of 450 rpm or less, more preferably, 50 to 450 rpm, and still more preferably, 150 to 450 rpm.

[80]

15 **[81]** Preferably, the culturing may control productivity of 2,3-butanediol by controlling an oxygen supply amount. As a method for controlling the oxygen supply amount during the culturing, for example, the culturing of the present invention may be performed while agitating, and the culturing may control
20 productivity of 2,3-butanediol by controlling the agitation speed during the culture. For example, when a concentration of acetoin is increased to be 5 g/L or more, preferably, 10 g/L or more, an agitation speed may be decreased, which increases concentration and productivity of 2,3-butanediol and inhibits
25 occurrence of by-products.

[82]

【Best Mode】

[83] Various advantages and features of the present invention and methods accomplishing thereof will become apparent from the following description of embodiments with reference to the accompanying drawings. However, the present invention is not limited to exemplary embodiment disclosed herein but will be implemented in various forms. The exemplary embodiments are provided by way of example only so that a person of ordinary skilled in the art can fully understand the disclosures of the present invention and the scope of the present invention. Therefore, the present invention will be defined only by the scope of the appended claims.

[84]

15 **[85]** Material and Method

[86] -2,3-butanediol concentration (g/L): Amount of 2,3-butanediol to be produced per unit volume

[87] -2,3-butanediol yield (g/g): Production amount (g) of 2,3-butanediol / carbon source (g) × 100

20 **[88]** -2,3-butanediol productivity (g/L/h): Amount of 2,3-butanediol to be produced per unit time and unit volume

[89] Selectivity (%) of 2,3-butanediol: Production amount (g) of 2,3-butanediol / (production amounts (g) of 2,3-butanediol, ethanol, acetoin, succinic acid, lactate formate, and acetic acid) × 100

25

[90]

[91] <Experimental Example 1> Production of recombinant microorganism

[92] In order to inactivate a target gene of *Klebsiella oxytoca* produced with a DNA fragment including a homologous region of the target gene, a recombinant mechanism of bacteria was used, and the homologous region of the gene to be removed was amplified by PCR. Then, the corresponding DNA fragment including the homologous region was transferred to bacteria, and then, the target gene was removed by the recombinant mechanism by recombinase between the homologous region of the gene in the DNA fragment and a gene in chromosome of *Klebsiella oxytoca*.

[93] First, in order to perform cloning of lactate dehydrogenase of *Klebsiella oxytoca*, a homologous region 1 (SEQ ID NO: 2) of *ldhA* (SEQ ID NO: 1) which is a target gene was amplified with primers of SEQ ID NOs: 3 and 4 by PCR. In addition, a homologous region 2 (SEQ ID NO: 5) thereof was amplified with primers of SEQ ID NOs: 6 and 7 by PCR. Then, both of the homologous regions 1 and 2 were simultaneously used as a template and amplified by PCR, thereby completing a DNA fragment (SEQ ID NO: 8) including the homologous regions 1 and 2.

[94] Meanwhile, in order to perform cloning of a homologous region of pyruvate-formate lyase of *Klebsiella oxytoca*, a

homologous region 1 (SEQ ID NO: 10) of pflB (SEQ ID NO: 9) which is a target gene was amplified with primers of SEQ ID NOS: 11 and 12 by PCR. In addition, a homologous region 2 (SEQ ID NO: 13) thereof was amplified with primers of SEQ ID NOS: 14
5 and 15 by PCR. Then, both of the homologous regions 1 and 2 were simultaneously used as a template and amplified by PCR, thereby completing a DNA fragment (SEQ ID NO: 16) including the homologous regions 1 and 2 (Table 1). In order to increase recombination probability of the target gene, the completed DNA
10 fragment may include an anti-biotic resistance gene, and the like. Further, the DNA fragment may include a sacB gene encoding a levansucrase enzyme, in order to remove the recombinant anti-biotic resistance gene in chromosome.

[95]

15 **[96]** [Table 1]

SEQ ID NO .	SEQUENCES
1	ATGAAAATCGCTGTGTATAGTACAAAACAGTACGACAAGAAGTATCTGCAGCATGTTAATGATG CATATGGCTTTGAACTGGAGTTTTTTGACTTCCTGCTAACCAGAAAAACCGCCAAAACCGCCA ACGGCTGTGAAGCGGTGTGTATCTTCGTAAACGATGACGGTAGCCGCCCGGTACTTGAAGAA CTGAAAGCCCCACGGCGTGCAGTACATCGCGCTGCGCTGCGCGGGTTCAACAACGTTGACC TCGATGCCGCCAAAGAGCTGGGCCTGCGGGTGGTGCGCGTCCCGGCCTACTCGCCGGAAG CGGTCGCTGAGCACGCGATCGGCATGATGATGTCGCTGAACCGCCGCATTACCGTGCCTA TCAGCGCACCCGCGACGCGAACTTCTCTCTGGAAGGGCTGACCGGTTTCACCATGCACGGT AAAACCGCCGGCGTTATTGGCACCGGTAAATCGCGCTCGCCGCGCTGCGCATTCTTAAAG GCTTCGGTATGCGTCTGCTGGCGTTTGATCCCTACCCAAGCGCCGCCGCGCTGGATATGGG CGTGGAGTATGTCGATCTTGAAACCCGTACCGGGAGTCCGATGTTATCTCACTGCACTGCC CACTGACCGATGAAAACCTACCATTTGCTGAACCATGCCGCTTCGATCGCATGAAAGACGGG GTGATGATCATCAACACCAGCCGCGCGCGCTCATCGATTGCGAGGCAGCGATCGACGCC TGAAGCATCAGAAAATTGGCGCGCTGGGGATGGACGTGTATGAGAACGAACGCGATCTGTTT TTTGAAGATAAGTCTAATGACGTGATTGAGGATGATGTGTTCCGCCGTCTCTCCGCCTGCCAT AACGTCTGTTTACCGGTCACCGGCGTTTCTGACCGCGGAAGCGTTGATCAGCATTTCGCA AACCACCTCGACAACCTGCGTCAAGTGGATGCAGGCGAAACCTGTCCTAACGCACTGGTCT GA
2	ATGACGTTGCTAAATCCTGCGCCGTATCTCGCTGCTGATCCCGGGCACCTCCGGGCTACT GCTGTTGCGGCACCCCTGGCATCGGCCAGCCCGGGACATTTCTGTTAATGTGGATGAGCGCC AGCCTCGGCGCTATCGGCGGATTCTGGCTCTCGTGGCTGACGGGCTACCGCTACCGGTACC ATCTGCATCGTATCCGCTGGCTTAATGCCGAACGCCTCGCTCGCGGCCAGTTGTTCTGCGC CGCCACGGCGCGTGGGCAGTCTTTTTAGCCGCTTTCTCTCTCGCTTCGCGCCACCGTGC CGCTGGTAACCGGCGCCAGCGGCACCTCTCTCTGGCAGTTTCAGCTCGCCAAACGTCAGCTC CGGGCTGCTCTGGCCGCTGATCCTGCTGGCGCCAGGCGCGTTAAGCCTCAGCTTTTGATGA

	AAGGTATTGTCTTTTAAAGAGATTCTTAACACCGGATATGCTCTAGAATTATTACTATAACCT GCTGATTAACTAGTTTTTAACATTTGTAAGATTATTTAATTATGCTACCGTGACGGTATTATCA CTGGAGAAAAGTCTTTTTTCCTTGCCCTTTTGTGC
3	Ko_ldhA_FP1 - CACGGATCCATGACGTTGCTAAATCCTGC
4	Ko_ldhA_RP1 - GCACAAAAGGGCAAGGAAAAAGACTTTTCTCCAGTGATA
5	TATCACTGGAGAAAAGTCTTTTTTCCTTGCCCTTTTGTGCTCCCCCTTCGCGGGGGGCACATT CAGATAATCCCCACAGAAATTGCCTGCGATAAAGTTACAATCCCTTCATTTATTAATACGATAA ATATTTATGGAGATTAAATGAACAAGTATGCTGCGCTGCTGGCGGTGGGAATGTTGCTATCGG GCTGCGTTTATAACAGCAAGGTGTCGACCAGAGCGGAACAGCTTCAGCACCCCGTTTTGTG CTGACCAGCGTTAACGGGCAGCCGCTGAATGCCGCGGATAAGCCGCAGGAGCTGAGCTTC GGCGAAAAGATGCCATTACGGGCAAGATGTCTGTTTCAGGTAATATGTGCAACCGCTCAG CGGCACGGGCAAAGTCTCTGACGGCGAGCTGAAGGTTGAAGAGCTGGCAATGACCCGCATG CTCTGCACGGACTCGCAGCTTAACGCCCTGGACGCCACGCTGAGCAAAATGCTGCGCGAAG GCGCGCAGGTCGACCTGACGGAAACGCAGCTAACGCTGGCGACCGCCGACCAGACGCTGG TGTATAAGCTCGCCGACCTGATGAATTAATAATTA
6	Ko_ldhA_FP2 - TATCACTGGAGAAAAGTCTTTTTTCCTTGCCCTTTTGTGC
7	Ko_ldhA_RP2 - CCTGCGGCCGCTAATTATTAATTCATCAGGTC
8	ATGACGTTGCTAAATCCTGCGCGTCATCTCGCTGCTGATCCCGGGCACCTCCGGGCTACT GCTGTTTCGGCACCCCTGGCATCGGCCAGCCCGGACATTTCTGTTAATGTGGATGAGCGCC AGCCTCGGCGCTATCGGCGGATTCTGGCTCTCGTGGCTGACGGGCTACCGCTACCGGTACC ATCTGCATCGTATCCGCTGGCTTAATGCCGAACGCCTCGCTCGCGGCCAGTTGTTCTCGCGC CGCCACGGCGCGTGGGCAGTCTTTTTAGCCGCTTTCTCTCCGCTTCGCGCCACCGTGC CGCTGGTAACCGGCGCCAGCGGCACCTCTCTGCGCAGTTTCAGCTCGCCAACGTCAGCTC

