

METHOD OF PRODUCING L-GLUTAMIC ACID USING MICROORGANISM HAVING CARBON DIOXIDE FIXATION PATHWAY

Technical Field

[0001] The present disclosure relates to a method of producing L- glutamic acid using a microorganism having an acetyl-CoA production cycle by the introduction of a carbon dioxide fixation pathway.

Background Art

[0002] Acetyl-CoA is one of significantly important intermediates in metabolic pathways of microorganisms. Various metabolites are produced via acetyl-CoA. Well-known examples of such substances produced via acetyl-CoA include amino acids such as L-glutamic acid, L-glutamine, L-proline, L-arginine, L-leucine, and L-isoleucine; organic acids such as acetic acid, propionic acid, butyric acid, caproic acid, citric acid, 3-hydroxybutyric acid, 3-hydroxyisobutyric acid, 3-aminoisobutyric acid, 2-hydroxyisobutyric acid, methacrylic acid, and poly-3-hydroxybutyric acid; alcohols such as isopropyl alcohol, ethanol, and butanol; acetone; and polyglutamic acids.

[0003] In most microorganisms, acetyl-CoA is produced using a sugar such as glucose as a carbon source. The sugar is first converted into pyruvate via a metabolic pathway called the glycolytic pathway, such as the Embden-Meyerhof pathway, Entner-Doudoroff pathway, or the pentose phosphate pathway. Subsequently, the pyruvate is converted into acetyl-CoA by actions of decarboxylase, pyruvate formate-lyase, and the like. In this process, carbon dioxide and formate are generated as byproducts, and some of the carbons derived from the sugar will be lost. Therefore, several studies have been carried out with the aim of achieving re-fixation of carbon dioxide in order to increase the yield of acetyl-CoA.

[0004] In microorganisms, there are several known pathways for fixing carbon dioxide as a carbon source (Appl. Environ. Microbiol. 77(6), 1925-1936, 2011). Specific examples of the pathways include the Calvin-Benson cycle, the reductive TCA cycle, the Wood-Ljungdahl pathway, the 3-hydroxypropionate cycle, and the 4-hydroxybutyrate cycle. The Calvin-Benson cycle is a CO₂ fixation pathway existing in plants and photosynthetic bacteria, and containing about 12 kinds of enzymes. In the Calvin-Benson cycle, CO₂ is fixed by ribulose-1,5-bisphosphate carboxylase (RubisCO) and, ultimately, glyceraldehyde 3-phosphate is produced. The reductive TCA cycle is found in microaerophilic bacteria and anaerobic bacteria including green sulfur bacteria, and contains 11 kinds of enzymes. This

cycle is characterized by CO₂ fixation enzymes (*i.e.*, acetyl-CoA carboxylase, 2-oxoglutarate synthase) that requires ferredoxin as a coenzyme. In the reductive TCA cycle, pyruvate is produced from CO₂ by the reverse reaction of the usual TCA cycle. The Wood-Ljungdahl pathway is found in anaerobic microorganisms such as acetic acid-producing bacteria, and contains 9 kinds of enzymes. In the Wood-Ljungdahl pathway, CO₂ and formate on a coenzyme are reduced by formate dehydrogenase, CO dehydrogenase, *etc.*, and, ultimately converted into acetyl-CoA. The 3-hydroxypropionate cycle is found in *Chloroflexus* bacteria and the like, and contains 13 kinds of enzymes. In the 3-hydroxypropionate cycle, CO₂ is fixed by the action of acetyl-CoA (propionyl-CoA) carboxylase and acetyl-CoA is produced via malonyl-CoA and the like. The 4-hydroxybutyrate cycle exists in archaeobacteria and the like. In the 4-hydroxybutyrate cycle, CO₂ is fixed by the actions of pyruvate synthase, acetyl-CoA (propionyl-CoA) carboxylase, and phosphoenolpyruvate carboxylase, whereby acetyl-CoA is produced via 4-hydroxybutyryl CoA and the like.

[0005] In order to produce a useful substance, several approaches have been reported as ideas to introduce a carbon dioxide fixation pathway to a useful-compound-producing microorganism. For example, International Publication WO 2009/094485 and WO 2010/071697 disclose approaches to producing acetyl-CoA from carbon dioxide, by using a microorganism to which a pathway similar to the Wood-Ljungdahl pathway of acetic acid bacteria was introduced. As an example of CO₂ fixation for producing a useful compound, WO 2009/046929 discloses an approach to producing lactic acid from carbon dioxide by using a microorganism to which hydrogenase and tetrahydrofolate lyase were introduced. WO 2011/099006 proposes a cycle in which CO₂ is fixed via a carbon dioxide fixation reaction onto acetyl-CoA or a malonyl-CoA reduction reaction. German Patent No. 102007059248 proposes production of acetyl-CoA by a pathway similar to the 4-hydroxybutyrate cycle.

SUMMARY OF INVENTION

Technical Problem

[0006] However, known carbon dioxide fixation cycles are not necessarily efficient from the viewpoints of CO₂ fixation and production of useful chemical products derived from acetyl-CoA. For example, the Calvin-Benson cycle is most famous as a carbon dioxide fixation cycle found in nature, but RubisCO involved in carbon dioxide fixation has a low reaction rate and causes side reactions such as oxidative degradation. Therefore, RubisCO is inefficient as an enzyme (Journal of Bioscience and Bioengineering 94(6) 497-505, 2002).

In the Wood-Ljungdahl pathway and the pathways described in WO 2009/094485, WO 2010/071697, WO 2009/046929 and the like, a pathway for reducing CO₂ into CO or formate is included. However, the reduction reaction hardly occurs under ordinal conditions, and the enzyme catalyzing this kind of strong reduction reaction often only acts under a reductive environment. Therefore, it is difficult to introduce this kind of pathway into microorganisms other than strictly anaerobic microorganisms. In the reductive TCA cycle, a reduction reaction by pyruvate synthase or 2-oxoglutarate synthase requires a strong reduction power from ferredoxin as an electron acceptor, and it is difficult to carry out the reaction. The 4-hydroxybutyrate cycle, 3-hydroxypropionate cycle, and the pathways described in WO 2011/099006, WO 2009/046929, and the like utilize the reduction reaction for carboxylic acid or a (thio)ester thereof, such as reduction of succinyl-CoA or reduction of malonyl-CoA. However, it is generally difficult to carry out this kind of reaction as an enzymatic reaction, and it is desirable to avoid them as fermentation pathways where possible (Atsumi et al., Nature, 451, (3), 86-89, 2008; Yim et al., Nat. Chem. Biol., 7, 445-452, 2011). The 4-hydroxybutyrate cycle proceeds via a dehydration reaction such as dehydration of 4-hydroxybutyryl CoA or dehydration of 3-hydroxypropionate, but this cycle has a disadvantage in that this kind of dehydration reaction often competes with the reverse reaction (hydration) in water. In the 4-hydroxybutyrate cycle, the 3-hydroxypropionate cycle, and the reductive TCA cycle, the acetyl-CoA produced is converted into other substances within the cycle by the action of malonyl-CoA synthase or pyruvate synthase. Therefore, these cycles are not necessarily efficient from the viewpoint of acetyl-CoA production.

[0007] When attempting to produce a certain substance by introducing this kind of cycle to a microorganism, it is necessary to consider the number of enzymes involved in the cycle and the number of enzymatic activities to be additionally imparted. That is, when the number of enzymes involved in the cycle or the number of enzymatic activities to be additionally imparted increases, regulation becomes more difficult and the burden on the microorganism increases. For example, in order to introduce the Wood-Ljungdahl pathway to *Escherichia coli*, it is required to introduce at least 9 kinds of genes. It would practically be a very difficult task to construct a substance-producing pathway and also introduce and regulate so many genes. It would clearly be advantageous to construct a cycle including a small number of enzymes by imparting a smaller number of enzymes, in terms of constructing the cycle and in terms of combination with another substance production pathway.

[0008] Accordingly, in order to fix CO₂ and convert it into acetyl-CoA, it would be ideal for (A) each enzyme involved in the pathway to have a sufficiently high activity; (B) the cycle to

not include an enzyme that consumes acetyl-CoA; and (C) the cycle to have a simple configuration and a small number of newly imparted enzymes. However, none of the cycles for producing acetyl-CoA from CO₂ reported so far satisfies all of the conditions (A) to (C), and, therefore, the possibility of realizing such cycles is low. In fact, as regards the proposals concerning the existing carbon dioxide fixation cycles, there have been almost no actual examples of converting CO₂ into acetyl-CoA or a substance derived from acetyl-CoA for use in fermentation by imparting an enzymatic activity to an industrially-usable microorganism.

[0009] The present disclosure provides a method of producing L-glutamic acid derived from acetyl-CoA using a microorganism having an acetyl-CoA production cycle by the introduction of a carbon dioxide fixation pathway.

Solution to Problem

[0010] The aspect of the present disclosure is as follows.

[1] A method of producing L-glutamic acid including providing a culture medium including 1 to 6 g/L of yeast extract; culturing a L-glutamic acid producing coryneform bacterium and increasing an amount of the produced L-glutamic acid in the culture medium by addition of yeast extract. The L-glutamic acid producing coryneform bacterium includes an acetyl-CoA production cycle obtained by imparting enzymatic activity of malate thiokinase, and optionally imparting at least one type of enzymatic activity selected from the group consisting essentially of malyl-CoA lyase, glyoxylate carboligase and 2-hydroxy-3-oxopropionate reductase to the L-glutamic acid producing coryneform bacterium that does not have any of: (a) a carbon dioxide fixation cycle having an enzymatic reaction from malonyl-CoA to malonate semialdehyde or 3-hydroxypropionate; (b) a carbon dioxide fixation cycle having an enzymatic reaction from acetyl-CoA and CO₂ to pyruvate; (c) a carbon dioxide fixation cycle having an enzymatic reaction from crotonyl-CoA and CO₂ to ethylmalonyl-CoA or glutaconyl-CoA; (d) a carbon dioxide fixation cycle having an enzymatic reaction from CO₂ to formate; or (e) at least one selected from the group consisting of malate thiokinase and malyl-CoA lyase, wherein none of (a), (b), (c), or (d) is imparted to the bacterium, or the bacterium exhibits none of the functions of (a), (b), (c), and (d) even though at least one of (a), (b), (c), or (d) is imparted.

[2] The method according to [1]. The L-glutamic acid producing coryneform bacterium is *Corynebacterium glutamicum*.

[3] The method according to [1] or [2]. The L-glutamic acid producing coryneform bacterium is modified by using a *yggB* gene so that L-glutamic acid-producing ability of the

strain is enhanced as compared to a non-modified strain.

[4] The method according to [3]. The coryneform bacterium is modified by introducing a mutant *yggB* gene.

[5] The method according to [1]-[4]. The coryneform bacterium is further modified so to decrease α -ketoglutarate dehydrogenase activity.

[6] The method according to [1]-[5]. The coryneform bacterium is further modified so to decrease lactate dehydrogenase activity.

[7] The method of [1]. The method includes providing the culture medium including more than 1 g/L and less than 4 g/L of the yeast extract.

Advantageous Effects of Invention

[0011] The present disclosure provides a method of producing L-glutamic acid using a microorganism having an acetyl-CoA production cycle by the introduction of a carbon dioxide fixation pathway.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Fig. 1 is a pathway diagram for illustrating the outline of the carbon dioxide fixation pathway according to an embodiment of the present disclosure.

Fig. 2 is a diagram showing a method of producing L-glutamic acid of the present disclosure.

Fig. 3 is a graph showing relative biomass and time of fermentation of a L-glutamic acid producing coryneform bacterium of the present disclosure in a culture medium with different complex supplements.

Fig. 4 is a table showing L-glutamic acid yield of a L-glutamic acid producing coryneform bacterium of the present disclosure in a culture medium with different complex supplements.

