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(54) Benævnelse: **Mutanter med evne til at producere 1,4-butandiol og fremgangsmåde til at fremstille 1,4-butandiol ved anvendelse heraf**

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DESCRIPTION

[Technical Field]

[0001] 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]

[0002] 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.

[0003] 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.

[0004] 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.

[0005] PCT Publication No. WO 2005/052135 is an example of a patent application disclosing a method of efficiently producing succinate, in which a Rumen 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.

[0006] 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]

[0007] 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]

[0008] 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 according to the independent claims. Preferred embodiments are disclosed in the subclaims.

[0009] 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.

[0010] Hereinafter, the present invention will be described in more detail.

[0011] 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.

[0012] 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.

[0013] The microorganism capable of producing succinate may exhibit high production of succinate, the microorganism is one selected from the group consisting of bacteria, for example, Rumen bacteria, *Corynebacterium species, Brevibacterium species* and *E.coli.*

[0014] The Rumen 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.

[0015] 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.

[0016] Further, the Rumen bacteria may 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.

[0017] Alternatively, the Rumen bacteria may 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.

[0018] The Rumen 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.

[0019] 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.

[0020] Among the above-mentioned microorganisms producing succinate in high concentration, the Rumen bacteria may be prepared in a method disclosed in PCT Publication No. WO 2005/052135. That is, a gene of lactic dehydrogenase (*ldhA*) 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.

[0021] 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.

[0022] 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.

[0023] Further, the gene of the enzyme converting succinate into 4-hydroxybutyrate is selected from the group consisting of a gene encoding succinyl-CoA transferase (Cat1), a gene encoding succinate semialdehyde dehydrogenase (SucD), and a gene encoding 4-hydroxybutyrate dehydrogenase (hbD or 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.

[0024] 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.

[0025] Further, effective use of succinate is very important to accomplish the object of the present invention, and thus succinic semialdehyde dehydrogenase (*GabD*) 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.

[0026] 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.

[0027] The genes of enzymes converting 4-hydroxybutyrate into 1,4-butanediol are genes encoding phosphotransbutyrylase, butyryl kinase and alcohol dehydrogenase reducing 4-hydroxybutyrate-CoA.

[0028] The gene encoding 4-hydroxybutyrate-CoA transferase may have a base sequence of SEQ ID NO: 5. Phosphotransbutyrylase (*ptb*; SEQ ID NO: 6) and butyryl kinase (*BuK*; SEQ ID NO: 7) convert 4-hydroxybutyrate into 4-hydroxybutyrate-CoA.

[0029] 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.

[0030] 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.

[0031] The present description also discloses 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.

[0032] 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 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.

[0033] The present invention further provides a method of preparing 1,4-butanediol comprising culturing the mutant in a medium containing a carbon source, wherein said carbon source is glucose, and obtaining 1,4-butanediol from the culture.

[Advantageous Effects]

[0034] 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]

[0035]

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]

[0036] 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.

[0037] While, in the present invention, a method of preparing 1, 4-butanediol uses Rumen 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 Rumen bacteria strain, and introducing and amplifying a gene associated with producing 1,4-butanediol.

[0038] 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 Rumen Bacteria Having High Production of Succinate

[0039] A microorganism, a Rumen bacterium, exhibiting high production of succinate according to the present invention was 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 Rumen 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

[0040] 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)**

[0041] 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

[0042] 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 *SacI*/*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

[0043] 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

[0044] 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

[0045] 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

[0046] 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.

[0047] 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

[0048] To construct reference expression vector pTacLacCat2, the amplified *cat2* gene was inserted into expression vector pTacLacI, which was cleaved with *Eco*RI/*Bam*HI.

[0049] 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

[0050] 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

[0051] 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°C. 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°C. 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°C. 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.

[0052] 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-butyrolactone 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°C, 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-Wax (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°C, 5 min

Program Rate: 10°C/min

Final Value & Time: 250°C, 5 min

Injector Temperature: 250°C

Detector Temperature: 250°C

Injector Split Ratio: 20/1

Injector Volume: 1.0ul

[0053] As shown in FIG. 3, it was confirmed that 1,4-butanediol was produced.

