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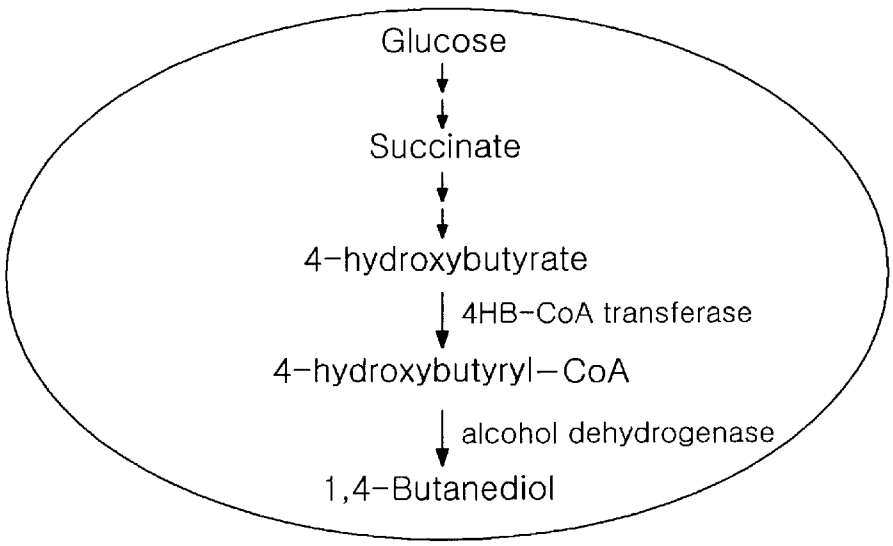
[Continued on next page]

(54) Title: MUTANTS HAVING CAPABILITY TO PRODUCE 1,4-BUTANEDIOL AND METHOD FOR PREPARING 1,4-BUTANEDIOL USING THE SAME

FIG. 2



(57) Abstract: A mutant capable of producing 1,4-butanediol and a method of preparing 1,4- butanediol using the same are provided. The mutant microorganism is prepared by introducing and amplifying genes encoding enzymes converting succinate into 4- hydroxybutyrate and 4-hydroxybutyrate into 1,4-butanediol in a microorganism capable of producing succinate. The method includes culturing the mutant in a medium containing carbohydrate and obtaining 1,4-butanediol from the culture. Thus, 1,4-butanediol, which is essential in chemical industry, can be prepared in a biological process.

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MUTANTS HAVING CAPABILITY TO PRODUCE 1,4-BUTANEDIOL AND METHOD FOR PREPARING 1,4-BUTANEDIOL USING THE SAME

【Technical Field】

The present invention relates to a mutant microorganism capable of producing 1,4-butanediol and a method of preparing 1,4-butanediol using the same.

【Background Art】

Biodegradable polymers have been suggested as an alternative to synthetic polymers, which are one of the major causes of serious environmental pollution. Among various biodegradable polymers currently being developed, poly-β-hydroxybutyrate, a biodegradable polymer stored by various microorganisms in a state of unbalanced nutrition, has excellent characteristics such as biodegradability, water-resistance, piezoelectricity and biocompatibility. In particular, 4-hydroxybutyrate, an example of polyhydroxyalkanoate (PHA), has polyester-like characteristics and exhibits a wide range of properties from those of crystalline plastic to highly elastic rubber. Therefore, a considerable amount of research into microbial biodegradable plastic is presently being conducted.

Further, 4-hydroxybutyrate can be easily converted into various chemicals having 4 carbon atoms, such as 1,4-butanediol, γ-butyrolactone (GBL) and THF. In particular, 1,4-butanediol is an important industrial chemical in various forms such as polymer, solvent and a fine chemical intermediate. Although most chemicals having 4 carbon atoms are currently synthesized from 1,4-butanediol, maleic anhydride and so on, increasing production costs caused by an increase in the price of oil is necessitating development of

another process for compensating and substituting a conventional chemical production process. A biological process has been suggested as such an alternative.

Meanwhile, succinate, dicarboxylic acid having 4 carbon atoms, is a kind of organic acid produced when a microorganism is cultured in an anaerobic condition. Now, various microorganisms are used as succinate-producing cells, and its production cost has become lower due to an effective fermentation process and development of a separation and purification process. Also, 4-hydroxybutyrate may be produced from succinate, and various organic acids having 4 carbon atoms can be derived from 4-hydroxybutyrate.

PCT Publication No. WO 2005/052135 is an example of a patent application disclosing a method of efficiently producing succinate, in which a Lumen bacterial mutant produces succinate in high concentration without producing other organic acids, and a method of preparing succinate using the mutant. In addition, a method of preparing an E. coli mutant capable of producing succinate in high concentration is disclosed in Korean Patent Application No. 10-2004-60149, and a method of preparing succinate using a novel gene is disclosed in Korean Patent Application Nos. 10-2005-0076301, 10-2005-0076317 and 10-2005-0076348.

As explained above, there is strong demand for a mutant capable of producing 1,4-butanediol, an industrially important chemical having 4 carbon atoms, and a biological method of preparing 1,4-butanediol using the mutant.

【Disclosure】

【Technical Problem】

The present invention is directed to providing a mutant microorganism capable of producing 1,4-butanediol with high efficiency and a method of preparing 1,4-butanediol using the same.

【Technical Solution】

In one aspect, a microorganism capable of producing succinate, and preferably, a mutant exhibiting high production of 1,4-butanediol, in which a gene encoding an enzyme converting succinate into 4-hydrozybutyrate and a gene encoding an enzyme converting 4-hydroxybutyrate into 1,4-butanediol are introduced or amplified, and a method of preparing 1,4-butanediol using the same, are provided.