Fig. 5 is a table showing L-glutamic acid yield of a L-glutamic acid producing coryneform bacterium of the present disclosure in a culture medium with different concentrations of yeast extract.

Fig. 6 is a real time fermentation monitoring data of a L-glutamic acid producing coryneform bacterium of the present disclosure.

DESCRIPTION OF EMBODIMENTS

[0013] Present disclosure provides a method of producing L-glutamic acid (glutamic acid) using a microorganism having an acetyl-CoA production cycle by the introduction of a carbon

dioxide fixation pathway. The microorganism used for the L-glutamic acid production of the present disclosure is a microorganism including an acetyl-CoA production cycle obtained by imparting at least one type of enzymatic activity selected from the group consisting of malate thiokinase, malyl-CoA lyase, glyoxylate carboligase and 2-hydroxy-3-oxopropionate reductase, to a microorganism that does not have any of the following (a), (b), (c), (d), or (e), in which none of (a), (b), (c), or (d) is imparted to the microorganism, or the microorganism exhibits none of the functions of (a), (b), (c), and (d) even though at least one of (a), (b), (c), or (d) is imparted.

[0014] (a) a carbon dioxide fixation cycle having an enzymatic reaction from malonyl-CoA to malonate semialdehyde or 3-hydroxypropionate;

(b) a carbon dioxide fixation cycle having an enzymatic reaction from acetyl-CoA and CO₂ to pyruvate;

(c) a carbon dioxide fixation cycle having an enzymatic reaction from crotonyl-CoA and CO₂ to ethylmalonyl-CoA or glutaconyl-CoA;

(d) a carbon dioxide fixation cycle having an enzymatic reaction from CO₂ to formate; or

(e) at least one selected from the group consisting of malate thiokinase and malyl-CoA lyase.

[0015] In the present disclosure, by imparting a predetermined enzymatic activity, there can be constructed a carbon dioxide fixation cycle that fixes CO₂ generated during carbohydrate metabolism or CO₂ supplied from outside, and provided a microorganism having an acetyl-CoA production cycle in which CO₂ is efficiently converted to acetyl-CoA and subsequently to L-glutamic acid.

CO₂ can be converted to acetyl-CoA by imparting at least one type of enzymatic activity selected from the group consisting of malate thiokinase, malyl-CoA lyase, glyoxylate carboligase and 2-hydroxy-3-oxopropionate reductase, to a microorganism that does not have any of:

(a) a carbon dioxide fixation cycle having an enzymatic reaction from malonyl-CoA to malonate semialdehyde or 3-hydroxypropionate;

(b) a carbon dioxide fixation cycle having an enzymatic reaction from acetyl-CoA and CO₂ to pyruvate;

(c) a carbon dioxide fixation cycle having an enzymatic reaction from crotonyl-CoA and CO₂ to ethylmalonyl-CoA or glutaconyl-CoA;

(d) a carbon dioxide fixation cycle having an enzymatic reaction from CO₂ to

formate; or

(e) at least one selected from the group consisting of malate thiokinase and malyl-CoA lyase,

in which none of (a), (b), (c), or (d) is imparted to the microorganism, or the microorganism exhibits none of the functions of (a), (b), (c), and (d) even though at least one of (a), (b), (c), or (d) is imparted.

[0016] Furthermore, by using the microorganism that converts CO₂ into acetyl-CoA, or by additionally imparting a predetermined enzymatic activity to the microorganism, substances including acetyl-CoA and useful metabolites derived from acetyl-CoA such as isopropyl alcohol, ethanol, acetone, citric acid, itaconic acid, acetic acid, butyric acid, (poly-)3-hydroxybutyric acid, 3-hydroxyisobutyric acid, 3-aminoisobutyric acid, 2-hydroxyisobutyric acid, methacrylic acid, (poly)glutamic acid, glutamic acid, arginine, ornithine, citrulline, leucine, isoleucine, or proline, can be efficiently produced.

[0017] The present disclosure uses the simplest and practical acetyl-CoA production cycle which fixes CO₂ and converts it into acetyl-CoA (Fig. 1). Preferable embodiments of the acetyl-CoA production cycle according to the present disclosure include the acetyl-CoA production cycle shown in Fig. 1 (hereinbelow may be referred to as "cycle of Fig. 1").

[0018] The cycle involves 8 to 10 types of enzymes, that is,
at least one selected from the group consisting of:

phosphoenolpyruvate carboxylase;
phosphoenolpyruvate carboxykinase, and
pyruvate carboxylase and pyruvate kinase;
malate dehydrogenase;
malate thiokinase;
malyl-CoA lyase;
glyoxylate carboligase;

at least one selected from the group consisting of:

hydroxypyruvate isomerase and hydroxypyruvate reductase; and
2-hydroxy-3-oxopropionate reductase;

at least one selected from the group consisting of:

glycerate 2-kinase; and
phosphoglycerate mutase and glycerate 3-kinase; and
enolase.

(Phosphoenol)pyruvate carboxylase is involved in carbon dioxide fixation.

(Phosphoenol)pyruvate carboxylase is a carbon dioxide-fixing enzyme having a high activity. For example, RubisCO used in photosynthesis in plants or the like is known to have a specific activity of about 3 U/mg to 20 U/mg (J. Biol. Chem. 274(8) 5078-82(1999), Anal. Biochem. 153(1) 97-101, 1986). On the other hand, (phosphoenol)pyruvate carboxylase is reported to have a specific activity of 30 U/mg in *Escherichia coli*, or as high as 100 U/mg to 150 U/mg (J. Biol. Chem. 247, 5785-5792 (1972); Biosci. Biotechnol. Biochem. 59, 140-142 (1995); Biochim Biophys Acta. 1475(3):191-206, 2000). In terms of malate thiokinase (mtk) that synthesizes malyl-CoA, malate thiokinase according to the present disclosure has a higher activity compared to that of the conventionally known enzymes (J. Biol. Chem. 248(21) 7295-303, 1973). The cycle of Fig. 1 is composed of 8 to 10 kinds of enzymes, and, therefore, the simplest cycle among the known acetyl-CoA production cycles. Only a small number of enzymes are required to be imparted to the microorganism. Furthermore, the cycle of Fig. 1 does not include an enzyme that consumes acetyl-CoA. Therefore, it can be said that the cycle of Fig. 1 can be an ideal cycle for fixing CO₂ and convert it into acetyl-CoA.

[0019] Another advantage of the cycle of Fig. 1 is that, since the cycle is independent from glycolytic pathways, the cycle can be freely combined with various glycolytic pathways. For example, the cycle of Fig. 1 can be easily combined with, which produces NADPH with a high production rate and is often used in production of substances (Japanese National-phase publication (JP-A) No. 2007-510411), since the cycle of Fig. 1 is independent from the pentose phosphate pathway.

[0020] In the cycle of Fig. 1, each of malate dehydrogenase (mdh), 2-hydroxy-3-oxopropionate reductase (glxR), and hydroxypyruvate reductase (ycdW) consumes NADH (or NADPH) as the reduction power; each of malate thiokinase (mtk), glycerate 3-kinase (glxK), glycerate 2-kinase (garK), and pyruvate carboxylase (pyc) consumes ATP; and pyruvate kinase (pyk) produces pyruvate.

[0021] In a case in which phosphoenolpyruvate is used as the starting substance, the balanced equation for the cycle of Fig. 1 is: "phosphoenolpyruvate + 2CoA + CO₂ + 3NAD(P)H + 3ATP → 2acetyl-CoA + 3NAD(P)⁺ + 3ADP".

In a case in which pyruvate is used as the starting substance, the balanced equation is: "pyruvate + 2CoA + CO₂ + 3NAD(P)H + 4ATP → 2acetyl-CoA + 3NAD(P)⁺ + 4ADP".

That is, the cycle of Fig. 1 requires supplementation of phosphoenolpyruvate (or pyruvate), NAD(P)H, and ATP for fixing CO₂ and converting it into acetyl-CoA.

[0022] Among fermentation pathways that produce acetyl-CoA as an intermediate, balanced

equations of pathways that consume oxygen during fermentation are listed in Table 1. It is assumed that, in these fermentation pathways, a reduced coenzyme such as NADH is produced during the pathway and the reduced coenzyme is reconverted into the oxidized form by the action of oxygen. Therefore, if it is possible to consume the produced reduced coenzyme by the cycle of Fig. 1 instead of consuming oxygen, it can be expected that the reduction power generated in fermentation could be efficiently used in the acetyl-CoA production cycles for fixing CO₂ and converting it into products.

[0023] Here, the reduced coenzyme refers to a coenzyme in the reduced state and involved in an oxidation-reduction reaction, and examples thereof include NADH, NADPH, FADH₂, FMNH₂, and a reduced quinone coenzyme. The reduced coenzyme is preferably NADH or NADPH, more preferably NADH. The oxidized coenzyme refers to the oxidized form of a reduced coenzyme, and examples thereof include NAD⁺, NADP⁺, FAD, FMN, and an oxidized quinone coenzyme. The oxidized coenzyme is preferably NAD⁺ or NADP⁺, more preferably NAD⁺.

[0023] Table 1

Compound Name	Fermentation equation
Glutamic acid	$C_6H_{12}O_6 + 3/2O_2 + NH_3 \rightarrow C_5H_9NO_4 + CO_2 + 3H_2O$
Isopropyl alcohol	$C_6H_{12}O_6 + H_2O + 3/2O_2 \rightarrow C_3H_8O + 3CO_2 + 3H_2O$
Acetone	$C_6H_{12}O_6 + 2O_2 \rightarrow C_3H_6O + 3H_2O + 3CO_2$
Glutamine	$C_6H_{12}O_6 + 3/2O_2 + 2NH_3 \rightarrow C_5H_{10}N_2O_3 + CO_2 + 4H_2O$
Arginine	$C_6H_{12}O_6 + 1/2O_2 + 4NH_3 \rightarrow C_6H_{14}N_2O_2 + 5H_2O$
Ornithine	$C_6H_{12}O_6 + 1/2O_2 + NH_3 \rightarrow C_5H_{12}N_2O_2 + 2H_2O + CO_2$
Citrulline	$C_6H_{12}O_6 + 1/2O_2 + 3NH_3 \rightarrow C_6H_{13}N_3O_3 + 4H_2O$
Proline	$C_6H_{12}O_6 + 1/2O_2 + NH_3 \rightarrow C_5H_9NO_2 + 3H_2O + CO_2$
Acetic acid	$C_6H_{12}O_6 + 2O_2 \rightarrow 2C_2H_4O_2 + 2CO_2 + 2H_2O$
(poly-)3-hydroxybutyric acid	$C_6H_{12}O_6 + 3/2O_2 \rightarrow C_4H_8O_3 + 2CO_2 + 2H_2O$
Itaconic acid	$C_6H_{12}O_6 + 1.5O_2 \rightarrow C_5H_6O_4 + CO_2 + 3H_2O$
Critic acid	$C_6H_{12}O_6 + 3/2O_2 \rightarrow C_6H_8O_7 + 2H_2O$
Butyric acid	$C_6H_{12}O_6 + 2O_2 \rightarrow C_3H_8O_2 + 2H_2O + 3CO_2$
Leucine (Isoleucine)	$3/2C_6H_{12}O_6 + 3/2O_2 + NH_3 \rightarrow C_6H_{13}NO_2 + 4H_2O + 3CO_2$
4-Aminobutyric acid	$C_6H_{12}O_6 + 3/2O_2 + NH_3 \rightarrow C_4H_9NO_2 + 3H_2O + 2CO_2$
4-hydroxybutyric acid	$C_6H_{12}O_6 + 3/2O_2 \rightarrow C_4H_8O_3 + 2H_2O + 2CO_2$

[0024] As shown in Table 1, fermentation in which oxygen is present on the left side of the

fermentation equation often requires a large amount oxygen. In such cases, extensive aeration and/or vigorous stirring may be required, which results in increases in equipment costs and electric power costs. Therefore, by introducing the cycle of Fig. 1, surplus reduction power can be consumed and excessive aeration/stirring can be moderated, and the cost of fermentative production can be expected to be reduced.