<110> LG CHEM, LTD. Korea Advanced Institute of Science and Technology

<120> Mutants having capability to produce 1,4-butanediol and method for preparing 1,4-butanediol using the same

<130> F2008-0085PC(X08029)

<150> KR10-2007-0091081
<151> 2007-09-07

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<210> 2
<211> 1419
<212> DNA
<213> SucD-coding gene

<400> 2

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tatgataatg cagaaatgtt tgcaaaagaa gcagttgaag aaacagaaat gggtgtttat 180
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gaaagtgctg atgtagttat tgctacaggc ggtgctggaa gagttaaagc tgcttactcc 600
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gaagcaaaag ttcctagcta tgaggaaata tggggataa 1419

<210> 3
<211> 1116
<212> DNA
<213> 4hbD-coding gene

<400> 3
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gaaccttctg atgaaatgat aaacaatata attaaggata ttggagataa acaatataat 240
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<210> 4 <211> 1460

<212> DNA
<213> GHB-coding gene

<400> 4

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cgagataacc ccgaaaagtc gctgtcagcc tgccacgcgg caagtttttg cgcgatgatc 120

ggctgaagcg gtcccgaggg ctccggaaac gcagtagtgc aggtccattg aaacccaaga 180

cagcgggcct ggcgagcatc cgctccaggc ccgtgcaaaa gacaatttgg cggcagatcc 240

cggcaggaga caagcaaaca tggcgtttat ctactatctg acccacatcc acctggattt 300

cggcgcggta agcctgctca agtccgaatg cgagcgcatc ggcatccgcc gcccgttgct 360

ggtgaccgac aagggcgtgg tcgccgcggg agtggcgcag cgtgccatcg atgcaatgca 420

gggcctgcag gttgcggtat tcgatgaaac cccgtcgaac ccgaccgagg ccatggtgcg 480

caaggccgcc gcacaatacc gcgaggccgg ctgcgacggg ctggtggcag tgggcggcgg 540

ctcgtcgatc gacctcgcca agggcatcgc catcctggcc acgcatgagg gcgagctgac 600

cacctatgcc accatcgaag gcggcagcgc caggatcacc gacaaggcgg cgccgctgat 660

cgcggtgccc accacctcgg gcaccggcag cgaggtggcg cgcggcgcca tcatcatcct 720

ggacgacggc cgcaagctgg gcttccattc ctggcatttg ctgcccaagt ccgccgtctg 780

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cctgcccacc gggctgcgtc agatgggtgt caccgaggac atgttcgaca aggtgattgc 1320

cggtgcgctg gtcgaccatt gccacaagac caacccgaaa gaagccagcg ccgcggatta 1380

tcggcgtatg cttgagcagt ccatgtagca cacagcggct tcccgccggt cagaccgacc 1440

aagcggctgt ccggcggccc 1460

<210> 5
<211> 1290
<212> DNA
<213> 4HB-CoA transferase-coding gene

<400> 5

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atagaaaacc atagcagggt agtttttgca catgcagtag gagaacccgt agatttagta 120
aatgcactag ttaaaaataa ggataattat ataggactag aaatagttca catggtagct 180

atgggcaaag gtgtatatac aaaagagggt atgcaaagac attttagaca taatgctttg 240
tttgtaggcg gatctactag agatgcagta aattcaggaa gagcagttta tacaccttgt 300
tttttctatg aagtgccaag tttgtttaaa gaaaaacgtt tgcctgtaga tgtagcactt 360
attcaggtaa gtgagccaga taaatatggc tactgcagtt ttggagtttc caatgactat 420
accaagccag cagcagaaag tgctaagctt gtaattgcag aagtgaataa aaacatgcca 480
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cacccattgt tagaattgca gcctcctaaa ttgggagatg tagaaaaagc cataggagaa 600
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catccaggca aaatagttgt aacattttta atgggaacaa aaaaattata tgattttgta 840
aacaataatc caatggtaga aacttattct gtagattatg taaataatcc actggtaatt 900
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tctagaaatg aagtagatta tgtagttact gaatatggtg ttgctcatct taagggcaaa 1200
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ttaatgaatg aatttaaaaa gagattttag 1290

<210> 6
<211> 906
<212> DNA
<213> Ptb-coding gene

<400> 6
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ggtattgcag atgctattct tgttggagac catgacgaaa tcgtgtcaat cgcgcttaaa 180
ataggaatgg atgtaaatga ttttgaaata gtaaacgagc ctaacgttaa gaaagctgct 240
ttaaaggcag tagagcttgt atcaactgga aaagctgata tggtaatgaa gggacttgta 300
aatacagcaa ctttcttaag atctgtatta aacaaagaag ttggacttag aacaggaaaa 360
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gatgttgctt tcaatactta tcctgaatta aaggaaaaaa ttgatatagt aaacaattca 480