In another aspect, a butyl-CoA dehydrogenase gene of SEQ ID NO: 8 or 9, which effectively produces 1,4-butanediol from 4-hydroxybutyl-CoA, and a recombinant vector having the same are provided.

Hereinafter, the present invention will be described in more detail.

As a result of efforts to prepare 1,4-butanediol using a microorganism capable of producing succinate, the present inventors developed a mutant microorganism producing 1,4-butanediol by inducing or amplifying a gene associated with 4-hydroxybutyrate biosynthesis and/or a gene associated with 1,4-butanediol biosynthesis in the microorganism capable of producing succinate, and found that the mutant microorganism effectively produced 1,4-butanediol. This finding led to the present invention.

The term "amplification" used herein means an increase in gene expression level compared to original expression level. If there is no gene to be amplified in a microorganism before mutation, the at least one gene may be introduced to the microorganism and then amplified. And if there is a gene to be amplified in a microorganism before mutation, the at least one gene may be introduced to the

microorganism by the same method described above, or a gene originally present in the microorganism may be manipulated by a genetic engineering technique to increase gene expression. For example, when a gene amplifying expression is present in a microorganism to be mutated, an original promoter for operating gene expression may be substituted with a stronger promoter, thereby amplifying gene expression.

The microorganism capable of producing succinate may exhibit high production of succinate, the microorganism being preferably one selected from the group consisting of bacteria, yeast and fungi, and more particularly, bacteria, for example, Lumen bacteria, *Corynebacterium species*, *Brevibacterium species* and *E.coli*.

The Lumen bacteria may have inactive genes encoding lactate dehydrogenase (*ldhA*) and pyruvate-formate lyase (*pfl*), and produce succinate in high concentration without other organic acids under anaerobic conditions.

The term "inactivation" used herein means that a gene is not transcribed due to mutation, or transcribed mRNA is not properly translated into original protein. In order to deactivate a gene, mutation may be conducted by missing a gene or changing a nucleic acid sequence of a gene.

Further, the Lumen bacteria may have inactive genes encoding lactate dehydrogenase (l*dhA*), pyruvate-formate lyase (*pfl*), phosphotransacetylase (*pta*) and acetate kinase (*ackA*), and produce succinate in high concentration without substantial production of other organic acids in an anaerobic condition.

Alternatively, the Lumen bacteria may have inactive genes encoding lactate dehydrogenase (l*dhA*), pyruvate-formate lyase (*pfl*) and phosphopyruvate carboxylase (*ppc*), and produce succinate in high concentration without substantial production of other organic acids in an anaerobic condition.

The Lumen bacteria may be selected from the group consisting of *Mannheimia sp.*, *Actinobacillus sp.* and *Anaerobiospirllum sp.*, but the present invention is not limited to these examples. *Mannheimia sp.* is preferable, and *Mannheimia succiniciproducens* MBEL55E (KCTC 0769BP), *Mannheimia sp.* LPK (KCTC 10558BP), LPK4 and LPK7 (KCTC 10626BP) are more preferable.

The E. coli may have inactive genes encoding glucose phosphotransferase (*ptsG*) and pyruvate kinase (*pykA* and *pykF*), and produce succinate in high concentration without substantial production of other organic acids in an anaerobic condition. In particular, the E. coli mutant is preferably W3110GFA disclosed in Korean Patent Publication No. 10-2006-0011345.

Among the above-mentioned microorganisms producing succinate in high concentration, the Lumen bacteria may be prepared in a method disclosed in PCT Publication No. WO 2005/052135. That is, a gene of lactic dehydrogenase (l*dhA*) and a gene of pyruvate-formate lyase (*pfl*) are inactivated in *Mannheimia succiniciproducens* 55E, thereby constructing a mutant strain, i.e., *Mannheimnia sp.* LPK (KCTC 10558BP). Then, in the LPK strains, genes of phosphotransacetylase gene (*pta*) and acetate kinase gene (*ackA*), and a gene of phosphopyruvate carboxylase (*ppc*), are independently inactivated, thereby constructing mutant strains (*Mannheimia sp.* LPK7 and LPK4) which are then cultured in an anaerobic condition to produce succinate with high yield.

In addition, among the microorganisms producing succinate in high concentration, E. coli may be constructed by a method disclosed in Korean Patent Publication No. 10-2006-0011345. That is, mutant E. coli strain W3110GFA is yielded by inactivating a gene encoding glucose phosphotransferase (*ptsG*) and two genes encoding pyruvate kinase (*pykA* and *pykF*) in W3110 strain transformed with a recombinant expression vector

expressing a bacteriophage red operon (exo-beta-gam). Then, when the mutant E.coli strain W3110GFA is cultured in an anaerobic condition, it can be confirmed that productivity of the mutant is greater than that of a mother strain W3110.

A gene of an enzyme converting the succinate into 4-hydroxybutyrate and a gene of an enzyme associated with conversion of the succinate semialdehyde into succinate may be derived from *Clostridium kluyveri*, and a gene of an enzyme converting the 4-hydroxybutyrate into 1,4-butanediol may be derived from *Clostridium acetobutylicum*. Although *Clostridium kluyveri* and *Clostridium acetobutylicum* do not produce 4-hydroxybutyrate and 1,4-butanediol, the enzymes cloned in these strains play an important role in producing 4-hydroxybutyrate and 1,4-butanediol.