[0025] In order to supply the reduction power to the cycle, the reduction power may be provided by adding a substance that can generate a reduction power, or imparting energies from outside. Specific examples thereof include using a substance that has a higher reduction degree (*e.g.*, hydrogen, sulfite, alcohols, or paraffin) as a substrate; supplying reduction energies directly by electric culture; and supplying a reduction power by a photochemical reaction in an organism. Other than the fermentation shown in Table 1, as long as the reduction power can be supplied from outside, it is possible to drive the intended carbon dioxide fixation pathway even in fermentation in which a reduced coenzyme is not produced.

[0026] The "CO₂ fixation" in the present disclosure refers to conversion of CO₂ generated in carbohydrate metabolism or CO₂ supplied from outside into an organic compound. The CO₂ may be HCO₃⁻. Here, "CO₂ fixation" may also be referred to as "carbon dioxide fixation".

[0027] The term "process" in the present specification encompasses an independent process, as well as a process that attains an intended effect of the process although it cannot be clearly distinguished from another process. In the present specification, a numerical range indicated using "to" means a range including numerical values given before and after "to" as a minimum value and a maximum value, respectively.

[0028] In the present disclosure, in the reference to the amount of each ingredient in the composition, when the composition includes plural substances corresponding to each ingredient, the amount of the each ingredient means the total amount of the plural substances unless otherwise specified.

[0029] As used herein, the term "inactivation" refers to a condition in which the activity of the enzyme (here, a factor that exhibit no enzymatic activity by themselves are also included in the scope of "enzyme," unless specifically indicated to be excluded) as measured by any existing measurement system is not higher than 1/10 of the activity in the microorganism before inactivation, assuming that the activity in the microorganism before inactivation is 100.

[0030] The "reduction" of an enzymatic activity in the present disclosure means a condition in which the activity of the enzyme is significantly reduced by a genetic recombination technique for a gene encoding the enzyme compared to those before such treatment.

[0030] The "enhancement" of an "activity" in the present disclosure broadly means that the enzymatic activity in microorganisms becomes higher after enhancement compared to the enzymatic activity before enhancement.

[0031] Methods for the enhancement are not particularly restricted as long as the activity of an enzyme possessed by microorganisms is enhanced. Examples thereof include enhancement by an enzyme gene introduced from outside the cell, enhancement by augmented expression of an enzyme gene inside the cell, and any combination thereof.

[0032] Specific examples of enhancement by an enzyme gene introduced from outside the cell include: introducing a gene encoding an active enzyme having a higher activity than the enzyme of host from outside the cell of the host microorganism by the genetic recombination technique, thereby adding the enzymatic activity of the introduced enzyme gene; substituting the introduced enzymatic activity for an intrinsic enzymatic activity that the host originally possesses; increasing the copy number of an enzyme gene of the host or an enzyme gene introduced from outside the cell to two or more; and any combination thereof.

[0033] Specific examples of enhancement by augmented expression of an enzyme gene in the microorganism include: introducing a base sequence that enhances the expression of an enzyme gene from the outside of the host microorganism into inside the microorganism; substitute another promoter for the promoter of an enzyme gene that the host microorganism possesses on its genome, thereby enhancing the expression of the enzyme gene; and any combination thereof.

[0034] The "imparting" of an "activity" in the present disclosure broadly means the provision of the activity of an intended enzyme by introducing, from the outside, a gene encoding the enzyme into an organism that does not possess a gene encoding the intended enzyme. The method of imparting an activity is not particularly limited as long as the activity of an intended enzyme can be imparted to a microorganism, and examples thereof include transformation with a plasmid harboring an enzyme gene, introduction of an enzyme gene into the genome, and any combination thereof.

[0035] The promoter to be used for "enhancing" or "imparting" of an "activity" is not particularly limited as long as the promoter allows the gene expression, and examples thereof include constitutive promoters and inducible promoters.

[0036] Whether or not the microorganism has the intended enzyme gene can be determined, with reference to, for example, the gene information of respective strains registered in KEGG (Kyoto Encyclopedia of Genes and Genomes; <http://www.genome.jp/kegg/>) or NCBI (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov/gene/>). In the

present disclosure, only the gene information of respective strains registered in KEGG or NCBI is used.

[0037] In the present disclosure, the enzymatic activity may be imparted by introducing, from the outside, a gene encoding the enzyme into the cell using the genetic recombination technique. In this case, the enzyme gene to be introduced may be either homologous or heterologous to the host cell.

[0038] Methods for preparation of a genomic DNA necessary to introduce a gene from outside the cell into the cell, cleavage and ligation of DNA, transformation, PCR (Polymerase Chain Reaction), the design and synthesis of oligonucleotides to be used as primers, *etc.* may be carried out by usual methods well known to those skilled in the art. These methods are described in Sambrook, J., et al., "Molecular Cloning A Laboratory Manual, Second Edition," Cold Spring Harbor Laboratory Press (1989), *etc.*

[0039] The expression "by the genetic recombination technique" in the present disclosure encompasses any alteration to the base sequence caused by the insertion of another DNA into the base sequence of a native gene, substitution or deletion of a certain site of a gene, or any combinations thereof. For example, the alteration may result from a mutation.

[0040] In the present disclosure, the microorganism in which the activity of a factor or enzyme is inactivated refers to a microorganism in which the native activity is impaired by a certain method applied from outside the cell to inside the cell. Such microorganism can be generated by, for example, disrupting a gene encoding the protein or enzyme (gene disruption).

[0041] Examples of the gene disruption in the present disclosure include introduction of a mutation to the base sequence of a gene, insertion of another DNA into the base sequence, or deletion of a certain part of a gene, which are carried out with a view to preventing the function of the gene from being performed. As a result of the gene disruption, for example, the gene becomes unable to be transcribed into mRNA, and the structural gene ceases to be translated. Alternatively, due to incompleteness of transcribed mRNA, the amino acid sequence of the translated structural protein is mutated or deleted, and, therefore, the intrinsic functions of the structural protein becomes unable to be realized.

[0042] The gene disruption variant may be prepared using any method as long as a disruption variant in which the target enzyme or protein is not expressed can be obtained. Various methods for gene disruption have been reported (natural breeding, addition of a mutagen, UV irradiation, radiation irradiation, random mutagenesis, transposons, site-directed gene disruption). In view of disrupting only a specific gene, gene disruption by homologous

recombination is preferable. Methods of gene disruption by homologous recombination are described in J. Bacteriol., 161, 1219-1221 (1985), J. Bacteriol., 177, 1511-1519 (1995), Proc. Natl. Acad. Sci. U.S.A., 97, 6640-6645 (2000), and the like, and those skilled in the art may easily perform homologous recombination using these methods or applying these methods.

[0043] The "host" in the present disclosure means a microorganism in a state that the effect of the present disclosure can be exerted as a result of the introduction of one or more genes from outside the microorganism.

[0044] More specifically, the "host" in the present disclosure means a microorganism that can be made to possess the ability to produce acetyl-CoA from a carbon source material by using a certain means, regardless of whether or not the microorganism intrinsically has the innate ability to produce acetyl-CoA from a carbon source material.

[0045] The "host" in the present disclosure may have a pathway for producing a useful metabolite. The "useful metabolite" in the present disclosure is a metabolite in the metabolic pathways of microorganisms, specifically L-glutamic acid. The microorganism may be any microorganism as long as it can be made to possess the ability to produce a useful metabolite by using any means, regardless of whether or not the microorganism intrinsically has the innate ability to produce the useful metabolite.

[0046] The "useful metabolite derived from acetyl-CoA" in the present disclosure refers to L-glutamic acid. The microorganism may be any microorganism as long as it can be made to possess the ability to produce a useful metabolite derived from acetyl-CoA by using a certain means, regardless of whether or not the microorganism intrinsically has the innate ability to produce the useful metabolite derived from acetyl-CoA.

[0047] The "production of acetyl-CoA" in the present disclosure refers to conversion of any substance into acetyl-CoA in a metabolic pathway. Since acetyl-CoA is a metabolic intermediate and quickly converted into various substances in metabolic pathways, the apparent amount of acetyl-CoA does not necessarily increase. However, the effect can be confirmed indirectly by detection of a CO₂-derived label in a substance derived from acetyl-CoA, by an increase in the yield of a substance derived from acetyl-CoA relative to sugar consumption, or the like. Since various factors (*e.g.*, the quantity of a coenzyme, the quantity of a substrate, or a change in metabolism caused by a feedback inhibition) are involved in conversion, the production amount of acetyl-CoA is not always proportional to the amount of each of the substances derived from acetyl-CoA. However, in a case in which a pathway to produce a specific substance from acetyl-CoA is enhanced or a case in which such pathway is intrinsically enhanced (for example, in the case of an isopropyl alcohol-producing

microorganism or a L-glutamic acid-producing microorganism described below), the efficiency of conversion from acetyl-CoA is hardly affected by external factors, and, therefore, the production efficiency of the specific substance can be regarded as an index of the production efficiency of acetyl-CoA.

[0048] The microorganism according to the present disclosure includes an acetyl-CoA production cycle obtained by imparting at least one type of enzymatic activity selected from the group consisting of malate thiokinase, malyl-CoA lyase, glyoxylate carboligase and 2-hydroxy-3-oxopropionate reductase, to a microorganism that does not have any of:

(a) a carbon dioxide fixation cycle having an enzymatic reaction from malonyl-CoA to malonate semialdehyde or 3-hydroxypropionate;

(b) a carbon dioxide fixation cycle having an enzymatic reaction from acetyl-CoA and CO₂ to pyruvate;

(c) a carbon dioxide fixation cycle having an enzymatic reaction from crotonyl-CoA and CO₂ to ethylmalonyl-CoA or glutaconyl-CoA;

(d) a carbon dioxide fixation cycle having an enzymatic reaction from CO₂ to formate; or

(e) at least one selected from the group consisting of malate thiokinase and malyl-CoA lyase,

in which none of (a), (b), (c), or (d) is imparted to the microorganism, or the microorganism exhibits none of the functions of (a), (b), (c), and (d) even though at least one of (a), (b), (c), or (d) is imparted.

[0049] In view of the production efficiency of acetyl-CoA, the microorganism is preferably imparted with the enzymatic activities of malate thiokinase and malyl-CoA lyase, more preferably imparted with the enzymatic activities of malate thiokinase, malyl-CoA lyase, and glyoxylate carboligase, still more preferably imparted with the enzymatic activities of malate thiokinase, malyl-CoA lyase, glyoxylate carboligase, and 2-hydroxy-3-oxopropionate reductase, and/or hydroxypyruvate reductase.