[97]

	CGGGCTGCTCTGGCCGCTGATCCTGCTGGCGCCAGGCGCGTTAAGCCTCAGCTTTTGATGA AAGGTATTGTCTTTTAAAGAGATTCTTAACACCGCGATATGCTCTAGAATTATTACTATAACCT GCTGATTAACTAGTTTTTAACATTTGTAAGATTATTTAATTATGCTACCGTGACGGTATTATCA CTGGAGAAAAGTCTTTTTCTTGCCCTTTTGTGCTCCCCCTTCGCGGGGGGCACATTCAGAT AATCCCCACAGAAATTGCCTGCGATAAAGTTACAATCCCTTCATTTATTAATACGATAAATATT ATGGAGATTAAATGAACAAGTATGCTGCGCTGCTGGCGGTGGGAATGTTGCTATCGGGCTGC GTTTATAACAGCAAGGTGTCGACCAGAGCGGAACAGCTTCAGCACCCCGTTTTGTGCTGAC CAGCGTTAACGGGCAGCCGCTGAATGCCGCGATAAGCCGCAGGAGCTGAGCTTCGGCGA AAAGATGCCCATACGGGCAAGATGTCTGTTTCAGGTAATATGTGCAACCGCTTCAGCGGCA CGGGCAAAGTCTCTGACGGCGAGCTGAAGGTTGAAGAGCTGGCAATGACCCGCATGCTCTG CACGGACTCGCAGCTTAACGCCCTGGACGCCACGCTGAGCAAAATGCTGCGCGAAGGCGC GCAGGTGACCTGACGGAAACGCAGCTAACGCTGGCGACCGCCGACCAGACGCTGGTGTA TAAGCTCGCCGACCTGATGAATTAATAATTA
9	ATGTCCGAGCTTAATGAAAAGTTAGCCACAGCCTGGGAAGGTTTTGCGAAAGGTGACTGGCA GAACGAAGTCAACGTCCGCGACTTCATCCAGAAAACTATACCCCGTACGAAGGTGACGAGT CCTTCCTGGCTGGCGCAACTGACGCGACCACCAAGCTGTGGGACACCGTAATGGAAGGCGT TAAACAGGAAAACCGCACTCACGCGCCTGTTGATTTTGATACTTCCCTGCATCCACCATCAC TTCTCATGACGCTGGCTACATCGAGAAAGGTCTCGAGAAAATCGTTGGTCTGCAGACTGAAG CTCCGCTGAAACGCGCGATTATCCCGTTCGGCGGCATCAAAATGGTCGAAGGTTCTGCAAA GCGTACGATCGCGAGCTGGACCCGATGCTGAAGAAAATCTTCACTGAATACCGTAAACTCA CAACCAGGGCGTGTTTGACGTTTACACCAAGACATCCTGAAGTCCCGTAAATCTGGTGTCT GACCGGTCTGCCGATGCCTATGGCCGTGGTCGTATCATCGGTGACTACCGTCGCGTTGCG CTGTACGGTATCGACTTCCTGATGAAAGACAAATACGCTCAGTTCGTTTCTCTGCAAGAGAAA CTGGAACGCGCAAGATCTGGAAGCAACCATCCGTCTGCGCGAAGAAATCTCTGAACAGCA

[98]

CCGCGCGCTGGGTCAGATCAAAGAAATGGCGGCTAAATATGGCTGCGATATCTCTGGTCCTG
 CTACCACCGCTCAGGAAGCTATCCAGTGGACCTACTTCGGTTACCTGGCTGCCGTAATCT
 CAGAACGGCGCGCAATGTCCTTCGGTCGTACCTCCAGCTTCTTGACATCTTCATCGAACG
 TGACCTGAAAGCCGGTAAATCACCGAGCAAGACGCACAGGAAATGATTGACCACCTGGTCA
 TGAAACTGCGTATGGTTCGTTTCCTGCGTACCCCTGAATATGATGAACTGTTCTCTGGCGACC
 CGATCTGGGCAACAGAATCTATCGGCGGTATGGGCGTTGACGGCCGTAATCTGGTCACCAA
 AACAGCTTCCGTTTCCTGAACACCCTGTACACCATGGGGCCGTCTCCGAGCCGAACATCA
 CCATTCTGTGGTCTGAAAACTGCCGCTGAGCTTCAAAAAATACGCCGCGAAAGTGTCCATC
 GATACCTCTTCTCTGCGTACGAGAACGATGACCTGATGCGTCCTGACTTCAACAACGATGAC
 TACGCTATCGCTTGTGCGTAAGCCCGATGGTTGTTGGTAAGCAAATGCAGTTCTTCGGCGC
 GCGTGCTAACCTGGCGAAAACCATGCTGTACGCAATCAACGGCGGCGTTGATGAAAACTGA
 AAATGCAGGTTGGTCTAAATCTGAACCGATCAAAGGCGACGTTCTGAACTTCGACGAAGTGA
 TGGACCGCATGGATCACTTCATGGACTGGCTGGCTAAACAGTACGTCACTGCGCTGAACATC
 ATCCACTACATGCACGACAAGTACAGCTACGAAGCTTCCCTGATGGCGCTGCACGACCGTGA
 TGTATCCGCACCATGGCATGTGGTATCGCAGGTCTTCCGTTGCGGCTGACTCCCTGTCTG
 CAATCAAATATGCGAAAGTTAAACCGATTCTGTGACGAAAACGGTCTGGCTGTGACTTCGAAA
 TCGAAGGCGAATACCCGAGTTTGGTAACAACGACTCTCGCGTCGATGATATGGCCGTTGAC
 CTGGTTGAACGTTTCATGAAGAAAATTGAGAACTGCACACCTACCGCAACGCTATCCCGACT
 CAGTCCGTTCTGACCATCACCTCTAACGTTGTGTATGGTAAGAAAACCGGCAACACCCCTGA
 CGGTCGTCGCGCTGGCGCTCCGTTCCGACCCAGGTGCTAACCCGATGCACGGCCGTGACCA
 GAAAGGCGCTGTTGCCTCTCTGACCTCCGTTGCAAACTGCCGTTTGCTTACGCGAAAGATG
 GTATTTCTTACACCTTCTCTATCGTGCCGAACGCGCTGGGTAAAGACGACGAAGTTCGTA
 CTAACCTCGCCGGCCTGATGGATGGTACTTCCACCACGAAGCGTCCATCGAAGGCGGTCA
 GCATCTGAACGTCAACGTTATGAACCGCGAAATGCTGCTCGACGCGATGGAAAACCCGGAAA

[99]