Further, the gene of the enzyme converting succinate into 4-hydroxybutyrate may be selected from the group consisting of a gene encoding succinyl-CoA transferase (Cat1), a gene encoding succinate semialdehyde dehydrogenase (SucD), a gene encoding 4-hydroxybutyrate dehydrogenase (hbD), and a gene encoding 4-hydroxybutyrate dehydrogenase (GHB). Preferably, the gene encoding Cat1 has a base sequence of SEQ ID NO: 1, the gene encoding SucD has a base sequence of SEQ ID NO: 2, the gene encoding 4hbD has a base sequence of SEQ ID NO: 3, and the gene encoding GHB has a base sequence of SEQ ID NO: 4.

For example, a mutant microorganism according to the present invention may have a gene encoding Cat1, a gene encoding SucD and a gene encoding 4hbD, or a gene encoding Cat1, a gene encoding SucD and a gene encoding GHB, but the present invention is not limited to these examples.

Further, effective use of succinate is very important to accomplish the object of the present invention, and thus succinic semialdehyde dehydrogenase (G*abD*) associated with

conversion of succinic semialdehyde into succinate may be removed from recombinant E. coli of the microorganisms producing succinate in high concentration. Therefore, the mutant microorganism according to the present invention may also have an inactive gene associated with conversion of succinate semialdehyde into succinate, which is preferably a gene encoding succinic *GabD*. The gene encoding *GabD* has a base sequence of SEQ ID NO: 10, but the present invention is not limited to the sequence.

Also, to effectively transport succinate in a microorganism, C4-dicarboxylate transport protein (DctA) enzyme associated with transport of succinate may be amplified. Thus, the mutant microorganism may further have a gene encoding Dct4 associated with transport of succinate, which is introduced thereinto or amplified, and a gene encoding Dct4 preferably has a base sequence of SEQ ID NO: 11.

The genes of enzymes converting 4-hydroxybutyrate into 1,4-butanediol may be genes encoding 4-hydroxybutyrate-CoA transferase and alcohol dehydrogenase reducing 4-hydroxybutyrate-CoA, or genes encoding phosphotransbutyrylase, butyryl kinase and alcohol dehydrogenase reducing 4-hydroxybutyrate-CoA.

The gene encoding 4-hydroxybutyrate-CoA transferase may have a base sequence of SEQ ID NO: 5, which may be substituted with phosphotransbutyrylase (*ptb*; SEQ ID NO: 6) and butyryl kinase (*BuK*; SEQ ID NO: 7) to convert 4-hydroxybutyrate into 4-hydroxybutyrate-CoA.

The alcohol dehydrogenase may be butyl-CoA dehydrogenase derived from Clostridium acetobutylicum, and the gene encoding butyl-CoA dehydrogenase preferably has a base sequence of SEQ ID NO: 8 or 9 (CAP0035 or CAP0162). The genes of SEQ. ID. NOs: 8 and 9 are very useful to produce 1, 4-butanediol in the mutant microorganism

according to the present invention. Accordingly, the present invention provides a gene encoding butyl-CoA dehydrogenase and a recombinant vector containing the same.

The term "vector" means a DNA construct containing a DNA sequence operably linked to a control sequence suitable for expressing DNA in a suitable host. In the present invention, the vector may comprise a plasmid vector, a bacteriophage vector, a cosmid vector, a Yeast Artificial Chromosome (YAC) vector, and preferably a plasmid vector. For example, the plasmid vector may have a constitution comprising (a) a replication origin for effective replication to have several hundreds of copies in one host cell, (b) an antibiotic-resistance gene for selecting a host cell transformed with the plasmid vector, and (c) a restriction enzyme site into which a foreign DNA fragment is capable of being inserted. Although there is no suitable restriction enzyme site, the vector may be easily ligated with the foreign DNA using a synthetic oligonucleotide adaptor or a linker according to a conventional method.

Therefore, the present invention provides a microorganism capable of producing succinate, and preferably, a mutant microorganism exhibiting high production of 1,4-butanediol in which a gene encoding GabD is inactivated, and all of a gene encoding Cat1, a gene encoding SucD, a gene encoding 4hbD (or GHB), a gene encoding 4-hydroxybutyrate-CoA transferase and a gene encoding butyl-CoA dehydrogenase are introduced or amplified.

Further, the present invention provides a microorganism capable of producing succinate, and preferably, a mutant microorganism exhibiting high production of 1,4-butanediol in which a gene encoding 4-hydroxybutyrate-CoA transferase (or a gene encoding phosphobutyrylase and a gene encoding butyryl kinase) and a gene encoding

butyl-CoA dehydrogenase are introduced or amplified, and a method of preparing 1,4-butanediol using the same.

The present invention further provides a method of preparing 1,4-butanediol comprising culturing the mutant in a medium containing a carbon source, and obtaining 1,4-butanediol from the culture.

【Advantageous Effects】

As described above in detail, the present invention provides a microorganism capable of producing succinate in high concentration, and more particularly, a mutant exhibiting high production of 1,4-butanediol that is a chemical having 4 carbon atoms having a wide range of important applications in chemical industry, and a biological method of preparing 1,4-butanediol using the same.

【Description of Drawings】

FIG. 1 is a schematic diagram of a pathway for producing 4-hydroxybutyrate from succinate;

FIG. 2 a schematic diagram of a pathway for producing 1,4-butanediol through 4-hydroxybutyrate produced from succinate; and

FIG. 3 shows GC analysis results of production of 1,4-butanediol.

【Modes of the Invention】

Hereinafter, the present invention will be described in more detail through examples. It will be clearly understood by those skilled in the art that the examples are provided merely to explain the present invention, not to limit its scope.