[0050] The expression "does not (naturally) have" herein means that the host microorganism does not intrinsically possess an attribute in nature. Here, the "carbon dioxide fixation cycle having an enzymatic reaction from malonyl-CoA to malonate semialdehyde or 3-hydroxypropionate" refers to the following cycles (1) to (7):

(1) the cycle shown in Fig. 1 of WO 2011/099006, in which acetyl-CoA is converted into malonyl-CoA, 3-hydroxypropionate, propionyl-CoA, malate, and malyl-CoA, which are again converted into acetyl-CoA;

(2) the cycle shown in Fig. 4A of WO 2011/099006, in which acetyl-CoA is converted into malonyl-CoA, malonate semialdehyde, β -alanine, malate, and malyl-CoA, which are again converted into acetyl-CoA;

(3) the cycle shown in Fig. 4B, 16, or 18 of WO 2011/099006, in which acetyl-CoA is converted into malonyl-CoA, hydroxypropionate, (R)-lactate or (S)-lactate, malate, and malyl-CoA, which are again converted into acetyl-CoA;

(4) the cycle shown in Fig. 8 of WO 2011/099006, in which acetyl-CoA is converted into malonyl-CoA, malonate semialdehyde or hydroxypropionate, pyruvate, malate, and malyl-CoA, which are again converted into acetyl-CoA;

(5) the cycle shown in Fig. 9A, 9B, or 9C of WO 2011/099006, in which acetyl-CoA is converted into malonyl-CoA, hydroxypropionate, 2-ketoglutarate, malate, and malyl-CoA, which are again converted into acetyl-CoA;

(6) the cycle shown in Fig. 9D or 9F of WO 2011/099006, in which acetyl-CoA is converted into malonyl-CoA, hydroxypropionate, methylmalonyl-CoA, malate, and malyl-CoA, which are again converted into acetyl-CoA; and

(7) the cycle shown in Fig. 17 of WO 2011/099006, in which acetyl-CoA is converted into malonyl-CoA, malonate semialdehyde or hydroxypropionate, methylmalonyl-CoA, pyruvate, oxaloacetate, malate, and malyl-CoA, which are again converted into acetyl-CoA.

[0051] All of carbon dioxide fixation cycles (1) to (7) described above have an enzymatic reaction from malonyl-CoA to malonate semialdehyde or from malonyl-CoA to 3-hydroxypropionate. This kind of reaction is catalyzed by malonate semialdehyde dehydrogenase or malonyl-CoA reductase (WO 2011/099006). It is thought that the reduction reaction of carboxylic acid or a (thio)ester thereof, such as reduction of succinyl-CoA or reduction of malonyl-CoA, is generally difficult to carry out as enzymatic reactions and should be avoided them as fermentation pathways where possible (Atsumi et al., *Nature*, 451,(3), 86-89, 2008; Yim et al., *Nat. Chem. Biol.*, 7, 445-452, 2011).

[0052] The "carbon dioxide fixation cycle having an enzymatic reaction from acetyl-CoA and CO₂ to pyruvate" in the present specification refers to the following cycles (8) to (10):

(8) the cycle shown in Fig. 1 of WO 2011/099006, in which acetyl-CoA is converted into pyruvate, phosphoenolpyruvate, oxaloacetate, malate, and malyl-CoA, which are again converted into acetyl-CoA;

(9) the cycle shown in Fig. 7C, 7D or 7E of WO 2011/099006, in which acetyl-CoA is converted into pyruvate, malate, and malyl-CoA, which are again converted into

acetyl-CoA; and

(10) the cycle shown in Fig. 9M of WO 2011/099006, in which acetyl-CoA is converted into pyruvate, 2-ketoglutarate, malate, and malyl-CoA, which are again converted into acetyl-CoA.

[0053] All of carbon dioxide fixation cycles (8) to (10) have an enzyme reaction converting acetyl-CoA and CO₂ into pyruvate. This reaction is catalyzed by pyruvate synthase (WO 2011/099006). The synthetic reaction of pyruvate by pyruvate synthase requires a strong reduction power from ferredoxin and proceeds slowly and proceeds only under strictly anaerobic conditions because the reaction is sensitive to oxygen.

[0054] The "carbon dioxide fixation cycle having an enzymatic reaction from crotonyl-CoA and CO₂ to ethylmalonyl-CoA or glutaconyl-CoA" in the present specification refers to the cycle shown in Fig. 9H or 9J of WO 2011/099006, in which acetyl-CoA is converted into crotonyl-CoA, ethylmalonyl-CoA or glutaconyl-CoA, oxaloacetate, malate, and malyl-CoA, which are again converted into acetyl-CoA.

[0055] The conversion of crotonyl-CoA and CO₂ to ethylmalonyl-CoA or glutaconyl-CoA is catalyzed by crotonyl-CoA carboxylase-reductase or methylcrotonyl-CoA carboxylase. Since the K_m value of crotonyl-CoA carboxylase-reductase for carbonates is high (14 mM; PNAS 104(25) 10631-10636, 2007), sufficient activity at low substrate concentration cannot be expected. Crotonyl-CoA, which is a substrate of crotonyl-CoA carboxylase-reductase, is produced from 3-hydroxybutyryl-CoA by a dehydration reaction. In general, an enzyme involved in a dehydration reaction predominantly catalyzes the reverse reaction (*i.e.*, hydration reaction) in an aqueous environment. Therefore, a sufficiently high production rate of crotonyl-CoA cannot be expected. Further, the reported specific activity of methylcrotonyl-CoA carboxylase is not so high (0.2 U/mg to 0.6 U/mg; Arch Biochem Biophys. 310(1) 64-75, 1994), and a sufficiently high production rate of crotonyl-CoA as a substrate cannot be expected for the same reason.

[0056] The "carbon dioxide fixation cycle having an enzymatic reaction from CO₂ to formate" in the present specification refers to the cycle shown in Figs. 5, 6, 13 or 14 of WO 2009/046929, that is, a cycle having a pathway in which the reaction proceeds from CO₂ via formate and serine, and oxaloacetate is converted into malate, malyl-CoA, and glycerate, which are again converted into oxaloacetate.

[0057] The enzymatic reaction from CO₂ to formate requires a strong reduction power, proceeds slowly, and proceeds only under strictly anaerobic conditions because the reaction is sensitive to oxygen.

[0058] In the present disclosure, "exhibits none of the functions" of the carbon dioxide fixation cycle "even though imparted" means that the carbon dioxide fixation cycle does not exhibit function even when the activity of the intended enzyme is imparted by introducing a gene encoding the enzyme having the activity is introduced to a microorganism that does not possess the gene encoding the intended enzyme. The fact that "the carbon dioxide fixation cycle does not function" can be confirmed indirectly, for example, by no detection of a label derived from CO₂ in a metabolite in the cycle or a substance derived from the metabolite in a test using a labeled CO₂, or by no increase in the yield of a substance derived from a metabolite in the cycle relative to sugar consumption.

[0059] The acetyl-CoA production cycle to be constructed in the microorganism includes malate thiokinase, malyl-CoA lyase, glyoxylate carboligase, or 2-hydroxy-3-oxopropionate reductase. An example of the acetyl-CoA production cycle is shown in Fig. 1. The acetyl-CoA production cycle does not include an enzyme that consumes acetyl-CoA, such as acetyl-CoA carboxylase or pyruvate synthase.

[0060] In the acetyl-CoA production cycle of Fig. 1, carbon dioxide is first bound to phosphoenolpyruvate or pyruvate by the action of phosphoenolpyruvate carboxylase (ppc), pyruvate carboxylase (pyc), or phosphoenolpyruvate carboxykinase (pck), and converted into oxaloacetate. The oxaloacetate is converted into malate by the action of malate dehydrogenase (mdh). The malate is converted into malyl-CoA (malate CoA) by the action of malate thiokinase (mtk). The malyl-CoA (malate CoA) is converted into acetyl-CoA and glyoxylate by the action of malyl-CoA lyase (Mcl). The glyoxylate is converted into 2-hydroxy-3-oxopropionate by the action of glyoxylate carboligase (gcl). The 3-hydroxy-2-oxopropionate is converted into glycerate by the action of 2-hydroxy-3-oxopropionate reductase (glxR), or alternatively, converted into hydroxypyruvate by the action of hydroxypyruvate isomerase (hyi) and then into glycerate by the action of hydroxypyruvate reductase (ycdW). The glycerate is converted into 3-phosphoglycerate by the action of glycerate 3-kinase (glxK) or converted into 2-phosphoglycerate by the action of glycerate 2-kinase (garK). The 3-phosphoglycerate is converted into 2-phosphoglycerate by the action of phosphoglycerate mutase (gpm). The 2-phosphoglycerate is converted into phosphoenolpyruvate by the action of enolase (eno). In a case in which pyruvate carboxylase is included in the cycle, the phosphoenolpyruvate is converted into pyruvate by the action of pyruvate kinase (pyk).

[0061] The enzymatic activity to be imparted to the microorganism having an acetyl-CoA production cycle is not particularly limited as long as the acetyl-CoA production cycle can be

functionally constructed thereby and may be appropriately selected within the scope described in the present specification depending on the host microorganism.

[0062] Among coryneform bacteria, for example, *Corynebacterium glutamicum* does not possess malate thiokinase, malyl-CoA lyase, glyoxylate carboligase, and 2-hydroxy-3-oxopropionate reductase, so that at least malate thiokinase, malyl-CoA lyase, glyoxylate carboligase, and 2-hydroxy-3-oxopropionate reductase, and/or hydroxypyruvate reductase need to be imparted.

[0063] In a microorganism in which a closed cycle cannot be formed with any of the pathways in Fig. 1 because of partial absence of the enzymes in the cycle of Fig. 1, the missing enzyme(s) need(s) to be supplied. For example, among *Escherichia* bacteria, *Escherichia coli* does not possess malate thiokinase and malyl-CoA lyase, so that at least these two enzymes need to be imparted.

[0064] *Pantoea* bacteria such as *Pantoea ananatis* does not possess malate thiokinase, malyl-CoA lyase, and glyoxylate carboligase, so that at least malate thiokinase, malyl-CoA lyase, and glyoxylate carboligase need to be imparted.

[0065] The enzyme that consumes acetyl-CoA as described above refers to an enzyme, other than phosphoenolpyruvate carboxylase and pyruvate carboxylase used in the cycle according to the present disclosure, that uses acetyl-CoA as a substrate and catalyzes the conversion of acetyl-CoA into another substance. Examples thereof include acetyl-CoA carboxylase, which is classified as enzyme code number: 6.4.1.2 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.) and catalyzes a reaction of converting acetyl-CoA into malonyl-CoA; and pyruvate synthase, which is classified as enzyme code number: 1.2.7.1 and catalyzes a reaction of converting acetyl-CoA into pyruvate.

[0066] The cycle including an enzyme that consumes acetyl-CoA as described above refers to a closed cycle in which acetyl-CoA is converted, via the cycle, into acetyl-CoA again by the action of an enzyme that consumes acetyl-CoA. In a case in which a substance produced by the conversion reaction of an enzyme that consumes acetyl-CoA is further converted into another product without being converted into acetyl-CoA again (for example, in a case in which the substance is converted via an isopropyl alcohol-producing pathway into the end-product, isopropyl alcohol), the cycle is not closed, and, therefore, the cycle is excluded from the "cycle including an enzyme that consumes acetyl-CoA".

[0067] The closed cycle refers to a pathway starting from an arbitrary substance in the cycle, in which the substance is converted via the cycle into another substance and, ultimately,

converted into the same substance as the initial substance.

[0068] Malate thiokinase is a generic name for enzymes which are classified as enzyme code number: 6.2.1.9 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which catalyze a reaction of binding of malate to CoA and converting malate into malyl-CoA. In this reaction, one molecule of ATP is consumed, and one molecule of ADP and one molecule of phosphate are produced. Malate thiokinase has a large subunit of approximately 400 amino acids and a small subunit of 300 amino acids. In the gene, the large subunit is usually followed by the small subunit. Here, for convenience, the large subunit is referred to as mtkB, and the small subunit is referred to as mtkA. It is reported that the specific activity of the purified malate thiokinase is, for example, 2.5 U/mg (Anal Biochem. 227(2), 363-367, 1995).

[0069] Malate thiokinase is mainly found in an assimilation pathway for C1 carbon sources such as methane (J. Bacteriol. 176(23), 7398-7404, 1994) and a 3-hydroxypropionate pathway (Arch. Microbiol., 151, 252-256, 1989), and is characterized in that malyl-CoA lyase is present in its vicinity in the genome. Such enzyme may be suitably used. An example of evaluation of the activity of a purified malate thiokinase is known in malate thiokinase derived from *Methylobacterium extorquens*, but there are only a few examples of comparing an activity with an actual sequence.