	AATATCCGCAGCTGACCATCCGCGTATCCGGCTACGCAGTACGTTTTAACTCCCTGACTAAAG AACAGCAGCAGGACGTTATTACTCGTACCTTCACTCAGACCATGTAA
10	GGGTCAACTGGCGAAAACTGGCTCAACGTCTATGTTGGTAACCTGATTGGTTGCTTACTGTT TGTAATTGCTGATGTGGCTTCAGGCGAATATATGACTGCCAACGGTCAATGGGGACTTAACGT TCTGCAAACCGCCGACCACAAAATGCACCATACTTTTGTGAAGCCGTGTGCCTGGGTATCCT GGCAAACCTGATGGTCTGCCTTGCGGTATGGATGAGTTACTCCGGCCGTAGCCTGATGGATA AAGCCATGATTATGGTTTTACCGGTGGCAATGTTTGTGCCAGCGGGTTTGAACACAGTATCG CGAACATGTTTATGATCCCGCTGGGTATCGTTATCCGCGACTTTGCAAGCCCGGAATTCTGGA CCGCAGTTGGTTCAACTCCGGAAGTTTCTCTACCTGACCGTCATGAACCTCATCACTGATA ACCTGATTCCGGTAACATCGGGAACATCATCGCGGTGGTCTGCTGGTTGGGTTGACATAC TGGGTCAATTAACCTGCGTGGCGACGACCATCACTAAGGGTTGTTTCAGGCAGTAAATAAAAA TCCACTTAAGAAGGTAGGTGTTACATGTCCGAGCTTAATGAAAAGTTACAGCAGCAGGACGTT ATTACTC
11	Ko_pflB_FP1 - ATCGGATCCGGTCAACTGGCGAAAACTGGCTCAACGT
12	Ko_pflB_RP1 - GAGTAATAACGTCCTGCTGCTGTAACITTTTCATTAAGCTCGGACAT
13	ATGTCCGAGCTTAATGAAAAGTTACAGCAGCAGGACGTTATTACTCGTACCTTCACTCAGACC ATGTAATGGTATTGACTGAAATCGTACAGTAAAAAGCGTACAATAAAGGCTCCACGCAAGTGG GGCCTTTTATGCAATATCATCCTGCCCCAGTCTCTTTGTCTGCTGTCTATACITTTATGGATAA CAGCCAAAACAGACTCGACATAGCCTTTGAGCTGTGCATCTACATAGGCCCCGGATGGGCCA AATTCGGAGATATCACCGCAATGTCAACAATTGGTCGCATTCACCTCTTGAATCCTGTGGCA CCGTCGATGGCCCGGGGATTGCTTTATCACCTTCTCCAGGGCTGCCTGATGCGCTGCCTC TATTGCCACAACCGCGATACCTGGGATACCCACGGCGGCAAAGAGATTACCGTTGAAGAGCT GATGAAAGAGGTGGTGACCTATCGCCACTTTATGAACGCTTCCGGCGGCGGCGTGACGGCA TCCGGCGGCGAGGCTATCCTGCAGGCCGAATTTGTTGCGGACTGGTCCGCGCCTGTAAGA AAGAAGGTATTACACCTGTCTCGATACCAACGGCTTTGTGCGCGCTACGATCCGGTTATTG ATGAACTGCTGGAGGTCACCGACCTGGTGATGCTCGATCTCAAGC

[100]

14	Ko_pflB_FP2 - ATGTCCGAGCTTAATGAAAAGTTACAGCAGCAGGACGTTATTACTC
15	Ko_pflB_RP2 - ACTGCGGCCGCGCTTGAGATCGAGCATCACCAGGTCGGTGA
16	GGGTCAACTGGCGAAAACTGGCTCAACGTCTATGTTGGTAACCTGATTGGTTGCTTACTGTT TGTATTGCTGATGTGGCTTTCAGGCGAATATATGACTGCCAACGGTCAATGGGGACTTAACGT TCTGCAAACCGCCGACCACAAAATGCACCATACTTTTGTGAAGCCGTGTGCCTGGGTATCCT GGCAAACCTGATGGTCTGCCTTGGGTATGGATGAGTTACTCCGGCCGTAGCCTGATGGATA AAGCCATGATTATGGTTTTACCGGTGGCAATGTTTGTGCCAGCGGGTTGAGCACAGTATCG CGAACATGTTTATGATCCCGCTGGGTATCGTTATCCGCGACTTTGCAAGCCCGGAATTCTGGA CCGCAGTTGGTTCAACTCCGGAAGTTTCTCTCACCTGACCGTCATGAACCTTCATCACTGATA ACCTGATTCCGGTAACTATCGGGAACATCATCGGCGGTGGTCTGCTGGTTGGGTTGACATAC TGGGTCATTTACCTGCGTGGCGACGACCATCACTAAGGGTTGTTTCAGGCAGTAAATAAAAAA TCCACTTAAGAAGGTAGGTGTTACATGTCCGAGCTTAATGAAAAGTTACAGCAGCAGGACGTT ATTACTCGTACCTTCACTCAGACCATGTAATGGTATTGACTGAAATCGTACAGTAAAAAGCGTA CAATAAAGGCTCCACGCAAGTGGGGCCTTTTTAGCAATATCATCCTGCCCCAGTCTCTTTTGT CTGCTGTCTATACTTTATGGATAACAGCCAAAACAGACTCGACATAGCCTTTGAGCTGTGCAT CTACATAGGCCCCGGATGGGCCAAATTCGGAGATATCACC GCAATGTCAACAATTGGTCGCA TTCACCTCCTTTGAATCCTGTGGCACCGTCGATGGCCCGGGGATTGCTTTATCACCTCTCTCC AGGGCTGCCTGATGCGCTGCCTCTATTGCCACAACCGCGATACCTGGGATACCCACGGCGG CAAAGAGATTACCGTTGAAGAGCTGATGAAAGAGGTGGTGACCTATGCCACTTTATGAACG CTTCCGGCGGCGGCGTGACGGCATCCGCGGCGAGGCTATCCTGCAGGCCGAATTTGTTC GCGACTGGTTCGCGCCTGTAAGAAAGAAGGTATTCATACCTGTCTCGATACCAACGGCTTT GTGCGCCGCTACGATCCGGTTATTGATGAACTGCTGGAGGTCACCGACCTGGTGATGCTCG ATCTCAAGC

[101]

[102]

[103] Development of recombinant microorganism

[104] The produced DNA fragments were transferred to *Klebsiella*
5 *oxytoca* by using electroporation (25 uF, 200 Ω , 18 kV/cm), and

the target gene was capable of being removed by using a recombinant mechanism of the microorganism.

[105] The DNA fragment including the homologous region of an *ldha* gene was transferred to a wild-type *Klebsiella oxytoca* to produce a recombinant *Klebsiella oxytoca* from which the *ldha* gene was removed (Comparative Example 2). Meanwhile, after the *ldhA* gene was removed from the wild-type *Klebsiella oxytoca*, the DNA fragment including the homologous region of a *pflB* gene was transferred to produce *Klebsiella oxytoca* from which the *pflB* gene was additionally removed together with the removal of the *ldha* gene (Example 1).

[106]

[107] Electroporation was performed, and the recombinant microorganisms were cultured at 30°C for 1 hour and stabilized. Then, the recombinant microorganisms were cultured by spreading them on an LB composite solid medium containing anti-biotics (such as kanamycin, chloramphenicol, and the like) at 37°C, respectively.

[108] Then, colony PCR was performed on the colonies grown on the solid medium, and it was confirmed that the corresponding genes were removed from the colonies (FIG. 2 illustrates deletion of *ldha* and FIG. 3 illustrates deletion of *pflB*, respectively). Here, in order to confirm that the *ldha* gene was removed, PCR was performed with primers of SEQ ID NOs: 17 and 18, and in order to confirm that the *pflB* gene was removed,

PCR was performed with primers of SEQ ID NOs: 19 and 20 (Table 2).

[109]

[110] [Table 2]

SEQ ID NO.	SEQUENCES
17	Ko_ldhA_Sc_FP - CCATCTGCATCGTATCCGCTGGCTTAAT
18	Ko_ldhA_Sc_RP - GCTGAAGCGGTTGCACATATTACCTG
18	Ko_pflB_Sc_FP - ACCATCACTAAGGGTTGTTTCAGGCAGTAA
20	Ko_pflB_Sc_RP - GCTAAAAAGGCCCACTTGCGTGGAGCCTT

5 [111]

[112] In next test, a wild-type *Klebsiella oxytoca* was used as Comparative Example 1. Meanwhile, a recombinant *Klebsiella oxytoca* from which *ldhA* and *adhE* genes were deleted was produced by additionally deleting the *adhE* gene from the recombinant *Klebsiella oxytoca* from which *ldhA* was deleted according to Comparative Example 2, the *adhE* gene being a gene encoding an alcohol dehydrogenation enzyme (aldehyde/alcohol dehydrogenase) directly involved in occurrence of ethanol. The produced recombinant *Klebsiella oxytoca* from which *ldhA* and *adhE* genes were deleted was tested as Comparative Example 3. The *adhE* gene was removed in a similar manner to the method for removing the *ldhA* gene or the *pflB* gene. The DNA fragment including the homologous region of the *adhE* gene was transferred to the *Klebsiella oxytoca* from which the *ldhA* was

removed, thereby producing *Klebsiella oxytoca* from which the adhE gene was additionally removed together with the removal of the ldhA gene.