While, in the present invention, a method of preparing 1, 4-butanediol uses Lumen bacteria such as mutants *Mannheimia sp.* LPK (KCTC 10558BP), LPK7 and LPK4, which

have an inactive gene derived from a *Mannheimia sp.* strain and produce succinate in high concentration, E. coli and mutant E. coli W3110GFA, it will be also clearly understood by those skilled in the art that 1,4-butanediol may be produced by yielding a mutant producing succinate in high concentration using another Lumen bacteria strain, and introducing and amplifying a gene associated with producing 1,4-butanediol.

Further, while the following example provides a specific medium and culture method, it will be clearly understood by those skilled in the art that, as disclosed in the literatures (Lee *et al.*, *Bioprocess Biosyst. Eng.*, 26:63, 2003; Lee *et al.*, *Appl. Microbiol. Biotechnol.*, 58:663, 2002; Lee *et al.*, *Biotechnol. Lett.*, 25:111, 2003; Lee *et al.*, *Appl. Microbiol. Biotechnol.*, 54:23, 2000; and Lee *et al.*, *Biotechnol. Bioeng.*, 72:41, 2001), a medium used herein may be different from a hydrolysate such as whey or corn steep liquor, or various culture methods such as fed-batch culture and continuous culture may be used.

Example 1: Method of Preparing Microorganism Exhibiting High Production of Succinate

1-1. Preparation of Lumen Bacteria Having High Production of Succinate

A microorganism, a Lumen bacterium, exhibiting high production of succinate according to the present inventionwas prepared by the method disclosed in PCT Publication No. WO 2005/052135. That is, a mutant strain *Mannheimia sp.* LPK (KCTC 10558BP) was prepared by inactivating a gene of lactate dehydrogenase (*ldhA*) and a gene of pyruvate-formate lyase (*pfl*) in *Mannheimia succiniciproducens* 55E, which is one of the Lumen bacteria species, and mutant strains (*Mannheimia sp.* LPK7 and LPK4) were prepared by inactivating a gene of phosphotransacetylase (*pta*), a gene of acetate kinase (*ackA*) and a gene of phosphopyruvate carboxylase (*ppc*) in the LPK strain.

1-2. Preparation of E. Coli Exhibiting High Production of Succinate

A microorganism, E. coli, exhibiting high production of succinate according to the present invention was prepared by the method disclosed in Korean Patent Publication No. 10-2006-0011345. That is, a mutant E. coli strain W3110GFA was yielded by inactivating a gene encoding glucose phototransferase (*ptsG*) and two genes encoding pyruvate kinase (*pykA* and *pykF*) in W3110 strain, which was transformed with a recombinant expression vector pTrcEBG expressing a bacteriophage red operon (exo-beta-gam).

Example 2: Cloning of 1,4-Butanediol Converting Enzyme

*2-1. Cloning of Genes Encoding 4-Hydroxybutyrate Converting Enzymes (*Cat1, SucD and 4hbD*)*

The present inventors amplified *cat1, sucD and 4hbD* genes by polymerase chain reaction (PCR) using oligonucleotide primers synthesized based on a known gene sequence (L21902) in order to clone operons for genes encoding Cat1, SucD and 4hbD derived from *Clostridium kluyveri* DSM 555. The primers used for PCR were as follows.

SEQ ID NO 12: Cat1f-SacI

5'-tttcccgagctc TGTGAGGCGATTAAATGAGTAAAGGGATAAAG

SEQ ID NO 13: 4hbDb-XabI

gc tctaga tta gat aaa aaa gag gac att tca caa tat gg

To construct expression vector pTacLac4HB1, the operon for the amplified *cat1, sucD* and *4hbD* genes were inserted into expression vector pTacLacI, which was cleaved with *Sac*I/*Xba*I. The vector pTacLacI was constructed by cleaving vector pTac99A (Park and Lee, *J. Bacteriol.* 185, 5391-5397, 2003) with *Ssp*I, and ligating the cleaved vector with pTrc991 (Amersham Pharmacia Biotech), which was also cleaved with *Ssp*I. The vector pTacLacI has the same sequence as pTrc99A, and loses an *Nco*I restriction enzyme

recognition site (restriction site) present in the pTrc99A from Multi Cloning sites (MCS). Here, the MCS started with an *EcoR*I site.

2-2. Cloning of Gene Encoding DctA Associated With Transport of Succinate

To clone a gene encoding DctA associated with transport of succinate in E. coli W3110, a *DctA* gene was amplified by DNA-PCR using oligonucleotide primers synthesized based on a known gene sequence (NC_000913). The primers used for PCR were as follows.

SEQ ID NO 14: DctAf-EcoRI

ggaattc ATGAAAACCTCTCTGTTTAAAAGC

SEQ ID NO 15: DctAb-XbaI

gc tctaga tta aga gga taa ttc gtg cgt ttt gcc

To construct expression vector p10499DctA, the amplified *DctA* gene was cleaved with EcoRI/XbaI and then inserted into expression vector p10499A (Park et al. (2002) FEMS Microbiol. Lett 214:217-222).

2-3. Cloning of Gene Encoding Enzyme Converting 4-Hydroxybutyrate Into 1,4-Butanediol

To clone genes encoding butyl-CoA dehydrogenase of SEQ ID NOs: 8 and 9, which are enzymes converting butyric acid into butanol in *Clostridium acetobutylicum*, cap0035 and cap0162 genes were amplified by DNA-PCR using oligonucleotide primers synthesized based on a known gene sequence (NC_003030). The primers used for PCR were as follows.