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gctgacagcc atgaaacaaa aatgaactct atagcacttg cagctttagt tgcaggcaat 900
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<210> 7
<211> 1068
<212> DNA
<213> Buk-coding gene

<400> 7

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aacactatat ttgatcaatt tcaattcaga aagaatgtaa ttttagatgc gttaaaagaa     180
gcaaacatag aagtaagttc tttaaatgct gtagttggaa gaggcggact cttaaagcca     240
atagtaagtg gaacttatgc agtaaatcaa aaaatgcttg aagaccttaa agtaggagtt     300
caaggtcagc atgcgtcaaa tcttggtgga attattgcaa atgaaatagc aaaagaaata     360
aatgttccag catacatagt tgatccagtt gttgtggatg agcttgatga agtttcaaga     420
atatcaggaa tggctgacat tccaagaaaa agtatattcc atgcattaaa tcaaaaagca     480
gttgctagaa gatatgcaaa agaagttgga aaaaaatacg aagatcttaa tttaatcgta     540
gtccacatgg gtggaggtac ttcagtaggt actcataaag atggtagagt aatagaagtt     600
aataatacac ttgatggaga aggtccattc tcaccagaaa gaagtggtgg agttccaata     660
ggagatcttg taagattgtg cttcagcaac aaatatactt atgaagaagt aatgaaaaag     720
ataaacggca aaggcggagt tgttagttac ttaaatacta tcgattttaa ggctgtagtt     780
gataaagctc ttgaaggaga taagaaatgt gcacttatat atgaagcttt cacattccag     840
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ttaacaggcg gaattgcgta caacgagcat gtatgtaatg ccatagagga tagagtaaaa     960
ttcatagcac ctgtagttag atatggtgga gaagatgaac ttcttgcact tgcagaaggt    1020
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<210> 8
<211> 2577
<212> DNA
<213> Butyl-CoA dehydrogenase(CAP0035)-coding gene

<400> 8

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gccgcagcta aagaaagaat aaacttagct aaattagcag tagaagaaac aggaataggt     180
cttgtagaag ataaaattat aaaaaatcat tttgcagcag aatatatata caataaatat     240
aaaaatgaaa aaacttgtgg cataatagac catgacgatt ctttaggcat aacaaaggtt     300
gctgaaccaa ttggaattgt tgcagccata gttcctacta ctaatccaac ttccacagca     360
attttcaaat cattaatttc tttaaaaaca agaaacgcaa tattcttttc accacatcca     420
cgtgcaaaaa aatctacaat tgctgcagca aaattaattt tagatgcagc tgttaaagca     480
ggagcaccta aaaatataat aggctggata gatgagccat caatagaact ttctcaagat     540
ttgatgagtg aagctgatat aatattagca acaggaggtc cttcaatggt taaagcggcc     600
tattcatctg gaaaacctgc aattggtgtt ggagcaggaa atacaccagc aataatagat     660
gagagtgcag atatagatat ggcagtaagc tccataattt tatcaaagac ttatgacaat     720
ggagtaatat gcgcttctga acaatcaata ttagttatga attcaatata cgaaaaagtt     780
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gaagcagaaa ttgaaaatct agctataaac tttatggata taagaaagag aatatgcaat    1740
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cctagaaaat taacagcagc aactggaata gatgcattag ttcatgctat agaagcatat    1980
gtttcggtta tggctacgga ttatactgat gaattagcct taagagcaat aaaaatgata    2040
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ttttataata cgctagataa aatgtcagag cttgcttttg atgaccaatg tacaacagct    2520
aatcctaggt atccacttat aagtgaactt aaggatatct atataaaatc attttaa       2577
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<210> 9
<211> 2589
<212> DNA
<213> Butyl-CoA dehydrogenase(CAP0162)-coding gene