[113]

5 **[114]** <Experimental Example 2>

[115] Batch culture was performed by using microorganisms of Example 1, Comparative Examples 1 and 2 prepared in Experimental Example 1 above. First, *Klebsiella oxytoca* strains were inoculated in 250 ml of composite medium including
10 9 g/L glucose (50 mM) and cultured at 37°C for 16 hours, and then the culture fluids were inoculated in 3L of composite medium and cultured. Here, the fermentation was performed under a micro-aerobic condition (an aerobic speed of 1 vvm and an agitation speed of 150 rpm), at an initial glucose
15 concentration of 90 g/L, pH 6.8, and a culture temperature of 37°C. 5N NH₄OH was used to control the pH during the fermentation. Samples for the *Klebsiella oxytoca* strains were taken during the fermentation, and growth rates were obtained by measuring optical density 600 (OD 600) of the taken samples.
20 Then, the taken samples were centrifuged at 13,000 rpm for 10 minutes, and metabolites of supernatants and concentration of 2,3-butanediol thereof were analyzed by liquid chromatography (HPLC).

[116]

[117] As a result, in the strain of Comparative Example 2, a production amount of 2,3-butanediol was 29.91 g/L, and a production yield of 2,3-butanediol (gram of 2,3-butanediol / gram of glucose) was 0.32. In addition, in the strain of Comparative Example 2, a productivity (g/L/h) of 2,3-butanediol was 1.07, and selectivity thereof was 59 %. As compared to Comparative Example 1 which is a wild-type *Klebsiella oxytoca*, it was confirmed that the recombinant strain of Comparative Example 2 improved all of production concentration, production yield, productivity and selectivity of 2,3-butanediol while decreasing lactic acid production and increasing producing ability of 2,3-butanediol. However, the recombinant *Klebsiella oxytoca* of Comparative Example 2 still produced excessive amounts of by-products including formic acid and ethanol, which is considered as a cause of inhibiting a production concentration, a production yield, selectivity, and the like, of 2,3-butanediol of Comparative Example 2 (FIG. 4 illustrates Comparative Example 1 and FIG. 5 illustrates Comparative Example 2).

[118]

[119] Meanwhile, as compared to Comparative Example 2 (*Klebsiella oxytoca* Δ ldhA, 2,3-BDO 29.91 g/L), in the recombinant strain of Example 1, a production amount of 2,3-butanediol was increased to be 39.17 ± 1.51 g/L. In addition, it was confirmed in the recombinant strain of Example 1 that a

production amount of formic acid was decreased by 90% or more, and a production amount of ethanol was decreased by 73% or more, and a production yield of 2,3-butanediol (gram of 2,3-butanediol / gram of glucose) was also significantly increased
5 from 0.32 to 0.45. Upon considering that a theoretical yield of 2,3-butanediol (a yield when it is assumed that all glucose supplied to *Klebsiella oxytoca* are converted to 2,3-butanediol) is 0.5, a yield of Example 1 (the theoretical yield thereof is 90%) was remarkably high.

10 **[120]**

[121] Accordingly, it could be confirmed that occurrence of by-products was remarkably decreased, and 2,3-butanediol was produced with high purity by simultaneously removing by-products such as formic acid, ethanol, and the like, through
15 removal of the *ldhA* and the *pflB* genes. Regarding this, it could be confirmed that the removal of the gene (*ldhA*) encoding the lactic acid dehydrogenation enzyme (lactate dehydrogenase) of *Klebsiella oxytoca* and the gene (*pflB*) encoding the pyruvate-formate lyase is significantly important for
20 production of 2,3-butanediol in various stages of pathways for producing 2,3-butanediol (FIG. 6) (Table 3).

[122]

[123] [Table 3]

[124]

25

Strain	2,3-butanediol				Concentration (g/L) of by-products				
	Concentration (g/L)	Yield (g/g)	Productivity (g/L/h)	Selectivity (%)	Lactate	Succinic Acid	Ethanol	Formic Acid	Acetic Acid
Comparative Example 1	17.31	0.18	0.58	27	32.20	1.92	1.61	3.88	1.27
Comparative Example 2	29.91	0.32	1.07	59	1.93	2.27	3.54	6.11	0.92
Example 1	39.17 ± 1.51	0.45	0.43 ± 0.02	83.33 ± 1.53	3.24	1.77	0.53	0.97	0.15

[125] <Experimental Example 3>

[126] Whether or not a producing ability of 2,3-butanediol was improved was tested at the time of removing a gene involved in occurrence of by-products in competition with biosynthesis of 2,3-butanediol in a 2,3-butanediol-producing microorganism. *adhE* is a gene encoding an alcohol dehydrogenation enzyme (aldehyde/alcohol dehydrogenase) directly involved in occurrence of ethanol which is a by-product in production of 2,3-butanediol. Accordingly, a recombinant *Klebsiella oxytoca* of Comparative Example 3 from which *ldhA* and *adhE* were removed was cultured and compared to that of Example 1. Here, culture conditions of the recombinant microorganism of Comparative Example 3 were the same as those of Experimental Example 2.

[127]

[128] As a result, in Comparative Example 3 from which *ldhA* and *adhE* were simultaneously removed from *Klebsiella oxytoca*, a production amount of 2,3-butanediol was rather decreased to be 25.96g/L as compared to Comparative Example 2 (*Klebsiella oxytoca* Δ *ldhA*, 2,3-BDO 29.91 g/L). In addition, in Comparative

Example 3, a production yield of 2,3-butanediol was 0.27, which was lower than that of Comparative Example 2 (0.32), and selectivity thereof was 55%, which was lower than that of Comparative Example 2 (59%), and productivity (g/L/h) was 0.36, which was remarkably lower than that of Comparative Example 2 (1.07). Further, in Comparative Example 3, a production amount of ethanol was decreased as compared to Comparative Example 2; however, a producing ability of 2,3-butanediol was rather deteriorated.

[129] In addition, although being compared to Example 1, it could be appreciated that a producing ability of 2,3-butanediol of Comparative Example 3 was remarkably decreased (Table 4). Accordingly, it could be confirmed that even though the gene involved in occurrence of by-products was removed, it was not favorable to the production of 2,3-butanediol.

[130]

[131] [Table 4]

[132]

Strain	2,3-butanediol				Concentration (g/L) of by-products				
	Concentration (g/L)	Yield (g/g)	Productivity (g/L/h)	Selectivity (%)	Lactate	Succinic Acid	Ethanol	Formic Acid	Acetic Acid
Comparative Example 1	17.31	0.18	0.58	27	32.20	1.92	1.61	3.88	1.2
Comparative Example 2	29.91	0.32	1.07	59	1.93	2.27	3.54	6.11	0.9
Comparative Example 3	25.96	0.27	0.36	55	2.71	2.32	0.89	4.13	6.4
Example 1	39.17 ± 1.51	0.45	0.43 ± 0.02	83.33 ± 1.53	3.24	1.77	0.53	0.97	0.1

[133] <Experimental Example 4> Change in production of 2,3-butanediol according to change in oxygen supply amount

[134] Effects of change in dissolved oxygen amount of a medium depending on an agitation speed during the culturing, on a production yield, a productivity, and selectivity of 2,3-butanediol were evaluated by using the recombinant *Klebsiella oxytoca* of Example 1.

[135]

[136] First, the recombinant microorganism of Example 1 was inoculated in 250 ml of composite medium including 9 g/L glucose (50 mM) and cultured at 37°C for 16 hours. Then, the obtained culture fluid was inoculated in 3L of composite medium, and batch fermentation was performed. The fermentation was performed under a micro-aerobic condition (aerobic speed of 1 vvm), at an initial glucose concentration of 90 g/L, pH 6.8, and a culture temperature of 37°C, with a variety of agitation speeds, for example, 150 rpm, 250 rpm, 350 rpm, and 450 rpm. 5N NH₄OH was used to control the pH during the fermentation. Samples for the recombinant *Klebsiella oxytoca* strains were taken during the fermentation, and growth rates were obtained by measuring optical density 600 (OD 600) of the taken samples. Then, the taken samples were centrifuged at 13,000 rpm for 10 minutes, and metabolites of supernatants and concentration of 2,3-butanediol (2,3-BDO) thereof were analyzed by liquid chromatography (HPLC).

[137]

[138] As a result, in the recombinant *Klebsiella oxytoca* strain of Example 1, productivity (g/L/h) of 2,3-butanediol was largely changed depending on change in agitation speed. That is, it could be confirmed that at the time of agitating at an agitation speed of 450 rpm, productivity of 2,3-butanediol was increased by 5 times or more as compared to agitating at an agitation speed of 150 rpm (Table 5, FIGS. 7 to 11). It could be confirmed that the change in oxygen supply amount depending on the agitation speed could improve productivity of Example 1.

[139]

[140] [Table 5]

[141]

agitation Speed (rpm)	2,3-Butanediol			
	Concentration (g/L)	Yield (g/g)	Productivity (g/L/h)	Selectivity (%)
150	39.17 ± 1.51	0.45	0.43 ± 0.02	83.33 ± 1.53
250	33.51 ± 0.23	0.39 ± 0.01	0.77 ± 0.02	84
350	31.39 ± 1.68	0.37 ± 0.02	1.40 ± 0.12	80 ± 1.00
450	30.79 ± 1.46	0.35 ± 0.02	2.71 ± 0.21	84 ± 4.58

[142] <Experimental Example 5> Production of 2,3-butanediol through fed-batch fermentation under aerobic condition (agitating at agitation speed of 450 rpm)

[143] Fed-batch fermentation for production of 2,3-butanediol was performed by using the strain of Example 1 while maintaining an agitation speed at 450 rpm which exhibited the

most improved productivity based on the results of Experimental Example 4.