[0070] In US 9,822,387, an example is disclosed in which malate thiokinase is actually cloned and expressed by a microorganism of another species, and the activity is correlated with the sequence.

[0071] Examples of malate thiokinase include those derived from *Methylobacterium* such as *Methylobacterium extorquens*, those derived from *Hyphomicrobium* such as *Hyphomicrobium methylovorum* or *Hyphomicrobium denitrificans*, those derived from *Rhizobium* such as *Rhizobium* sp. NGR234, those derived from *Granulibacter* such as *Granulibacter bethesdensis*, those derived from *Nitrosomonas* such as *Nitrosomonas europaea*, those derived from *Methylococcus* such as *Methylococcus capsulatus*, and those derived from Gammaproteobacteria.

[0072] In view of the production efficiency of useful substances produced via acetyl-CoA, specific examples of the preferable amino acid sequence include the amino acid sequences derived from *Hyphomicrobium*, amino acid sequences derived from *Rhizobium*, amino acid sequences derived from *Nitrosomonas*, amino acid sequences derived from *Methylococcus*, and amino acid sequences derived from Gammaproteobacteria.

[0073] The malate thiokinase derived from *Hyphomicrobium*, malate thiokinase derived

from *Rhizobium* and malate thiokinase derived from *Nitrosomonas* share 65% to 80% sequence homology with one another. The malate thiokinase derived from *Methylococcus* has 70% to 80% sequence homology with the malate thiokinase derived from Gammaproteobacteria.

[0074] Malate thiokinases having at least 70% amino acid sequence homology with each of the amino sequences of malate thiokinase derived from *Hyphomicrobium*, malate thiokinase derived from *Rhizobium*, malate thiokinase derived from *Nitrosomonas*, malate thiokinase derived from *Methylococcus*, and malate thiokinase derived from Gammaproteobacteria disclosed here, and having the malate thiokinase activity may be suitably used for the production of acetyl-CoA or the production of a useful product derived from acetyl-CoA according to the present disclosure.

[0075] As a gene of malate thiokinase (*mtk*), a DNA having the base sequence of a gene encoding malate thiokinase obtained from each of the above-mentioned enzyme origin organisms, or a synthetic DNA sequence that is synthesized based on a known base sequence of the gene, may be used.

[0076] Preferable examples thereof include a DNA having the base sequence of a gene derived from *Methylobacterium* such as *Methylobacterium extorquens*, *Hyphomicrobium* such as *Hyphomicrobium methylovorum* or *Hyphomicrobium denitrificans*, *Rhizobium* such as *Rhizobium* sp. NGR234, *Granulibacter* such as *Granulibacter bethesdensis*, *Nitrosomonas* such as *Nitrosomonas europaea*, *Methylococcus* such as *Methylococcus capsulatus*, or Gammaproteobacteria.

[0077] In view of the production efficiency of acetyl-CoA, a DNA having the base sequence of a gene derived from *Hyphomicrobium*, *Rhizobium*, *Granulibacter*, *Nitrosomonas*, *Methylococcus*, or Gammaproteobacteria is preferable.

[0078] In particular, the base sequence of a gene derived from *Hyphomicrobium*, *Rhizobium* of which codon usage is optimized, *Nitrosomonas*, *Methylococcus*, or Gammaproteobacteria is preferable.

[0079] Malyl-CoA lyase is an enzyme which is classified as enzyme code number: 4.1.3.24 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which catalyzes a reaction of producing glyoxylate and acetyl-CoA from malyl-CoA. Examples of malyl-CoA lyase include those derived from *Methylobacterium* such as *Methylobacterium extorquens*, *Hyphomicrobium* such as *Hyphomicrobium methylovorum* or *Hyphomicrobium denitrificans*, *Chloroflexus* such as *Chloroflexus aurantiacus*, *Nitrosomonas* such as *Nitrosomonas europaea*, or *Methylococcus* such as

Methylococcus capsulatus.

[0080] In view of the production efficiency of acetyl-CoA, specific examples of the preferable amino acid sequences include an amino acid sequence derived from *Methylobacterium*, *Hyphomicrobium*, *Nitrosomonas*, or *Methylococcus*.

[0081] It is reported that the specific activity of purified malyl-CoA lyase in *Methylobacterium extorquens* is, for example, 28.1 U/mg (Biochem. J. 139, 399-405, 1974).

[0082] As a gene of malyl-CoA lyase (*mcl*), a DNA having the base sequence of a gene encoding malyl-CoA lyase obtained from any of the above-listed enzyme origin organisms, or a synthesized DNA sequence that is synthesized based on a known base sequence of the gene, may be used. Preferable examples thereof include a DNA having the base sequence of a gene derived from *Methylobacterium* such as *Methylobacterium extorquens*, *Hyphomicrobium* such as *Hyphomicrobium methylovorum* or *Hyphomicrobium denitrificans*, or *Chloroflexus* such as *Chloroflexus aurantiacus*. In view of the production efficiency of acetyl-CoA, more preferable examples thereof include a DNA having the base sequence of a gene derived from *Methylobacterium* and a gene having the base sequence of a gene derived from *Hyphomicrobium*.

[0083] Specific examples of the preferable base sequence of the gene derived from *Methylobacterium* include the base sequence of a gene derived from *Methylobacterium extorquens*. Specific examples of the preferable base sequence of a gene derived from *Hyphomicrobium* include the base sequence of a gene derived from *Hyphomicrobium methylovorum* or *Hyphomicrobium denitrificans*. Specific examples of the preferable base sequence of a gene derived from *Nitrosomonas* include the base sequence of a gene derived from *Nitrosomonas europaea*. Specific examples of the preferable base sequence of the gene derived from *Methylococcus* include the base sequence of a gene derived from *Methylococcus capsulatus*.

[0084] Acetyl-CoA carboxylase is a general name for enzymes which are classified as enzyme code number: 6.4.1.2 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which catalyze a reaction of converting acetyl-CoA and CO₂ into malonyl-CoA.

[0085] Malonate semialdehyde dehydrogenase is classified as enzyme code number: 1.2.1.18 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which catalyze a reaction of converting malonyl-CoA into malonate semialdehyde.

[0086] Malonyl-CoA reductase is a generic name for enzymes that catalyze a reaction of

converting malonyl-CoA into malonate semialdehyde or 3-hydroxypropionate.

[0087] Crotonyl-CoA carboxylase-reductase is a generic name for enzymes which are classified as enzyme code number: 1.3.1.85 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which catalyze the conversion of crotonyl-CoA into ethylmalonyl-CoA.

[0088] Methylcrotonyl-CoA carboxylase is a generic name for enzymes which are classified as enzyme code number: 6.4.1.4 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which catalyze a reaction of converting crotonyl-CoA into glutaconyl-CoA.

[0089] Pyruvate synthase is a generic name for enzymes which are classified as enzyme code number: 1.2.7.1 based on the report of the enzyme commission of International Union of Biochemistry (I.U.B.), and which catalyze a reaction of converting acetyl-CoA into pyruvate.

[0090] It is preferable that, in the microorganism having an acetyl-CoA production cycle by the introduction of a carbon dioxide fixation pathway, the activity of at least one enzyme selected from the group consisting of lactate dehydrogenase, 2-ketoglutarate dehydrogenase, malate synthase, and fumarate hydratase is inactivated or reduced. As a result, acetyl-CoA can be produced more efficiently.

[0091] Among the above-mentioned lactate dehydrogenase, 2-ketoglutarate dehydrogenase, malate synthase, and fumarate hydratase, each of which is the target enzyme whose activity may be inactivated or reduced, malate synthase catalyzes a reaction of converting acetyl-CoA and glyoxylate into malate. This reaction is the reverse reaction of a reaction catalyzed by malate thiokinase and malyl-CoA lyase. Therefore, it is preferable to inactivate or reduce the activity of malate synthase, since the reaction of converting acetyl-CoA and glyoxylate into malate again can be blocked or reduced and the yield of acetyl-CoA is improved.

[0092] Among lactate dehydrogenase, malate synthase, and fumarate hydratase, the activity of which may be inactivated or reduced, it is preferable to inactivate fumarate hydratase in view of the production efficiency of acetyl-CoA. Inactivating fumarate hydratase prevents a conversion of malate into other substances including fumarate and a reduction in the amount of malate, and hence improves the yield of acetyl-CoA.

[0093] Lactate dehydrogenase (*ldhA*) is a generic name for enzymes which are classified as enzyme code number: 1.1.1.28 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which catalyze a reaction of converting pyruvate into lactate or converting lactate into pyruvate.

[0094] 2-ketoglutarate dehydrogenase (*sucA*, *odhA*) or α -ketoglutarate dehydrogenase or

oxoglutarate dehydrogenase is a generic name for enzymes which are classified as enzyme code number: 1.2.4.2 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which catalyze a reaction of converting 2-ketoglutarate to succinyl-CoA. 2-ketoglutarate is the precursor for production of glutamic acid in the cell. Inactivating 2-ketoglutarate dehydrogenase thus prevents the conversion of 2-ketoglutarate to other substances.

[0095] Isocitrate lyase (*aceA*) is classified under enzyme code number: 4.1.3.1 based on the Report of the Commission on Enzymes, International Union of Biochemistry (I.U.B.), and is a generic name for enzymes that catalyze a reaction of converting isocitrate into succinate and glyoxylate.

[0096] Malate synthase (*aceB* and *glcB*) is a generic name for enzymes which are classified as enzyme code number: 2.3.3.9 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which catalyze a reaction of converting acetyl-CoA and glyoxylate into malate and CoA. Depending on the microorganisms, plural isomers of malate synthase is encoded in the genome. Most *Escherichia coli* strains possess two genes named *aceB* and *glcB*, respectively, and both are described in the present specification. Each of *Pantoea ananatis* and *Corynebacterium glutamicum* possesses a single type of gene corresponding to *aceB* or *glcB*, and the gene is collectively described as *aceB* in the present specification, for convenience.

[0097] Fumarate hydratase (*fum*) is a generic name for enzymes which are classified as enzyme code number: 4.2.1.2 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which catalyze a reaction of converting malate into fumarate. Depending on the microorganisms, plural isomers of fumarate hydratase is encoded in the genome. For example, *Escherichia coli* has three types of fumarate hydratase, *fumA*, *fumB*, and *fumC*. *Pantoea ananatis* has *fumA* and *fumC*, and *Corynebacterium glutamicum* has *fumC*.

[0098] Phosphoenolpyruvate carboxylase is a generic name for enzymes which are classified as enzyme code number: 4.1.1.31 based on the report of the enzyme commission of International Union of Biochemistry (I.U.B.), and which catalyze a reaction of converting phosphoenolpyruvate and carbon dioxide into oxaloacetate and phosphate. Examples of phosphoenolpyruvate carboxylase include those derived from *Corynebacterium* bacteria such as *Corynebacterium glutamicum*, *Escherichia* bacteria such as *Escherichia coli*, *Pantoea* bacteria such as *Pantoea ananatis*, *Hyphomicrobium* bacteria such as *Hyphomicrobium methylovorum*, *Starkeya* bacteria such as *Starkeya novella*, *Rhodopseudomonas* bacteria such

as *Rhodopseudomonas sp.*, or *Streptomyces* bacteria such as *Streptomyces coelicolor*.

[0099] As a gene of phosphoenolpyruvate carboxylase (*ppc*), a DNA having the base sequence of a gene encoding phosphoenolpyruvate carboxylase obtained from any of the above-listed enzyme origin organisms, or a synthesized DNA sequence that is synthesized based on a known base sequence of the gene, may be used. Preferable examples thereof include a DNA having the base sequence of a gene derived from *Corynebacterium* bacteria such as *Corynebacterium glutamicum*, *Escherichia* bacteria such as *Escherichia coli*, *Pantoea* bacteria such as *Pantoea ananatis*, *Hyphomicrobium* bacteria such as *Hyphomicrobium methylovorum*, *Starkeya* bacteria such as *Starkeya novella*, *Rhodopseudomonas* bacteria such as *Rhodopseudomonas sp.*, or *Streptomyces* bacteria such as *Streptomyces coelicolor*.