<400> 9

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gcagcaatcg acgcaaggat agagctagca aaagcagctg ttttggaaac cggtatgggc 180

ttagttgaag acaaggttat aaaaaatcat tttgcaggcg aatacatcta taacaaatat 240

aaggatgaaa aaacctgcgg tataattgaa cgaaatgaac cctacggaat tacaaaaata 300

gcagaaccta taggagttgt agctgctata atccctgtaa caaaccccac atcaacaaca 360

atatttaaat ccttaatatc ccttaaaact agaaatggaa ttttcttttc gcctcaccca 420

agggcaaaaa aatccacaat actagcagct aaaacaatac ttgatgcagc cgttaagagt 480

ggtgccccgg aaaatataat aggttggata gatgaacctt caattgaact aactcaatat 540

ttaatgcaaa aagcagatat aacccttgca actggtggtc cctcactagt taaatctgct 600

tattcttccg gaaaaccagc aataggtgtt ggtccgggta acaccccagt aataattgat 660

gaatctgctc atataaaaat ggcagtaagt tcaattatat tatccaaaac ctatgataat 720

ggtgttatat gtgcttctga acaatctgta atagtcttaa aatccatata taacaaggta 780

aaagatgagt tccaagaaag aggagcttat ataataaaga aaaacgaatt ggataaagtc 840

cgtgaagtga tttttaaaga tggatccgta aaccctaaaa tagtcggaca gtcagcttat 900

actatagcag ctatggctgg cataaaagta cctaaaacca caagaatatt aataggagaa 960

gttacctcct taggtgaaga agaacctttt gcccacgaaa aactatctcc tgttttggct 1020

atgtatgagg ctgacaattt tgatgatgct ttaaaaaaag cagtaactct aataaactta 1080

ggaggcctcg gccatacctc aggaatatat gcagatgaaa taaaagcacg agataaaata 1140

gatagattta gtagtgccat gaaaaccgta agaacctttg taaatatccc aacctcacaa 1200

ggtgcaagtg gagatctata taattttaga ataccacctt ctttcacgct tggctgcgga 1260

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aagttcggtt gtcttcaatt tgctttaaaa gatttaaaag atctaaagaa aaaaagagcc 1440

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aaaaccataa aaaaagcaac tgaagaaatg tcctccttta tgccagacac tataatagct 1620

ttaggtggta cccctgaaat gagctctgca aagctaatgt gggtactata tgaacatcca 1680

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tctgaggtta ctccttttgc tttagtaact gacaataaca ctggaaataa gtacatgtta 1860

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ctaaaaaaag ctttaaatat accaacttca ataaaggatg caggtgtttt ggaggaaaac 2460

ttctattcct cccttgatag aatatctgaa cttgcactag atgatcaatg cacaggcgct 2520

aatcctagat ttcctcttac aagtgagata aaagaaatgt atataaattg ttttaaaaaa 2580

caaccttaa 2589

<210> 10
<211> 1449
<212> DNA
<213> GabD-coding gene

<400> 10

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REFERENCES CITED IN THE DESCRIPTION

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Patentkrav

1. Mutant, som udviser høj produktion af 1,4-butandiol, og som fremstilles ved at indføre eller amplificere gener, som koder for enzymer, der omdanner succinat til 4-hydroxybutyrat og 4-hydroxybutyrat til 1,4-butandiol, i en mikroorganisme i stand til at producere succinat,

hvor mikroorganismen i stand til at producere succinat er en bakterie, og

hvor genet, som koder for enzymet, som omdanner succinat til 4-hydroxybutyrat, er valgt fra gruppen bestående af gener, som koder for succinyl-CoA-transferase, succinatsemialdehyddehydrogenase, og 4-hydroxyburatdehydrogenase, og

genet, som koder for enzymet, der omdanner 4-hydroxybutyrat til 1,4-butandiol er et gen, der koder for phosphotransbutyrylase, et gen, som koder for butyrylkinase og et gen, som koder for alkoholdehygrogenasereducerende 4-hydroxybutyrat-CoA.

2. Mutant ifølge krav 1, hvor bakterien er valgt fra gruppen bestående af vomflora, *Corynebacterium sp.*, *Brevibacterium sp.* og *E. coli*.

3. Mutant ifølge krav 2, hvor vomflorabakterierne har inaktive gener, som koder for i) lactatdehydrogenase (*ldhA*) og pyruvatformatlyase (*pff*), ii) lactatdrhydrogenase (*lhdA*), pyruvatformatlyase (*Pff*), phosphotranacetylase (*pta*) og acetatkinase (*ackA*), eller iii) lactatdehydrogenase (*lhdA*), pyruvatformatlyase (*Pff*) og phosphopyruvatcarboxylase (*Ppc*) og producerer succinat i en høj koncentration uden væsentlig produktion i anaerob tilstand af andre organiske syrer.