[144] First, the recombinant *Klebsiella oxytoca* strain of Example 1 was inoculated in 250 ml of composite medium including 9 g/L glucose (50 mM) and cultured at 37°C for 16 hours, and then the obtained culture fluid was inoculated in 3L of composite medium and fed-batch culture was performed. Here, the fermentation was performed under a micro-aerobic condition (aerobic speed of 1 vvm), at an initial glucose concentration of 90 g/L, pH 6.8, and a culture temperature of 37°C. The agitation speed was continuously maintained at 450 rpm. 5N NH₄OH was used to control the pH during the fermentation. When a glucose concentration was decreased to be 10 g/L or less during the fermentation, a glucose solution of 700 g/L or more was fed. A sample for the recombinant *Klebsiella oxytoca* strain was taken during the fermentation, and a growth rate was obtained by measuring optical density 600 (OD 600) of the taken sample. Then, the taken sample was centrifuged at 13,000 rpm for 10 minutes, and a metabolite of supernatant and concentration of 2,3-butanediol thereof were analyzed by liquid chromatography (HPLC).

[145] As a result, it could be confirmed that when the agitation speed was continuously maintained at 450 rpm, a producing ability of 2,3-butanediol was not continuously maintained (Table 6). In particular, it could be confirmed that when a

concentration of acetoin was over 10 g/L, the producing ability of 2,3-butanediol was remarkably decreased (FIG. 12). It could be appreciated that the agitation speed of the fed-batch culture was required to be controlled based on results of the fed-batch culture performed at the agitation speed maintained at 450 rpm, and a time point for controlling the agitation speed was required to be determined based on the concentration of acetoin to be accumulated.

[146]

10 [147] [Table 6]

[148]

Strain	2,3-Butanediol						
	Concentration (g/L)	Productivity (g/L/h)	Yield (g/g)	Selectivity (%)	Acetoin (g/L)	Succinic Acid (g/L)	Acetic Acid (g/L)
Example 1	57.7	1.65	29	63	13.44	12.38	5.32

[149] <Experimental Example 6> Production of 2,3-butanediol through fed-batch fermentation

[150] The recombinant *Klebsiella oxytoca* strain of Example 1 was inoculated in 250 ml of composite medium including 9 g/L glucose (50 mM) and cultured at 37°C for 16 hours. Then, the obtained culture fluid was inoculated in 3L of composite medium, and fed-batch culture was performed. Here, the fermentation was performed under a micro-aerobic condition (aerobic speed of 1 vvm), at an initial glucose concentration of 90 g/L, pH 6.8, a culture temperature of 37°C, and an agitation speed of 450 rpm. 5N NH₄OH was used to control the pH

during the fermentation. When a glucose concentration was decreased to be 10 g/L or less during the fermentation, a glucose solution of 700 g/L or more was fed. In addition, at a time point at which the concentration of acetoin which is one
5 of the by-products, is 7 g/L, the fermentation was performed by changing the agitation speed from 450 rpm to 350 rpm. A sample for the recombinant *Klebsiella oxytoca* strain was taken during the fermentation, and a growth rate was obtained by measuring optical density 600 (OD 600) of the taken sample. Then, the
10 taken sample was centrifuged at 13,000 rpm for 10 minutes, and a metabolite of supernatant and concentration of 2,3-butanediol thereof were analyzed by liquid chromatography (HPLC).

[151]

[152] As a result, as compared to the fed-batch culture
15 performed by uniformly maintaining the agitation speed at 450 rpm in Experimental Example 5, all of the concentration, the productivity, the yield, and the selectivity of 2,3-butanediol were largely increased by 74.5%, 29.7%, 55.2%, and 27.0%, respectively. Therefore, it could be confirmed that the
20 production of 2,3-butanediol using the recombinant strain of Example 1 was largely affected by the control of the dissolved oxygen amount depending on the agitation speed. Accordingly, it was determined that the productivity of 2,3-butanediol could be improved by controlling the agitation speed to control the
25 dissolved oxygen amount in the medium (Table 7, FIG. 13).

[153]

[154] [Table 7]

[155]

Strain	2,3-Butanediol					
	Concentration (g/L)	Productivity (g/L/h)	Selectivity (%)	Acetoin (g/L)	Succinic Acid (g/L)	Acetic Acid (g/L)
Example 1	100.66	2.14	80	5.19	14.76	2.73

【INDUSTRIAL APPLICABILITY】

5 **[156]** The present invention relates to a recombinant microorganism having enhanced 2,3-butanediol producing ability, wherein a pathway for converting pyruvate to acetyl-CoA, a pathway for converting pyruvate to formic acid, or a pathway for converting pyruvate to lactate is inhibited in a
10 microorganism having acetyl-CoA and lactate biosynthetic pathways. The recombinant microorganism of the present invention may produce 2,3-butanediol with high selectivity and concentration.

[157]

15

Claims:

1. A recombinant microorganism having enhanced 2,3-butanediol producing ability in comparison to a wild type microorganism, wherein a pathway for converting pyruvate to acetyl-CoA via pyruvate-formate lyase, a pathway for converting pyruvate to formic acid via pyruvate-formate lyase, and a pathway for converting pyruvate to lactate via lactate dehydrogenase are inhibited in the recombinant microorganism having acetyl-CoA and lactate biosynthetic pathways, and the recombinant microorganism is *Klebsiella*.
2. The recombinant microorganism of claim 1, wherein the pathway for converting pyruvate to acetyl-CoA, and the pathway for converting pyruvate to formic acid are inhibited by inhibiting pyruvate-formate lyase.
3. The recombinant microorganism of claim 1, wherein the pathway for converting pyruvate to lactate is inhibited by inhibiting lactate dehydrogenase.
4. The recombinant microorganism of claim 1, wherein any one of *pflB* and *ldhA* is deleted or inhibited, the *pflB* being a gene encoding pyruvate-formate lyase and the *ldhA* being a gene encoding lactate dehydrogenase.
5. The recombinant microorganism of claim 1, wherein a producing ability of formic acid, acetic acid, or ethanol is inhibited.
6. The recombinant microorganism of claim 1, wherein a gene encoding alcohol dehydrogenation enzyme (*adhE*) for converting acetyl-CoA to ethanol is not deleted.
7. The recombinant microorganism of claim 1, wherein selectivity of 2,3-butanediol calculated as follows:

Selectivity (%) of 2,3-butanediol: $\text{Production amount (g) of 2,3-butanediol} / (\text{production amounts (g) of 2,3-butanediol, ethanol, acetoin, succinic acid, lactate formate, and acetic acid}) \times 100$ is 70% or more based on batch culture.

8. The recombinant microorganism of claim 1, wherein a yield of 2,3-butanediol is at least 0.35 gram per gram of carbon source based on batch culture.

9. The recombinant microorganism of claim 1, wherein selectivity of 2,3-butanediol calculated as follows:

Selectivity (%) of 2,3-butanediol: $\text{Production amount (g) of 2,3-butanediol} / (\text{production amounts (g) of 2,3-butanediol, ethanol, acetoin, succinic acid, lactate formate, and acetic acid}) \times 100$

is 70% or more based on fed-batch culture.

10. The recombinant microorganism of claim 1, wherein selectivity of 2,3-butanediol calculated as follows:

Selectivity (%) of 2,3-butanediol: $\text{Production amount (g) of 2,3-butanediol} / (\text{production amounts (g) of 2,3-butanediol, ethanol, acetoin, succinic acid, lactate formate, and acetic acid}) \times 100$

is 80% or more based on fed-batch culture.

11. A method for producing 2,3-butanediol, the method comprising:

culturing the recombinant microorganism of any one of claims 1 to 11; and

recovering 2,3-butanediol from a culture in which the recombinant microorganism is cultured.

12. The method of claim 11, wherein the culturing is performed under aerobic condition.

13. The method of claim 11, wherein the culturing is performed while agitating at an agitation speed of 450 rpm or less.

14. The method of claim 11, wherein the culturing is performed while agitating, and when a concentration of acetoin during the culturing is increased to be 5 g/L or more, an agitation speed is decreased.

15. The method of claim 11, wherein the culturing controls productivity of 2,3-butanediol by controlling an oxygen supply amount.

16. The method of claim 11, wherein the culturing is performed while agitating, and the culturing controls productivity of 2,3-butanediol by controlling an agitation speed.