SEQ ID NO: 16: CAP0035f-SacI

tttcccgagctc atgaaagttacaaatcaaaaa

SEQ ID NO: 17: CAP0035b-XbaI

gc tctaga tta aaa tgc ttt tat ata gat

SEQ ID NO: 18: CAP0162f-EcoRI

GGA ATT C atgaaagtcacaacagtaaag

SEQ ID NO: 19: CAP0162b-XbaI

gc tctaga tta agg ttg ttt ttt aaa

To construct expression vectors pTacLacCAP35 and pTacLacCAP 162, the amplified *cap0035* and *cap0162* genes were independently inserted into expression vectors pTacLacI, which were cleaved with *Sac*I/*Xba*I and *Eco*RI/*Xba*I.

To convert 4-hydroxybutyrate into 4-hydroxybutyrate-CoA, an operon of a *Cat2* gene of SEQ ID NO: 5 was amplified by DNA-PCR using oligonucleotide primers synthesized based on the sequence of SEQ ID NO: 5. The primers for PCR were as follows.

SEQ ID NO: 20: cat2f-EcoRI

ggaattc ATGGAGTGGGAAGAGATATATAAAGAG

SEQ ID NO: 21: cat2b-BamHI

cg ggatcc tta aaa tct ctt ttt aaa ttc att cat taa tg

To construct expression vector pTacLacCat2, the amplified *cat2* gene was inserted into expression vector pTacLacI, which was cleaved with *Eco*RI/*Bam*HI.

To convert 4-hydroxybutyrate into 4-hydroxybutyrate-CoA, operons for *ptb* and *buk* genes of SEQ ID NOs: 6 and 7 were amplified by DNA-PCR using oligonucleotide primers synthesized based on the sequences of SEQ ID NOs: 6 and 7. The primers used for PCR were as follows.

SEQ ID NO: 22: ptbf-RcoRI

ggaattc ATGATTAAGAGTTTTAATGAAATATCATG

SEQ ID NO: 23: bukb-XbaI

gc tctaga tta ttt gta ttc ctt agc ttt ttc ttc tcc

To construct an expression vector, operons for the amplified *ptb* and *buk* genes were inserted into expression vector pTacLacI, which was cleaved with *Eco*RI/*Xba*I, thereby obtaining pTacLacPtbBuk. The vector pTacLacPtbBuk was cleaved with *Ssp*I to obtain a gene fragment including a tac promoter, the *ptb* and *buk* genes and a transcription terminator, and the gene fragment was inserted into vector pBBR1MCS2 (Kovach et al., Gene. 166:175, 1995) which was cleaved with *Eco*RV, thereby obtaining vector pMCS2TacPtbBuk.

Example 3: Yield of 1,4-BDO

Vectors pTacCAP162 and pMCS2Tacptbbuk were simultaneously transformed with E. coli XL1-Blue by electroporation and then plated on a LB plate containing 100ug/ml ampicillin and 50ug/ml kinamycin and cultured overnight at 37℃. The cultured colony was inoculated into a 15ml tube (Falcon, USA) having 3ml LB liquid medium containing 100ug/ml ampicillin, and grown in a shaking incubator overnight at 200rpm and 37℃. The incubated cells were inoculated into a fresh LB liquid medium containing 100ml of 2% glucose and 100ug/ml ampicillin, and then grown in a shaking incubator at 200rpm and 37℃. When OD_{600} reached 0.7, IPTG was added at a final concentration of 1mM to induce protein expression and the cells were cultured overnight.

Afterward, the culture was centrifuged and the supernatant was removed therefrom. Then, the cell pellet was washed with an MR medium once, resuspended in an MR medium containing 50ml of 2% glucose, and 2% gamma-hydroxbutyrolactone and 1mM IPTG, and fuzzed using gas mixture of 5% H_2, 5% CO_2 and N_2 balance for 30 minutes to

set up an anaerobic condition. The culture was grown in a shaking incubator overnight for about 3 days at 200rpm and 37℃, and then centrifuged to obtain a supernatant. The obtained supernatant was concentrated two times, and used as a GC analysis sample for analysis to confirm production of 1,4-butanediol. The analysis was conducted under the following conditions, and the results are shown in FIG. 3.

Column: AT-Waw (0.53 mm ID x 15ml, 1.2 um u.f. capillary)

Gas Flow Rate: Column (He): 4.0 ml/min

Oven Temperature: Initial Value & Time: 50℃, 5 min

Program Rate: 10℃/min

Final Value & Time: 250℃, 5 min

Injector Temperature: 250℃

Detector Temperature: 250℃

Injector Split Ratio: 20/1

Injector Volume: 1.0ul

As shown in FIG. 3, it was confirmed that 1,4-butanediol was produced.

While the invention has been shown and described with reference to certain examples thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

【CLAIMS】

【Claim 1】

A mutant exhibiting high production of 1,4-butanediol, which is prepared by introducing or amplifying genes encoding enzymes converting succinate into 4-hydroxybutyrate, and 4-hydroxybutyrate into 1,4-butanediol, in a microorganism capable of producing succinate.

【Claim 2】

The mutant according to claim 1, wherein the microorganism capable of producing succinate is selected from the group consisting of bacterium, yeast and fungi that exhibit high production of succinate.

【Claim 3】

The mutant according to claim 2, wherein the bacterium is selected from the group consisting of Lumen bacteria, *Corynebacterium species*, *Brevibacterium species* and *E. coli*.

【Claim 4】

The mutant according to claim 3, wherein the Lumen bacteria have inactive genes encoding lactate dehydrogenase (*ldhA*) and pyruvate-formate lyase (*pfl*), and produce succinate in high concentration without substantial production of other organic acids in an anaerobic condition.