[0100] Phosphoenolpyruvate carboxykinase is a generic name for enzymes which are classified as enzyme code number: 4.1.1.32, enzyme code number: 4.1.1.38, or enzyme code number: 4.1.1.49 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which catalyze a reaction of converting phosphoenolpyruvate and carbon dioxide into oxaloacetate. Among the enzyme code numbers, the enzymes classified as enzyme code number: 4.1.1.32 are involved in a reaction of converting GDP into GTP; the enzymes classified as enzyme code number: 4.1.1.38 are involved in a reaction of converting phosphate into pyrophosphate; and the enzymes classified as enzyme code number: 4.1.1.49 are involved in a reaction of converting ADP into ATP. Examples of phosphoenolpyruvate carboxykinase include those derived from *Actinobacillus* bacteria such as *Actinobacillus succinogenes*, *Mycobacterium* bacteria such as *Mycobacterium smegmatis*, or *Trypanosoma* bacteria such as *Trypanosoma brucei*.

[0101] As a gene of phosphoenolpyruvate carboxykinase (*pck*), a DNA having the base sequence of a gene encoding phosphoenolpyruvate carboxykinase obtained from any of the above-listed enzyme origin organisms, or a synthesized DNA sequence that is synthesized based on a known base sequence of the gene, may be used. Preferable examples thereof include a DNA having the base sequence of a gene derived from *Actinobacillus* such as *Actinobacillus succinogenes*, *Mycobacterium* bacteria such as *Mycobacterium smegmatis*, or *Trypanosoma* bacteria such as *Trypanosoma brucei*.

[0102] Pyruvate carboxylase is a generic name for enzymes which are classified as enzyme code number: 6.4.1.1 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which catalyze a reaction of converting pyruvate and carbon dioxide into oxaloacetate. The reaction consumes ATP and produces ADP and phosphate. Examples of pyruvate carboxylase include those derived from *Corynebacterium* bacteria such

as *Corynebacterium glutamicum*, *Escherichia* bacteria such as *Escherichia coli*, *Pantoea* bacteria such as *Pantoea ananatis*, or *Mycobacterium* bacteria such as *Mycobacterium smegmatis*.

[0103] As a gene of pyruvate carboxylase (*pyc*), a DNA having the base sequence of a gene encoding phosphoenolpyruvate carboxylase obtained from any of the above-listed enzyme origin organisms, or a synthesized DNA sequence that is synthesized based on a known base sequence of the gene, may be used. Preferable examples thereof include a DNA having the base sequence of a gene derived from *Corynebacterium* bacteria such as *Corynebacterium glutamicum*, *Escherichia* bacteria such as *Escherichia coli*, *Pantoea* bacteria such as *Pantoea ananatis*, or *Mycobacterium* bacteria such as *Mycobacterium smegmatis*.

[0104] Malate dehydrogenase is a generic name for enzymes which are classified as enzyme code number: 1.1.1.37 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which use NADH as a coenzyme and catalyze a reaction of producing malate from oxaloacetate. Examples of malate dehydrogenase include those derived from *Corynebacterium* bacteria such as *Corynebacterium glutamicum*, or *Escherichia* bacteria such as *Escherichia coli*.

[0105] As a gene of malate dehydrogenase (*mdh*), a DNA having the base sequence of a gene encoding malate dehydrogenase obtained from any of the above-listed enzyme origin organisms, or a synthesized DNA sequence that is synthesized based on a known base sequence of the gene, may be used. Preferable examples thereof include a DNA having the base sequence of a gene derived from *Corynebacterium* bacteria such as *Corynebacterium glutamicum*, *Escherichia* bacteria such as *Escherichia coli*, or *Pantoea* bacteria such as *Pantoea ananatis*.

[0106] Glyoxylate carboligase is a generic name for enzymes which are classified as enzyme code number: 4.1.1.47 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which catalyze a reaction of converting two molecules of glyoxylate into one molecule of 2-hydroxy-3-oxopropionate. This reaction is accompanied by decarboxylation of one molecule of carbon dioxide. Examples of glyoxylate carboligase include those derived from *Corynebacterium* bacteria such as *Corynebacterium glutamicum*, *Escherichia* bacteria such as *Escherichia coli*, or *Rhodococcus* bacteria such as *Rhodococcus jostii*.

[0107] As a gene of glyoxylate carboligase (*gcl*), a DNA having the base sequence of a gene encoding glyoxylate carboligase obtained from any of the above-listed enzyme origin organisms, or a synthesized DNA sequence that is synthesized based on a known base

sequence of the gene, may be used. Preferable examples thereof include a DNA having the base sequence of a gene derived from *Rhodococcus* bacteria such as *Rhodococcus jostii*, or *Escherichia* bacteria such as *Escherichia coli*.

[0108] 2-hydroxy-3-oxopropionate reductase is a generic name for enzymes which are classified as enzyme code number: 1.1.1.60 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which use NADH as a coenzyme and catalyze a reaction of converting 2-hydroxy-3-oxopropionate into glycerate. Examples of 2-hydroxy-3-oxopropionate reductase include those derived from *Corynebacterium* bacteria such as *Corynebacterium glutamicum*, or *Escherichia* bacteria such as *Escherichia coli*.

[0109] As a gene of 2-hydroxy-3-oxopropionate reductase (*glxR*), a DNA having the base sequence of a gene encoding 2-hydroxy-3-oxopropionate reductase obtained from any of the above-listed enzyme origin organisms, or a synthesized DNA sequence that is synthesized based on a known base sequence of the gene, may be used. Preferable examples thereof include a DNA having the base sequence of a gene derived from *Escherichia* bacteria such as *Escherichia coli*.

[0110] Hydroxypyruvate isomerase is a generic name for enzymes which are classified as enzyme code number: 5.3.1.22 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which catalyze a reaction of isomerizing 2-hydroxy-3-oxopropionate to hydroxypyruvate. Examples of hydroxypyruvate isomerase include those derived from *Corynebacterium* bacteria such as *Corynebacterium glutamicum*, *Escherichia* bacteria such as *Escherichia coli*, or *Pantoea* bacteria such as *Pantoea ananatis*.

[0111] As a gene of hydroxypyruvate isomerase (*hyi*), a DNA having the base sequence of a gene encoding hydroxypyruvate isomerase obtained from any of the above-listed enzyme origin organisms, or a synthesized DNA sequence that is synthesized based on a known base sequence of the gene, may be used. Preferable examples thereof include a DNA having the base sequence of a gene derived from *Corynebacterium* bacteria such as *Corynebacterium glutamicum*, *Escherichia* bacteria such as *Escherichia coli*, or *Pantoea* bacteria such as *Pantoea ananatis*.

[0112] Hydroxypyruvate reductase is a generic name for enzymes which are classified as enzyme code number: 1.1.1.81 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which use NADH or NADPH as a coenzyme and catalyze a reaction of converting hydroxypyruvate into glycerate.

[0113] Examples of hydroxypyruvate reductase include those derived from *Escherichia* bacteria such as *Escherichia coli*, or *Pantoea* bacteria such as *Pantoea ananatis*.

[0114] As a gene for hydroxypyruvate reductase (*ycaW*), a DNA having the base sequence of a gene encoding hydroxypyruvate reductase obtained from any of the above-listed enzyme origin organisms, or a synthetic DNA sequence synthesized based on a known base sequence of the gene, may be used. Preferable examples thereof include a DNA having the base sequence of a gene derived from *Escherichia* bacteria such as *Escherichia coli*, or *Pantoea* bacteria such as *Pantoea ananatis*.

[0115] Glycerate 3-kinase is a generic name for enzymes which are classified as enzyme code number: 2.7.1.31 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which catalyze a reaction of converting glycerate into 3-phosphoglycerate. In this reaction, one molecule of ATP is consumed, and one molecule of ADP and one molecule of phosphate are produced. Examples of the glycerate 3-kinase include those derived from *Corynebacterium* bacteria such as *Corynebacterium glutamicum*, or *Escherichia* bacteria such as *Escherichia coli*.

[0116] As a gene of glycerate 3-kinase (*glxK*) according to the present disclosure, a DNA having the base sequence of a gene encoding glycerate 3-kinase obtained from any of the above-listed enzyme origin organisms, or a synthesized DNA sequence that is synthesized based on a known base sequence of the gene, may be used. Preferable examples thereof include a DNA having the base sequence of a gene derived from *Corynebacterium* bacteria such as *Corynebacterium glutamicum*, *Escherichia* bacteria such as *Escherichia coli*, or *Pantoea* bacteria such as *Pantoea ananatis*.

[0117] Glycerate 2-kinase is a generic name for enzymes which are classified as enzyme code number: 2.7.1.165 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which catalyze a reaction of converting glycerate into 2-phosphoglycerate. In this reaction, one molecule of ATP is consumed, and one molecule of ADP and one molecule of phosphate are produced. Examples of glycerate 2-kinase include those derived from *Corynebacterium* bacteria such as *Corynebacterium glutamicum*, or *Escherichia* bacteria such as *Escherichia coli*.

[0118] As a gene of glycerate 2-kinase (*garK*) according to the present disclosure, a DNA having the base sequence of a gene encoding glycerate 2-kinase obtained from any of the above-listed enzyme origin organisms, or a synthesized DNA sequence that is synthesized based on a known base sequence of the gene, may be used. Preferable examples thereof include a DNA having the base sequence of a gene derived from *Corynebacterium* bacteria such as *Corynebacterium glutamicum*, *Escherichia* bacteria such as *Escherichia coli*, or *Pantoea* bacteria such as *Pantoea ananatis*.

[0119] Phosphoglycerate mutase is a generic name for enzymes which are classified as enzyme code number: 5.4.2.1 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which catalyze a reaction of converting

3-phosphoglycerate into 2-phosphoglycerate. Examples of the phosphoglycerate mutase include those derived from *Corynebacterium* bacteria such as *Corynebacterium glutamicum*, *Escherichia* bacteria such as *Escherichia coli*, or *Pantoea* bacteria such as *Pantoea ananatis*.

[0120] As a gene of phosphoglycerate mutase (*gpm*), a DNA having the base sequence of a gene encoding phosphoglycerate mutase obtained from any of the above-listed enzyme origin organisms, or a synthesized DNA sequence that is synthesized based on a known base sequence of the gene, may be used. Preferable examples thereof include a DNA having the base sequence of a gene derived from *Corynebacterium* bacteria such as *Corynebacterium glutamicum*, *Escherichia* bacteria such as *Escherichia coli*, or *Pantoea* bacteria such as *Pantoea ananatis*.

[0121] Enolase is a generic name for enzymes which are classified as enzyme code number: 4.2.1.11 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which catalyze a reaction of converting 2-phosphoglycerate into phosphoenolpyruvate. Examples of enolase include those derived from *Corynebacterium* bacteria such as *Corynebacterium glutamicum*, *Escherichia* bacteria such as *Escherichia coli*, or *Pantoea* bacteria such as *Pantoea ananatis*.

[0122] As a gene of enolase (*eno*), a DNA having the base sequence of a gene encoding enolase obtained from any of the above-listed enzyme origin organisms, or a synthesized DNA sequence that is synthesized based on a known base sequence of the gene, may be used. Preferable examples thereof include a DNA having the base sequence of a gene derived from *Corynebacterium* bacteria such as *Corynebacterium glutamicum*, *Escherichia* bacteria such as *Escherichia coli*, or *Pantoea* bacteria such as *Pantoea ananatis*.