【DRAWINGS】

Fig.1

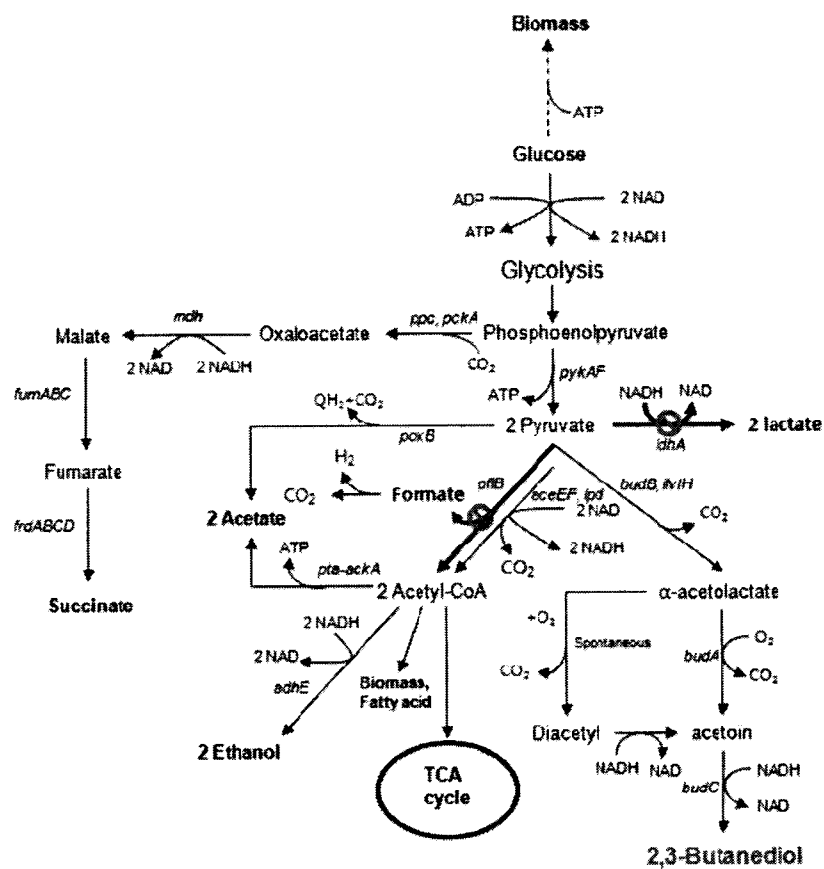


Fig.2

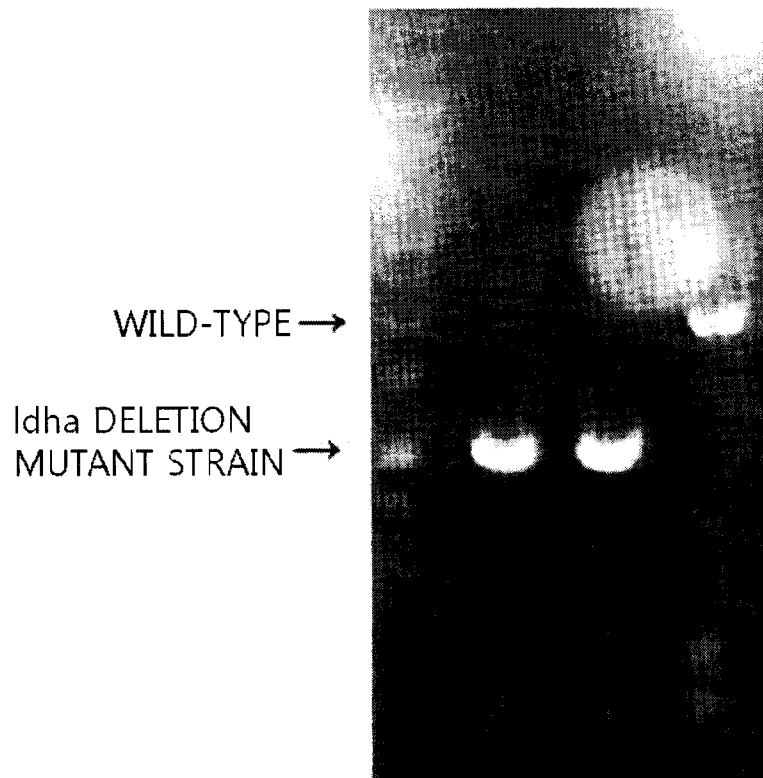


Fig.3

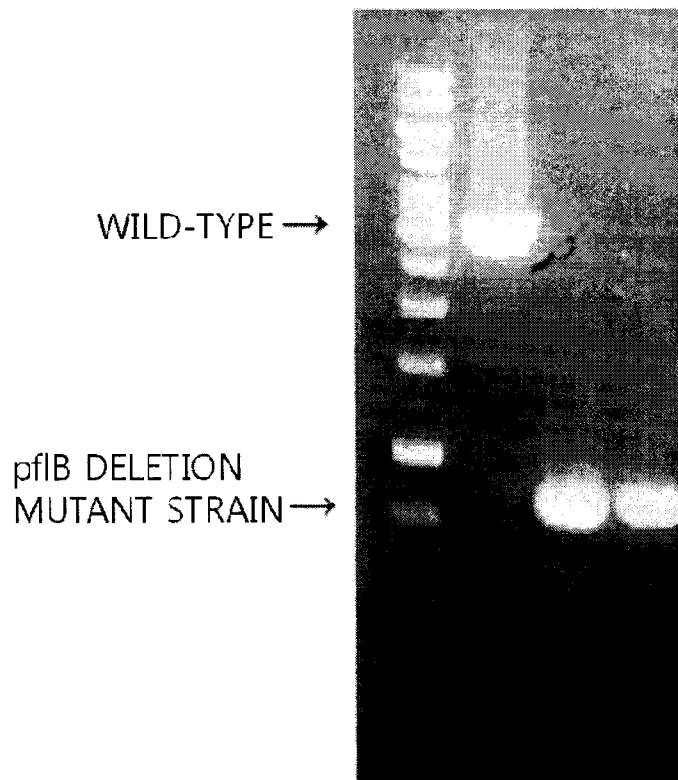


Fig.4

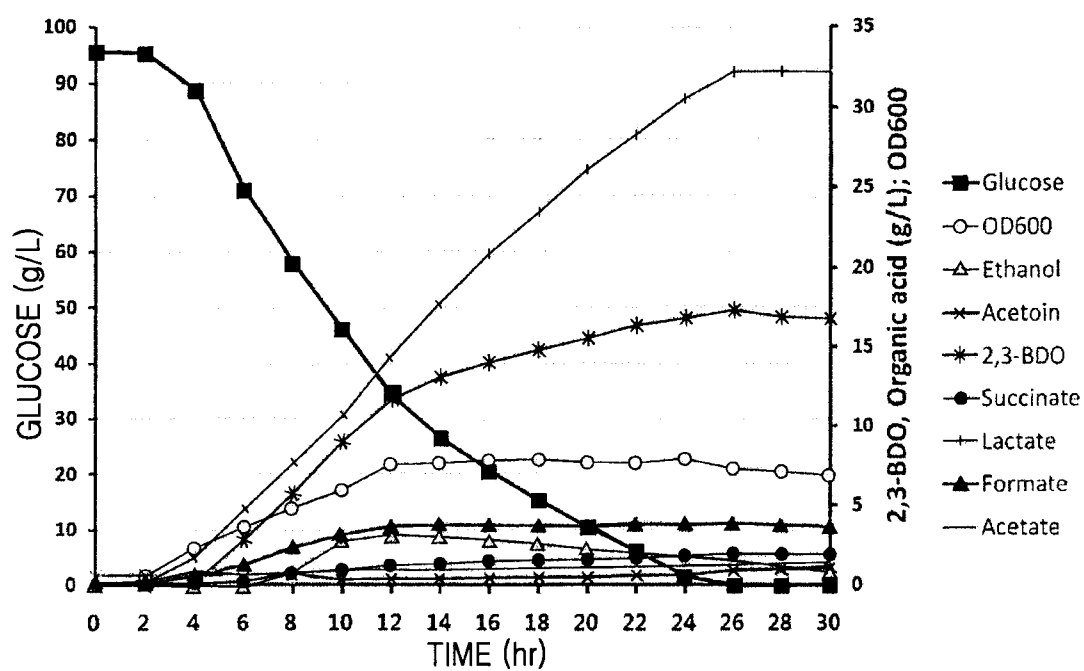