【Claim 5】

The mutant according to claim 3, wherein the Lumen bacteria have inactive genes encoding lactate dehydrogenase (*ldhA*), pyruvate-formate lyase (*pfl*), phosphotransacetylase (*pta*) and acetate kinase (*ackA*), and produce succinate in high

concentration without substantial production of other organic acids in an anaerobic condition.

【Claim 6】

The mutant according to claim 3, wherein the Lumen bacteria have inactive genes encoding lactate dehydrogenase (*ldhA*), pyruvate-formate lyase (*pfl*) and phosphopyruvate carboxylase (*ppc*), and produce succinate in high concentration without substantial production of other organic acids in an anaerobic condition.

【Claim 7】

The mutant according to claim 3, wherein the Lumen bacteria are selected from the group consisting of *Mannheimia species*, *Actinobacillus species* and *Anaerobiospirillum species*.

【Claim 8】

The mutant according to claim 7, wherein the Lumen bacteria are *Mannheimia species*.

【Claim 9】

The mutant according to claim 8, wherein the Lumen bacteria are selected from the group consisting of *Mannheimia succiniciproducens* MBEL55E (KCTC 0769BP), and *Mannheimia species* LPK (KCTC 10558BP), LPK4 and LPK7 (KCTC 10626BP).

【Claim 10】

The mutant according to claim 3, wherein the E. coli has inactive genes encoding glucose phosphotransferase (*ptsG*) and pyruvate kinase (*pykA* and *pykF*), and produces succinate in high concentration without substantial production of other organic acids in an anaerobic condition.

【Claim 11】

The mutant according to claim 10, wherein the E. coli mutant is W3110GFA.

【Claim 12】

The mutant according to claim 1, wherein the gene encoding the enzyme converting succinate into 4-hydroxybutyrate is derived from *Clostridium kluyveri*.

【Claim 13】

The mutant according to claim 1, wherein the gene encoding the enzyme converting succinate into 4-hydroxybutyrate is selected from the group consisting of genes encoding succinyl-CoA transferase (Cat1), succinate semialdehyde dehydrogenase (SucD), 4-hydroxybutyrate dehydrogenase (4hbD) and 4-hydroxybutyrate dehydrogenase (GHB).

【Claim 14】

The mutant according to claim 13, wherein the gene encoding Cat1 has a base sequence of SEQ ID NO: 1, the gene encoding SucD has a base sequence of SEQ ID NO: 2, the gene encoding 4hbD has a base sequence of SEQ ID NO: 3, and the gene encoding GHB has a base sequence of SEQ ID NO: 4.

【Claim 15】

The mutant according to claim 13, wherein the mutant comprises a gene encoding Cat1; a gene encoding SucD; and a gene encoding 4hbD or a gene encoding GHB.

【Claim 16】

The mutant according to claim 1, wherein the gene encoding the enzyme converting 4-hydroxybutyrate into 1,4-butanediol is derived from *Clostridium acetobutylicum*.

【Claim 17】

The mutant according to claim 1, wherein the gene encoding the enzyme converting 4-hydrxoybutyrate into 1,4-butanediol is a gene encoding 4-hydroxybutyrate-CoA transferase and a gene encoding alcohol dehydrogenase reducing 4-hydroxybutyrate-CoA; or a gene encoding phosphotransbutyrylase, a gene encoding butyryl kinase and a gene encoding alcohol dehydrogenase reducing 4-hydroxybutyrate-CoA.

【Claim 18】

The mutant according to claim 17, wherein the gene encoding 4-hydroxybutyrate-CoA transferase has a base sequence of SEQ ID NO: 5.

【Claim 19】

The mutant according to claim 17, wherein the gene encoding phosphotransbutyrylase and the gene encoding butyryl kinase have base sequences of by SEQ ID NOs: 6 and 7, respectively.

【Claim 20】

The mutant according to claim 17, wherein the alcohol dehydrogenase is butyl-CoA dehydrogenase derived from *Clostridium acetobutylicum*.

【Claim 21】

The mutant according to claim 20, wherein the gene encoding butyl-CoA dehydrogenase has a base sequence of SEQ ID NO: 8 or 9.

【Claim 22】

The mutant according to claim 1, wherein the mutunt has an inactive gene associated with conversion of succinate semialdehyde into succinate.

【Claim 23】

The mutant according to claim 22, wherein the gene associated with conversion of succinate semialdehyde into succinate is a gene encoding succinic semialdehyde dehydrogenase (GabD).

【Claim 24】

The mutant according to claim 23, wherein the gene encoding GabD has a base sequence of SEQ ID NO: 10.

【Claim 25】

The mutant according to claim 1, wherein a gene encoding C4-dicarboxylate transport protein (DctA) associated with transport of succinate is further introduced or amplified in the mutant.

【Claim 26】

The mutant according to claim 25, wherein the gene encoding DctA has a base sequence of SEQ ID NO: 11.

【Claim 27】

A mutant exhibiting high production of 1,4-butanediol, which is prepared by introducing or amplifying a gene encoding Cat1; a gene encoding SucD; a gene encoding 4hbD or GHB; a gene encoding 4-hydroxybutyrate-CoA transferase, or a gene encoding Ptb and a gene encoding Buk; and a gene encoding butyl-CoA dehydrogenase, in a microorganism capable of producing succinate.

【Claim 28】

The mutant according to claim 27, wherein a gene encoding GabD is inactivated in the mutant.