4. Mutant ifølge krav 2 eller 3, hvor vomflorabakterierne er valgt fra gruppen bestående af *Mannheimia sp.*, *Actinobacillus sp.* og *Anaerobiospirillum sp.*

5. Mutant ifølge krav 4, hvor vomflorabakterierne er valgt fra gruppen bestående af *Mannheimia succiniciproducens* MBEL55E (KCTC 0769BP), og *Mannheimia sp.* LPK (KCTC 10558BP), LPK4 og LPK7 (KCTC 10626BP).

6. Mutant ifølge krav 2, hvor E. coli har inaktive gener, som koder for glucose-phosphotransferase (*ptsG*) og pyruvatkinase (*pykA* og *pykF*) og producerer succinat i høj koncentration uden væsentlig produktion af andre organiske syrer ved anaerobe betingelser.

7. Mutant ifølge krav 6, hvor E. coli-mutanten er W3110GFA.

8. Mutant ifølge krav 1, hvor genet, som koder for enzymet, der omdanner succinat til 4-hydroxybutyrat, stammer fra *Clostridium kluyveri*.

9. Mutant ifølge krav 1, hvor genet, som koder for enzymet, der omdanner 4-hydroxybutyrat til 1,4-butandiol, stammer fra *Clostridium acetobutylicum*.

10. Mutant ifølge krav 1, hvor alkoholdehydrogenasen er butyl-CoA-dehydrogenase stammende fra Clostridium acetobutylicum.

11. Mutant ifølge krav 1, hvor mutanten har et inaktivt gen associeret med omdannelsen af succinatsemialdehyd til succinat.

12. Mutant ifølge krav 11, hvor genet associeret med omdannelse af succinatsemialdehyd til succinat er et gen, som koder for succinsemialdehyddehydrogenase.

13. Mutant ifølge krav 1, hvor et gen, som koder for C4-dicarboxy sent transportprotein (DctA) associeret med transport af succinat, yderligere indføres i eller amplificeres i mutanten.

14. Fremgangsmåde til at fremstille 1,4-butandiol omfattende:

at dyrke mutanten ifølge et vilkårligt af kravene 1 til 13 i et medie indeholdende glucose, og

opnå 1,4-butandiol fra mediet.

DRAWINGS

FIG. 1

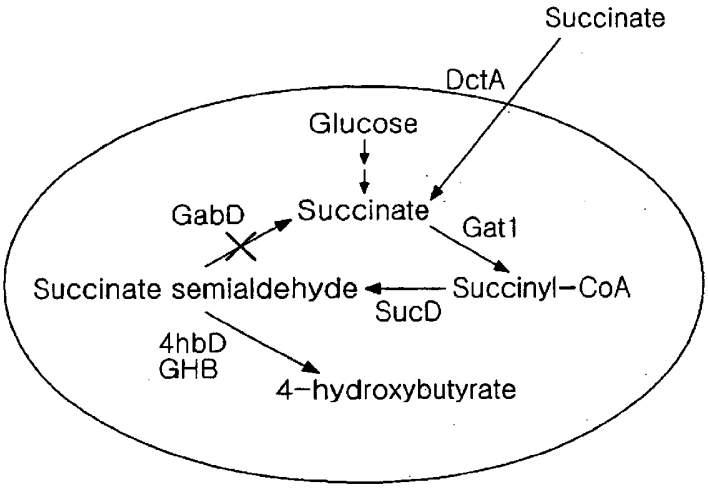

FIG. 2

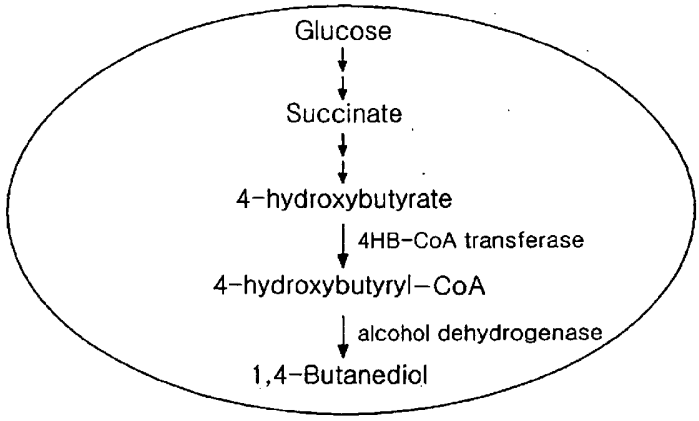
Glucose
Succinate
4-hydroxybutyrate
4HB-CoA transferase
4-hydroxybutyryl-CoA
alcohol dehydrogenase
1,4-Butanediol

FIG. 3