Fig.5

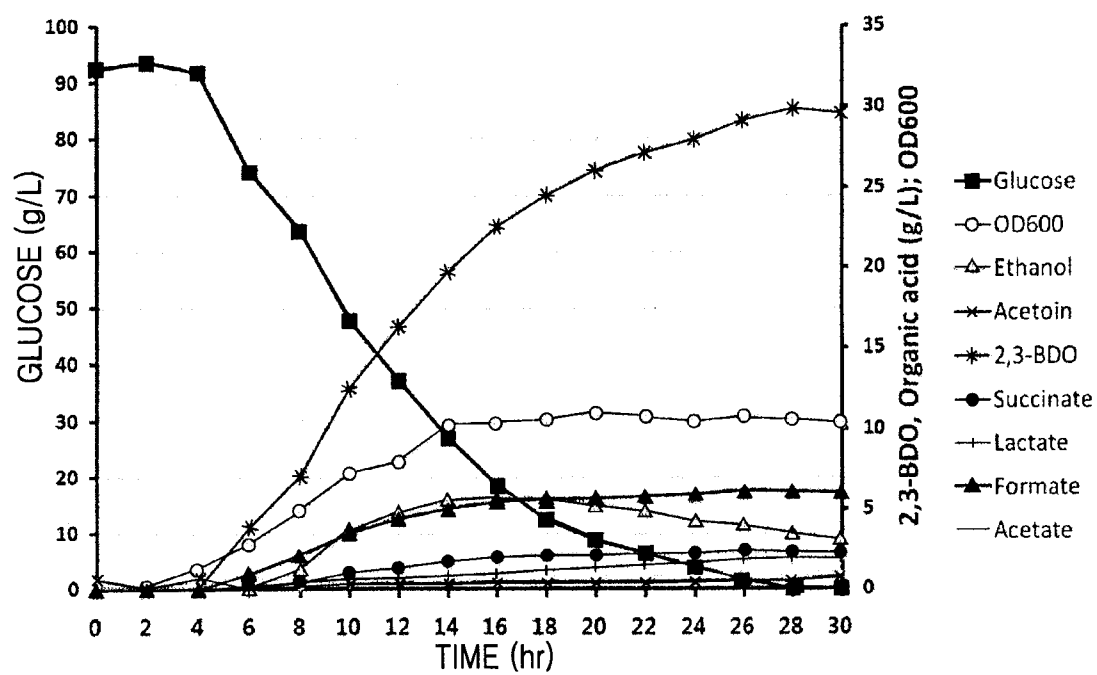


Fig.6

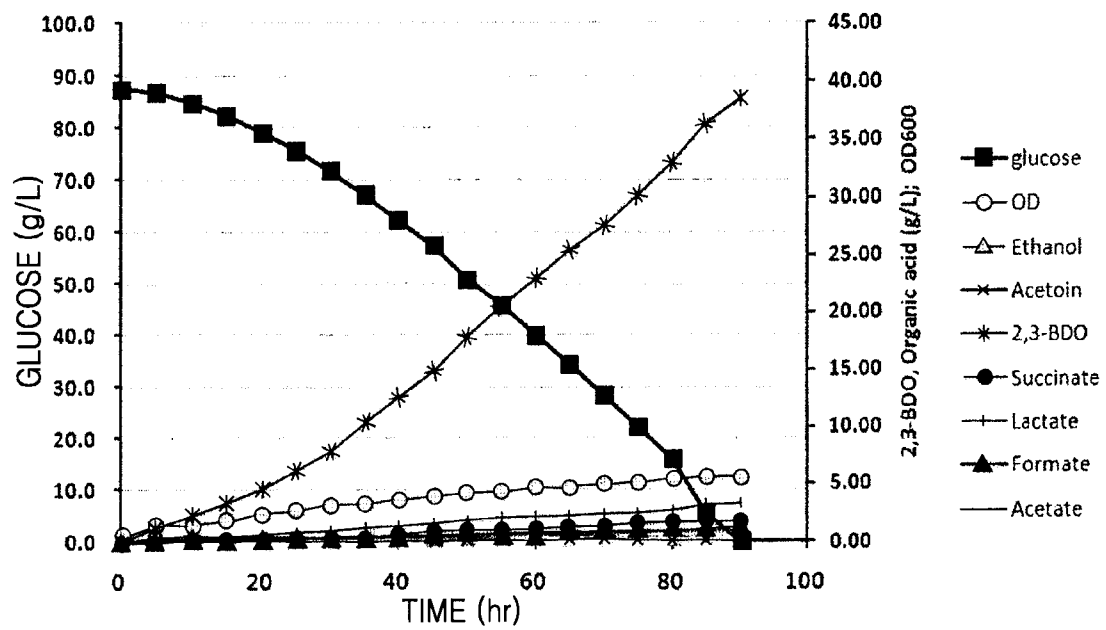


Fig.7

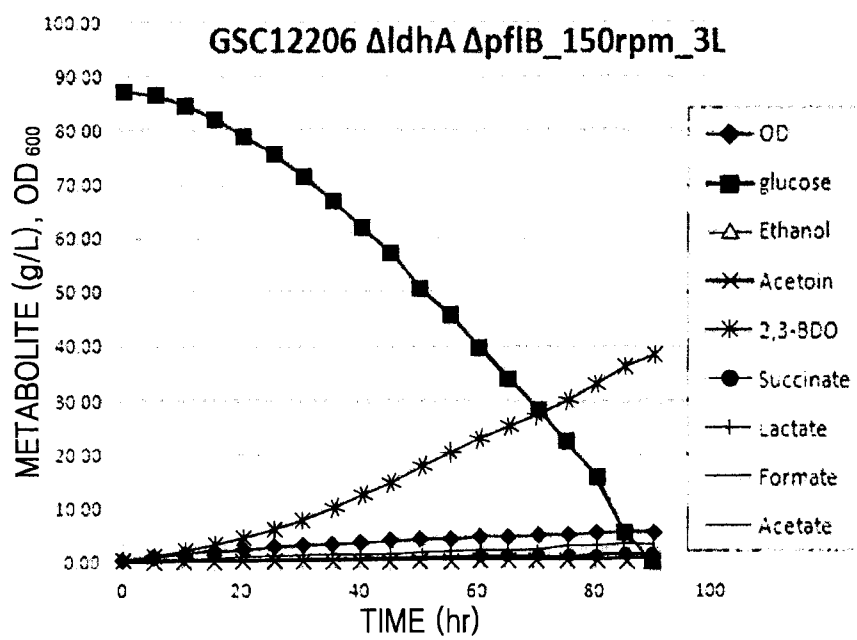


Fig.8

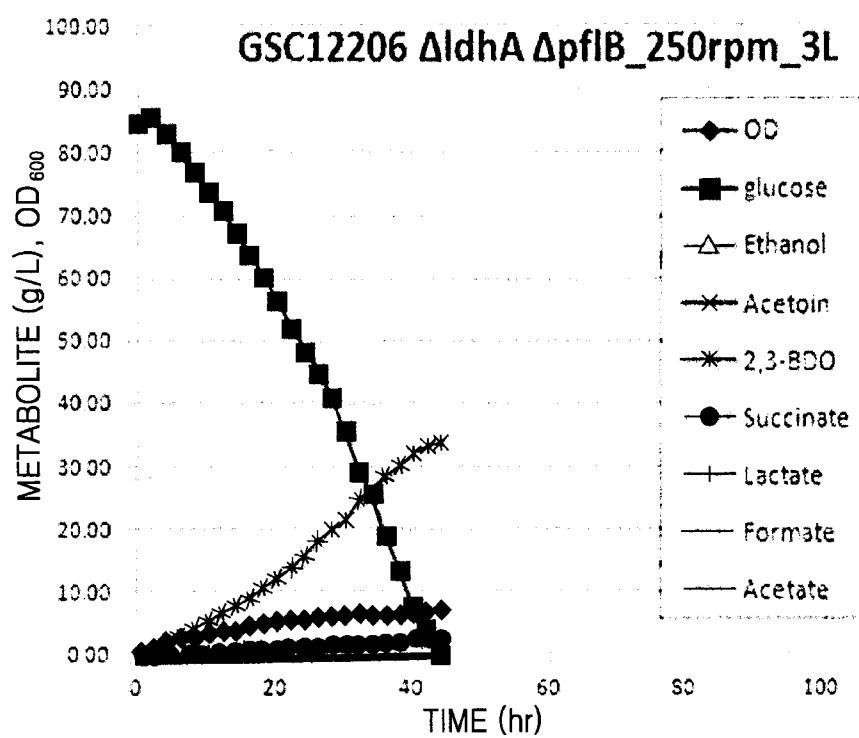