【Claim 29】

The mutant according to claim 27, wherein a gene encoding DctA associated with transport of succinate is introduced or amplified in the mutant.

【Claim 30】

A method of preparing 1,4-butanediol, comprising:

culturing the mutant according to any one of claims 1 to 29 in a medium containing a carbon source; and

obtaining 1,4-butanediol from the medium.

【Claim 31】

A butyl-CoA dehydrogenase gene having a base sequence of SEQ ID NO: 8 or 9.

【Claim 32】

A recombinant vector containing the gene according to claim 31.

FIG. 1

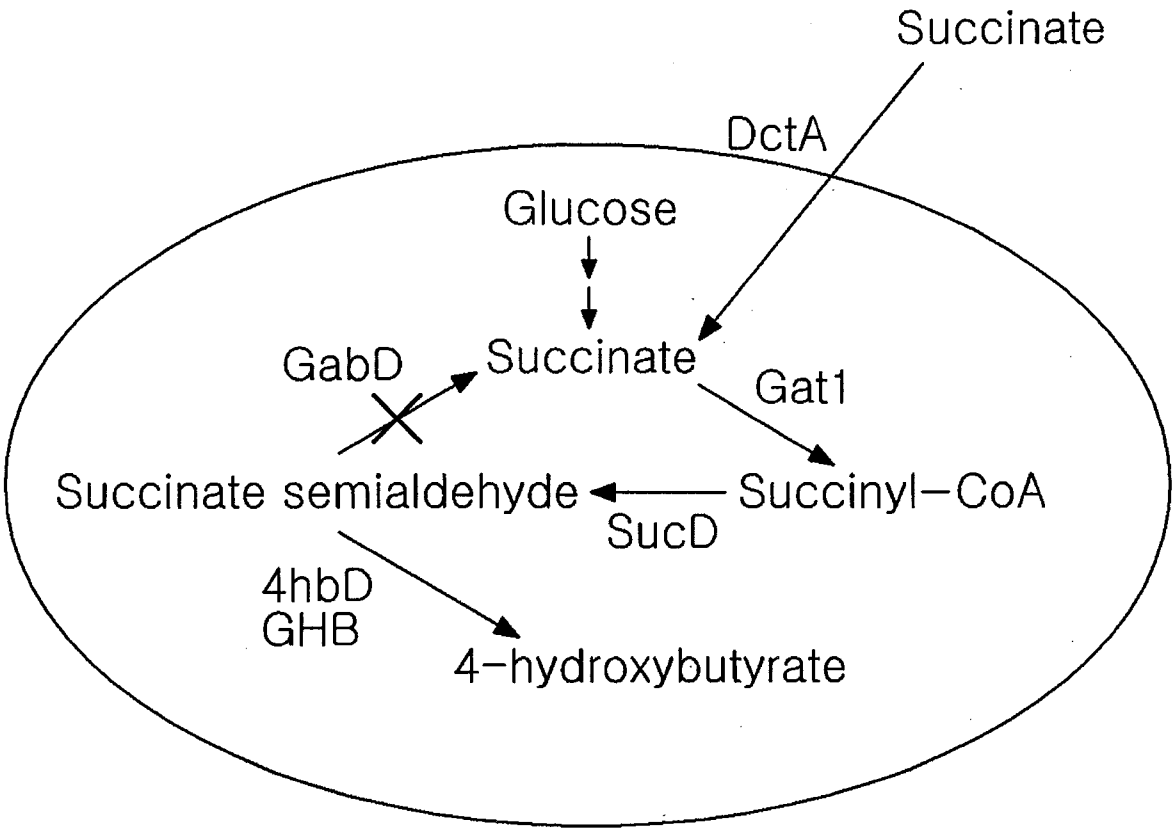

Succinate
DctA
Glucose
GabD
Succinate
Gat1
Succinate semialdehyde
Succinyl-CoA
SucD
4hbD
GHB
4-hydroxybutyrate