[0123] Pyruvate kinase is a generic name for enzymes which are classified as enzyme code number: 2.7.1.40 based on the report of the Enzyme Commission of International Union of Biochemistry (I.U.B.), and which catalyze a reaction of converting phosphoenolpyruvate and ADP into pyruvate and ATP. Examples of pyruvate kinase include those derived from *Corynebacterium* bacteria such as *Corynebacterium glutamicum*, *Escherichia* bacteria such as *Escherichia coli*, or *Pantoea* bacteria such as *Pantoea ananatis*.

[0124] As a gene of pyruvate kinase (*pyk*), a DNA having the base sequence of a gene encoding pyruvate kinase obtained from any of the above-listed enzyme origin organisms, or a synthesized DNA sequence that is synthesized based on a known base sequence of the gene,

may be used. Preferable examples thereof include a DNA having the base sequence of a gene derived from *Corynebacterium* bacteria such as *Corynebacterium glutamicum*, *Escherichia* bacteria such as *Escherichia coli*, or *Pantoea* bacteria such as *Pantoea ananatis*.

[0125] A microorganism having an acetyl-CoA production cycle by the introduction of a carbon dioxide fixation pathway may be a microorganism having, in addition to the pathway for converting acetyl-CoA into a useful metabolite, a pathway that produces another metabolite by using acetyl-CoA as a raw material, or may be a microorganism whose enzymatic activity involved in a pathway that produces another metabolite is enhanced. As a result, the useful metabolites derived from acetyl-CoA can be produced from a carbon source material and carbon dioxide, and the productivity of the useful metabolite derived from acetyl-CoA can be increased.

[0126] The microorganism used in the present disclosure is not particularly limited as long as the microorganism does not have any of:

- (a) a carbon dioxide fixation cycle having an enzymatic reaction from malonyl-CoA to malonate semialdehyde or 3-hydroxypropionate;

- (b) a carbon dioxide fixation cycle having an enzymatic reaction from acetyl-CoA and CO₂ to pyruvate;

- (c) a carbon dioxide fixation cycle having an enzymatic reaction from crotonyl-CoA and CO₂ to ethylmalonyl-CoA or glutaconyl-CoA;

- (d) a carbon dioxide fixation cycle having an enzymatic reaction from CO₂ to formate; or

- (e) at least one selected from the group consisting of malate thiokinase and malyl-CoA lyase.

[0127] Examples of the microorganism include microorganisms belonging to *Enterobacteriaceae* and microorganisms belonging to coryneform bacteria. Specific examples of the microorganism include microorganisms belonging to coryneform bacteria such as *Corynebacterium* bacteria and *Brevibacterium* bacteria, microorganisms belonging to *Enterobacteriaceae* such as *Escherichia* bacteria and *Pantoea* bacteria; filamentous fungi; and actinomycetes.

[0128] Examples of the *Corynebacterium* bacteria include *Corynebacterium glutamicum*. Specific examples of the microorganisms belonging to *Enterobacteriaceae* include bacteria belonging to *Enterobacter*, *Erwinia*, *Escherichia*, *Klebsiella*, *Pantoea*, *Providencia*, *Salmonella*, *Serratia*, *Shigella*, *Morganella*, or *Erwinia*. Among these, microorganisms belonging to *Escherichia* and microorganisms belonging to *Pantoea* are preferable from the

viewpoint of efficient production of useful metabolites.

[0129] The *Escherichia* bacteria are not particularly limited, and examples thereof include *Escherichia coli*. Specific examples of the *Escherichia coli* include *Escherichia coli* W3110 (ATCC 27325) and *Escherichia coli* MG1655 (ATCC 47076), derived from the prototype wild-type strain K12.

[0130] The "coryneform bacteria" in the present disclosure refers to the microorganisms belonging to *Corynebacterium*, *Brevibacterium*, or *Microbacterium*, as defined in Bergey's Manual of Determinative Bacteriology, 8, 599 (1974).

[0131] Examples of the coryneform bacteria further include microorganisms which had been classified into *Brevibacterium* but was reclassified later into *Corynebacterium* (Int. J. Syst. Bacteriol., 41, 255, 1991), and related bacteria such as microorganisms belonging to *Brevibacterium*. Examples of the coryneform bacteria are listed below. That is, examples thereof include *Corynebacterium acetoacidophilum*, *Corynebacterium acetoglutamicum*, *Corynebacterium alkanolyticum*, *Corynebacterium callunae*, *Corynebacterium glutamicum*, *Corynebacterium lilium*, *Corynebacterium melassecola*, *Corynebacterium thermoaminogenes*, *Corynebacterium herculis*, *Brevibacterium divaricatum*, *Brevibacterium flavum*, *Brevibacterium immariophilum*, *Brevibacterium lactofermentum*, *Brevibacterium roseum*, *Brevibacterium saccharolyticum*, *Brevibacterium thiogenitalis*, *Corynebacterium ammoniagenes*, *Brevibacterium album*, *Brevibacterium cerinum*, and *Microbacterium ammoniaphilum*.

[0132] Specific examples thereof include the following strains. *Corynebacterium acetoacidophilum* ATCC 13870, *Corynebacterium acetoglutamicum* ATCC 15806, *Corynebacterium alkanolyticum* ATCC 21511, *Corynebacterium callunae* ATCC 15991, *Corynebacterium glutamicum* ATCC 13869 or DSM 1412, ATCC 13020, ATCC 13032 and ATCC 13060, *Corynebacterium lilium* ATCC 15990, *Corynebacterium melassecola* ATCC 17965, *Corynebacterium thermoaminogenes* AJ 12340 (FERM BP-1539), *Corynebacterium herculis* ATCC13868, *Brevibacterium divaricatum* ATCC 14020, *Brevibacterium flavum* ATCC 13826, ATCC 14067, and AJ 12418 (FERM BP-2205), *Brevibacterium immariophilum* ATCC 14068, *Brevibacterium lactofermentum* (*Corynebacterium glutamicum*) ATCC 13869, *Brevibacterium roseum* ATCC 13825, *Brevibacterium saccharolyticum* ATCC 14066, *Brevibacterium thiogenitalis* ATCC 19240, *Corynebacterium ammoniagenes* ATCC 6871 and ATCC 6872, *Brevibacterium album* ATCC 15111, *Brevibacterium cerinum* ATCC 15112, and *Microbacterium ammoniaphilum* ATCC 15354.

[0133] Specific examples thereof also includes *Corynebacterium glutamicum* 2256 Δ ldhA

Δ sucA yggB* which is a glutamic acid producing strain derived from *C. glutamicum* 2256 (ATCC 13869). The strain 2256 Δ ldhA Δ sucA yggB* is deficient in *ldhA* gene and *sucA* gene, and has an IS mutation (V419::IS) in *yggB* gene encoding a mechanosensitive channel.

[0134] Both *Escherichia* bacteria and *Pantoea* bacteria belong to Enterobacteriaceae and are closely related to each other (J. Gen. Appl. Microbiol. 43(6) 355-361 (1997); International Journal of Systematic Bacteriology, p1061-1067, 1997). In recent years, some bacteria belonging to *Enterobacter* have been reclassified into *Pantoea agglomerans*, *Pantoea dispersa*, or the like (International Journal of Systematic Bacteriology, July 39(3) 337-345, 1989). Further, some bacteria belonging to *Erwinia* have been reclassified into *Pantoea ananatis* or *Pantoea stewartii* (International Journal of Systematic Bacteriology, 43(1), 162-173, 1993).

[0135] Examples of the *Enterobacter* bacteria include *Enterobacter agglomerans* and *Enterobacter aerogenes*. More specifically, strains exemplified in European Patent Laid-open No. 952221 may be used. Examples of representative strains of *Enterobacter* include the *Enterobacter agglomerans* ATCC 12287.

[0136] Examples of representative strains of the *Pantoea* bacteria include *Pantoea ananatis*, *Pantoea stewartii*, *Pantoea agglomerans*, and *Pantoea citrea*. Specific examples of the strains include the following.

·*Pantoea ananatis* AJ13355 (FERM BP-6614) (European Patent Laid-open No. 0952221)

·*Pantoea ananatis* AJ13356 (FERM BP-6615) (European Patent Laid-open No. 0952221)

[0137] Although these strains are described as *Enterobacter agglomerans* in European Patent Laid-open No. 0952221, the strains were reclassified into *Pantoea ananatis* as described above based on base sequence analysis of 16S rRNA and the like.

[0138] Each of the activities of these enzymes in the present disclosure may be an activity introduced from outside the cell into the cell, or an activity obtained by overexpression of the enzyme gene that the host bacterium possesses on its genome via enhancement of the promoter activity for the enzyme gene or replacement of the promoter with another promoter.

[0139] L-glutamic acid is produced from acetyl-CoA in a pathway. Preferable example of the microorganism having a pathway that produces L-glutamic acid or the microorganism whose enzymatic activity involved in a pathway that produces L-glutamic acid is enhanced include a microorganism obtained by imparting or enhancing, or inactivating or reducing any of the above-described enzymatic activities by using a microorganism having a pathway that efficiently produces a L-glutamic acid (hereinafter sometimes referred to as "L-glutamic acid-producing microorganism") as a host.

[0140] Examples of the L-glutamic acid-producing microorganism include the above-mentioned microorganisms having an ability to produce L-amino acids. Specific examples of the L-glutamic acid-producing microorganism include, but not limited thereto, coryneform bacteria such as *Corynebacterium glutamicum*, *Enterobacteriaceae* bacteria such as *Escherichia* bacteria or *Pantoea* bacteria.

[0141] The L-glutamic acid-producing microorganism may be any microorganism that allows the introduction or modification of a gene for imparting the L-glutamic acid production ability. It is more preferable that the L-glutamic acid-producing microorganism is a coryneform bacterium or *Pantoea* bacterium to which L-glutamic acid production ability has been imparted in advance. By using this kind of microorganism, L-glutamic acid can be more efficiently produced.

[0142] Examples of a method of imparting the L-glutamic acid production ability to a microorganism include modifying the microorganism such that expression of a gene encoding an enzyme involved in L-glutamic acid biosynthesis is increased and/or is overexpressed. Examples of the enzyme involved in L-glutamic acid biosynthesis include glutamate dehydrogenase, glutamine synthetase, glutamate synthase, isocitrate dehydrogenase, aconitate hydratase, citrate synthase, phosphoenolpyruvate carboxylase, pyruvate carboxylase, pyruvate dehydrogenase, pyruvate kinase, phosphoenolpyruvate synthase, enolase, phosphoglyceromutase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, triose-phosphate isomerase, fructose-bisphosphate aldolase, phosphofructokinase, and glucose-phosphate isomerase.

[0143] Examples of the L-glutamic acid-producing microorganism include a L-glutamic acid-producing microorganism described in Japanese Patent Application Laid-Open (JP-A) No. 2005-278643 and US20150307907A1.

[0144] The L-glutamic acid-producing microorganism to be used may be a microorganism having an ability to accumulate L-glutamic acid in an amount exceeding the saturation concentration of L-glutamic acid in a liquid medium when the microorganism was cultured under acidic conditions (hereinafter referred to as "L-glutamic acid-accumulating ability under acidic conditions"). For example, a variant having increased resistance to L-glutamic acid in a low-pH environment may be obtained by a method described in European Patent Laid-open No. 1078989, whereby the ability to accumulate L-glutamic acid in an amount exceeding the saturation concentration is imparted.