Fig.9

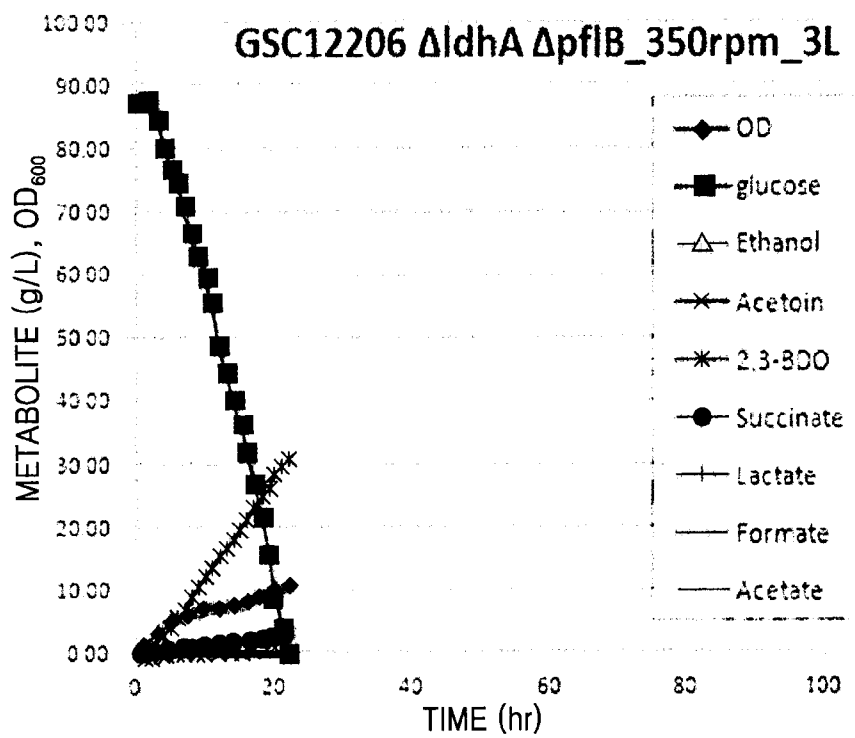


Fig.10

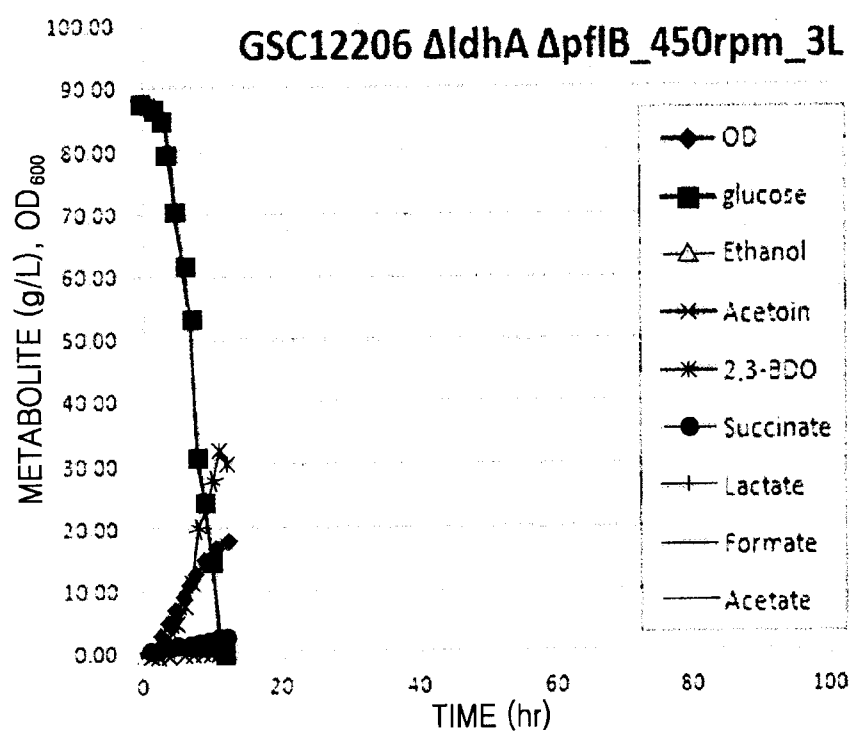


Fig.11

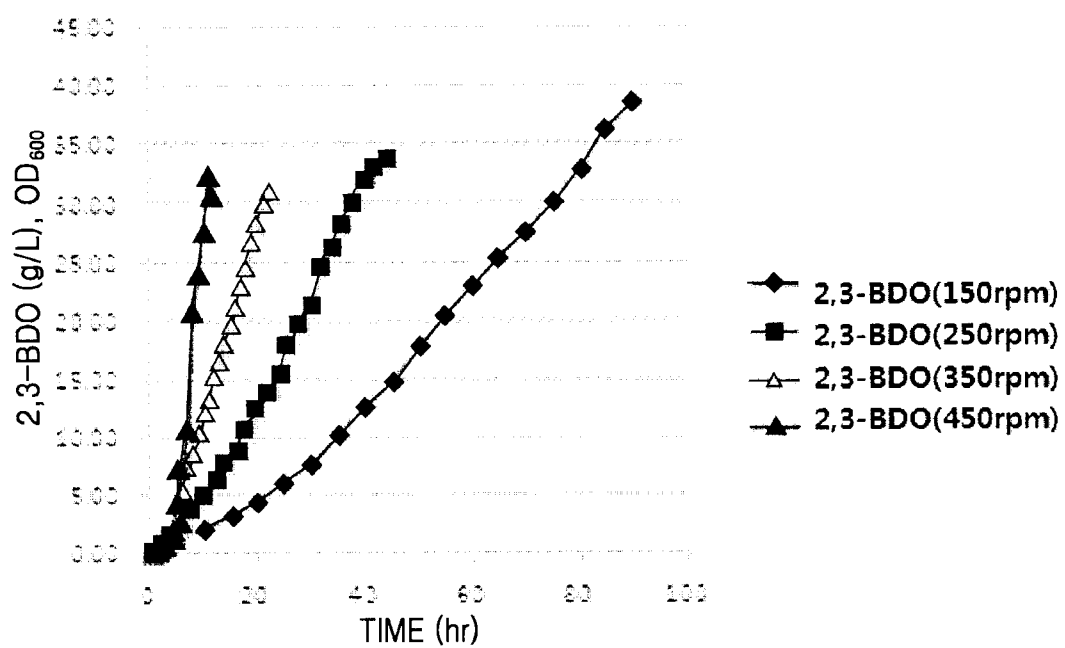


Fig.12

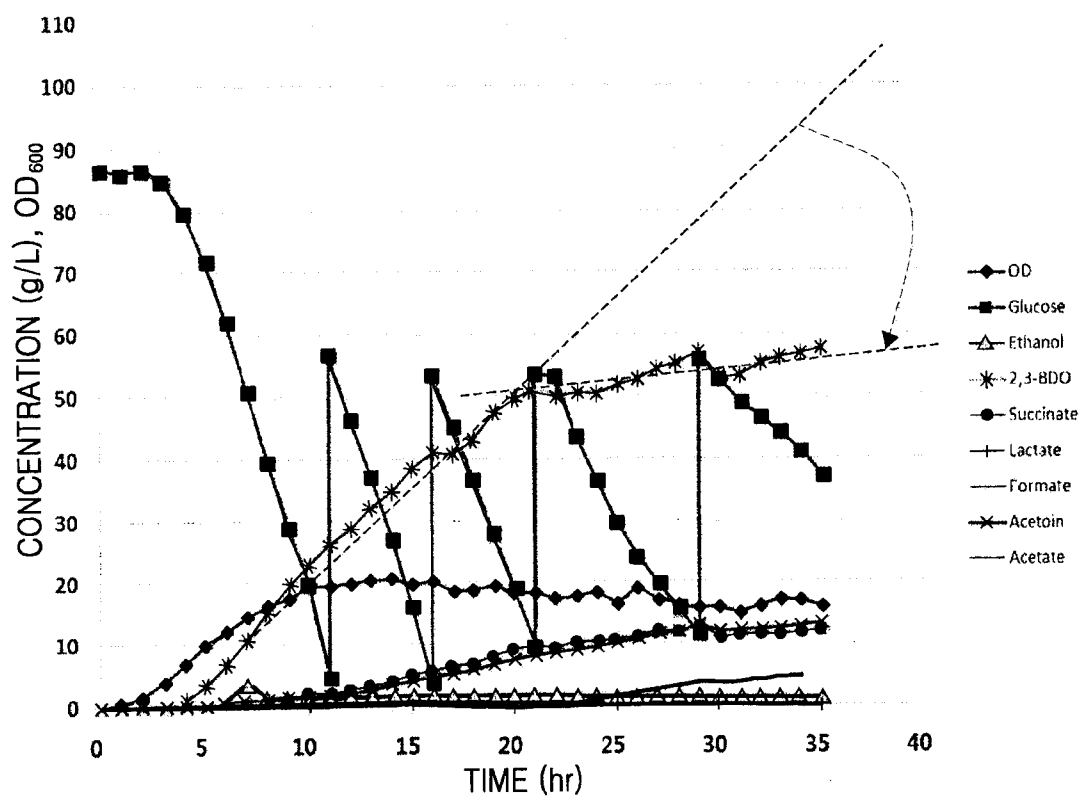


Fig.13