FIG. 2

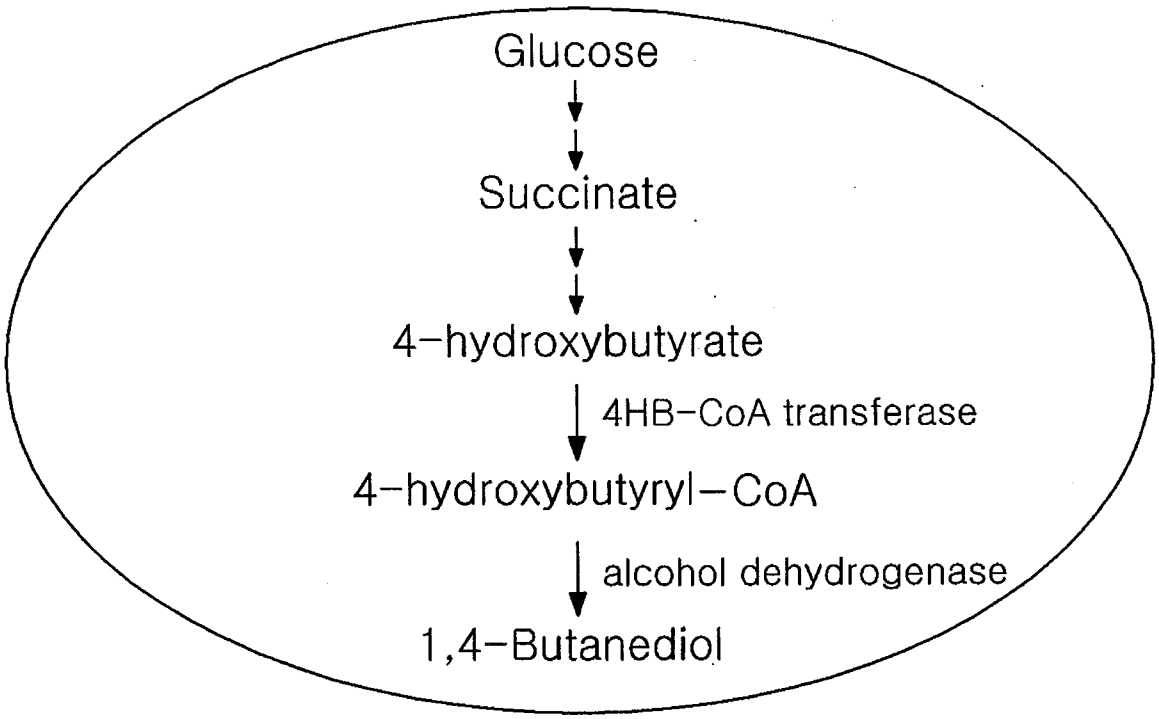
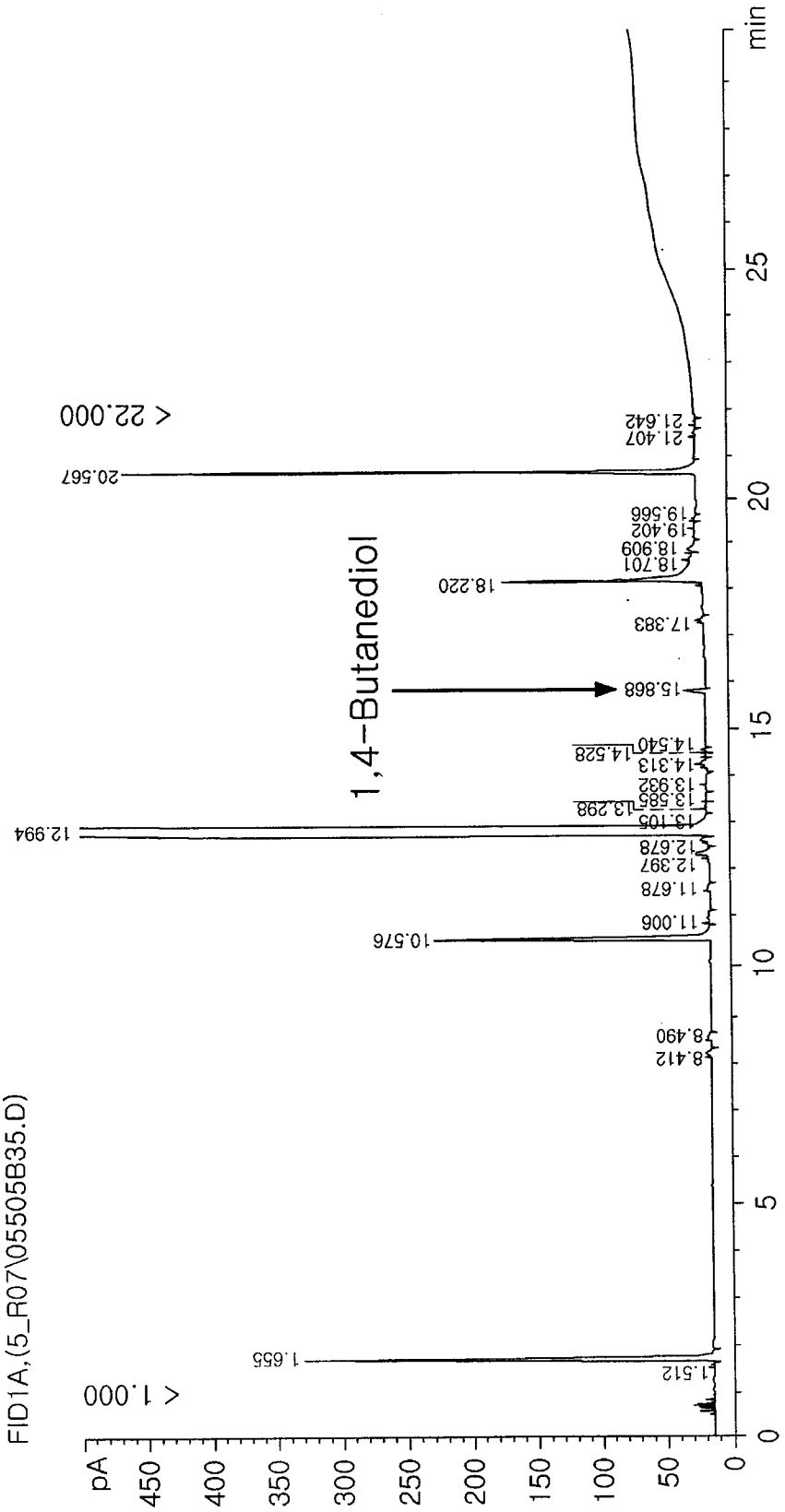

Glucose
Succinate
4-hydroxybutyrate
4HB-CoA transferase
4-hydroxybutyryl-CoA
alcohol dehydrogenase
1,4-Butanediol

FIG. 3


FID1A,(5_R07\05505B35.D)
pA
min
< 1.000
< 22.000
1,4-Butanediol
1.512
1.655
8.412
8.490
10.576
11.006
11.678
12.397
12.678
12.994
13.105
13.298
13.585
13.932
14.313
14.528
14.540
15.868
17.383
18.220
18.701
18.909
19.402
19.566
20.567
21.407
21.642