[0145] Specific examples of the microorganism having an intrinsic L-glutamic acid-accumulating ability under acidic conditions include *Pantoea ananatis* AJ13356 (FERM

BP-6615) and AJ13601 (FERM BP-7207) (see European Patent Laid-open No. 0952221). *Pantoea ananatis* AJ13356 was deposited with National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (present name: International Patent Organism Depository, National Institute of Technology and Evaluation (IPOD, NITE); address: Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan) under accession No. FERM P-16645 on February 19, 1998, and then transferred to an international depository authority under the Budapest Treaty under accession No. FERM BP-6615 on January 11, 1999. This strain was identified as *Enterobacter agglomerans* and deposited as *Enterobacter agglomerans* AJ13355 when first isolated, but, according to recent base sequence analysis of 16S rRNA and the like, the strain was reclassified as *Pantoea ananatis* (see Examples below). Similarly, AJ13356 and AJ13601 mentioned below induced from AJ13355 were deposited to the above depository as *Enterobacter agglomerans*, but these strains are described as *Pantoea ananatis* in the present specification. AJ13601 was deposited with National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry (present name: International Patent Organism Depository, National Institute of Technology and Evaluation (IPOD, NITE) under accession No. FERM P-17156 on August 18, 1999, and then transferred to an international depository authority under the Budapest Treaty under accession No. FERM BP-7207 on July 6, 2000.[0143] Other examples of the method of imparting or enhancing the L-glutamic acid production ability include a method of imparting resistance to an organic acid analogue or a respiratory inhibitor, and a method of imparting sensitivity to a cell wall synthesis inhibitor. Specific examples of the method include imparting resistance to monofluoroacetic acid (JP-A No. S50-113209), imparting resistance to adenine or resistance to thymine (JP-A No. S57-065198), weakening urease (JP-A No. S52-038088), imparting resistance to malonic acid (JP-A No. S52-038088), imparting resistance to benzopyrone or naphthoquinones (JP-A No. S56-001889), imparting resistance to HOQNO (JP-A No. S56-140895 A), imparting resistance to α -ketomalonic acid (JP-A No. S57-002689 A), imparting resistance to guanidine (JP-A No. S56-035981), and method of imparting resistance to penicillin (JP-A No. H04-088994).

[0146] Specific examples of the resistant microorganisms include the following strains.

- Brevibacterium flavum* AJ3949 (FERM BP-2632; see JP-A No. S50-113209)
- Corynebacterium glutamicum* AJ11628 (FERM P-5736; see JP-A No. S57-065198)
- Brevibacterium flavum* AJ11355 (FERM P-5007; see JP-A No. S56-001889)
- Corynebacterium glutamicum* AJ11368 (FERM P-5020; see JP-A S56-001889)

- Brevibacterium flavum* AJ11217 (FERM P-4318; see JP-A No. S57-002689)
- Corynebacterium glutamicum* AJ11218 (FERM P-4319; see JP-A No. S57-002689)
- Brevibacterium flavum* AJ11564 (FERM P-5472; see JP-A No. S56-140895)
- Brevibacterium flavum* AJ11439 (FERM P-5136; see JP-A No. S56-035981)
- Corynebacterium glutamicum* H7684 (FERM BP-3004; see JP-A No. H04-088994)
- Brevibacterium lactofermentum* AJ11426 (FERM P-5123; see JP-A No. S56-048890)
- Corynebacterium glutamicum* AJ11440 (FERM P-5137; see JP-A No. S56-048890)
- Brevibacterium lactofermentum* AJ11796 (FERM P-6402; see JP-A No. S58-158192)

[0147] The microorganism in the present disclosure is a microorganism that is constructed to have the acetyl-CoA production cycle of Fig. 1. by imparting at least both malate thiokinase and malyl-CoA lyase. Therefore, microorganisms intrinsically having malate thiokinase and malyl-CoA lyase are not included in the microorganism of the present disclosure.

[0148] Examples of the microorganisms intrinsically having Mtk and mcl include methanotrophic microorganisms such as *Methylobacterium extorquens*. Since vector systems suitable for methanotrophic microorganisms or techniques for modification of genomic genes of methanotrophic microorganisms have not been developed, genetic manipulation of microorganisms is more difficult than industrial microorganisms such as *Escherichia coli* and *Corynebacterium*. Further, these microorganisms grow slowly in many cases and therefore are not suitable for producing useful metabolites.

[0149] A specific example of the microorganism having an L-glutamic acid production ability of the present disclosure is *Corynebacterium glutamicum* AJ3. *Corynebacterium glutamicum* AJ3 strain in this disclosure is the same strain as *Corynebacterium glutamicum* 2256 Δ ldhA Δ sucA yggB* described in US20150307907A1.

[0150] A method of producing L-glutamic acid according to the present disclosure includes producing L-glutamic acid as the product of interest from a carbon source material using the above-described microorganism. Specifically, the method of producing L-glutamic acid includes culturing the microorganism having an acetyl-CoA production cycle by the introduction of a carbon dioxide fixation pathway in a state in which the microorganism having an acetyl-CoA production cycle by the introduction of a carbon dioxide fixation pathway contacts with a carbon source material (hereinafter, culture process), and collecting the product of interest (L-glutamic acid) obtained by the contact (hereinafter, collection process).

[0151] According to the method of producing L-glutamic acid, since the microorganism having an acetyl-CoA production cycle by the introduction of a carbon dioxide fixation

pathway is cultured in a state in which the microorganism having an acetyl-CoA production cycle by the introduction of a carbon dioxide fixation pathway contacts with a carbon source material, the carbon source material is assimilated by the microorganism having an acetyl-CoA production cycle by the introduction of a carbon dioxide fixation pathway, and the product of interest can be efficiently produced while carbon dioxide is fixed.

[0152] The culture medium to be used for culture of the microorganism having an acetyl-CoA production cycle by the introduction of a carbon dioxide fixation pathway includes a carbon source, a nitrogen source, and an inorganic salt; and an organic trace nutrient such as an amino acid or vitamins as necessary. Either a synthetic culture medium or natural culture medium may be used. The carbon source and nitrogen source used in the culture medium may be of any type that can be utilized by the microorganisms to be cultured.

[0153] Examples of the carbon source material which may be used include sugars such as glucose, glycerol, fructose, sucrose, maltose, mannose, galactose, starch hydrolysates, and molasses; and organic acids such as acetic acid or citric acid, and alcohols such as ethanol may be used singly or in combination with other carbon sources.

[0154] The culture medium of the present disclosure further includes yeast extract (CAS No. 8013-01-02) as a complex supplement. The yeast extract is available from commercial suppliers, examples of which include BD Biosciences and Sigma-Aldrich.

[0155] As shown in Fig. 2, a method of producing L-glutamic acid of the present disclosure includes providing a culture medium including 1 to 6 g/L of yeast extract; culturing an L-glutamic acid producing coryneform bacterium and increasing the L-glutamic acid yield in the culture medium by the yeast extract. The L-glutamic acid producing coryneform bacterium includes an acetyl-CoA production cycle obtained by imparting enzymatic activity of malate thiokinase, and optionally imparting at least one type of enzymatic activity selected from the group consisting essentially of malyl-CoA lyase, glyoxylate carboligase and 2-hydroxy-3-oxopropionate reductase, to the L-glutamic acid producing coryneform bacterium that does not have any of: (a) a carbon dioxide fixation cycle having an enzymatic reaction from malonyl-CoA to malonate semialdehyde or 3-hydroxypropionate; (b) a carbon dioxide fixation cycle having an enzymatic reaction from acetyl-CoA and CO₂ to pyruvate; (c) a carbon dioxide fixation cycle having an enzymatic reaction from crotonyl-CoA and CO₂ to ethylmalonyl-CoA or glutaconyl-CoA; (d) a carbon dioxide fixation cycle having an enzymatic reaction from CO₂ to formate; or (e) at least one selected from the group consisting of malate thiokinase and malyl-CoA lyase. None of (a), (b), (c), or (d) is imparted to the bacterium, or the bacterium exhibits none of the functions of (a), (b), (c), and (d) even

though at least one of (a), (b), (c), or (d) is imparted.

[0156] The yeast extract is added to the culture medium at 1-6 g/L of the culture medium in one embodiment. The yeast extract is added at 3 g/L of the culture medium in one embodiment. When the culture medium includes the yeast extract at 1-6 g/L or at 3 g/L of the culture medium, the biomass of the microorganism is increased and/or the L-glutamic acid yield is increased.

[0157] In the culture process, the contact between the microorganism having an acetyl-CoA production cycle by the introduction of a carbon dioxide fixation pathway and carbon source is generally made by culturing the microorganism having an acetyl-CoA production cycle by the introduction of a carbon dioxide fixation pathway in a culture medium containing the carbon source. The contact density between the carbon source and the microorganism having an acetyl-CoA production cycle by the introduction of a carbon dioxide fixation pathway may be varied depending on the activity of the microorganism. In general, the concentration of the carbon source in the culture medium may be such that the initial sugar concentration in terms of glucose may be set to be 100 g/L or lower of the culture medium. From the viewpoint of sugar tolerance of the microorganism having an acetyl-CoA production cycle by the introduction of a carbon dioxide fixation pathway, the initial sugar concentration is preferably set to be 80 g/L or lower of the culture medium or about 60 g/L of the culture medium. Other components may be added in usual addition amounts for microorganism culture media, without particular limitation.

[0158] The culture is preferably carried out at a fermentation temperature of 20°C to 45°C at a pH of 3 to 9 under aeration. For adjusting the pH, an inorganic or organic, acidic or alkaline substance, ammonia gas, *etc.* may be used. In one embodiment, the pH is maintained at about 5-8. In one embodiment, the pH is maintained at 7-7.5. In one embodiment, the pH is maintained at 7.28-7.32.

[0159] L-glutamic acid (L-amino acid) is accumulated in the culture medium or in the cells by culturing the microorganism for 10 hours to 120 hours under these culture conditions. In one embodiment, L-glutamic acid is accumulated in the culture medium or in the cells by culturing the microorganism for 20 hours to 40 hours under these culture conditions. In one embodiment, L-glutamic acid is accumulated in the culture medium or in the cells by culturing the microorganism 25 hours to 36 hours under these culture conditions. In one embodiment, L-glutamic acid is accumulated in the culture medium or in the cells by culturing the microorganism for about 30 hours under these culture conditions.

[0160] In one embodiment, the fermentation may be carried out such that L-glutamic acid

produced is precipitated and accumulated in the culture medium, by using a liquid medium whose conditions are adjusted to precipitate L-glutamic acid. For example, the pH to culture and produce L-glutamic acid may be a pH of 7.0 to 8.0, preferably a pH of 7.0 to 7.5, more preferably a pH of 7.28 to 7.32, while the pH to precipitate L-glutamic acid may be a pH of 3.0 to 5.0, preferably a pH of 3.0 to 4.0, and more preferably a pH of 3.2 which is the isoelectric point of L-glutamic acid

[0161] The aeration volume into the culture medium is not particularly restricted. When air alone is used as the gas, the aeration volume is generally 0.02 vvm to 2.0 vvm (vvm: aeration volume [mL]/liquid volume [mL]/time [min]) at 500 to 1,000 rpm. From the viewpoint of suppressing physical damage to the microorganism, the aeration is carried out preferably at 0.1 vvm to 2.0 vvm, more preferably at 0.1 vvm to 1.0 vvm.

[0162] The culture process may be continued from the beginning of the culturing until the carbon source material in the mixture is exhausted, or until the activity of the microorganism having an acetyl-CoA production cycle by the introduction of a carbon dioxide fixation pathway disappears. The duration of the culture process may be varied with the number and the activity of the microorganism having an acetyl-CoA production cycle by the introduction of a carbon dioxide fixation pathway in the mixture and the amount of the carbon source material. In general, the duration may be at least one hour, and preferably at least four hours. The duration of the culture process may be unlimitedly continued by a new addition of the carbon source material or the microorganism having an acetyl-CoA production cycle by the introduction of a carbon dioxide fixation pathway. However, from the viewpoint of process efficiency, the duration may generally be set 5 days or less, preferably 72 hours or less. With regard to other conditions, conditions employed for usual cultivation may be applied as they are.

[0163] Fig. 6 illustrates real time fermentation monitoring data of one embodiment. Fig. 6 shows an agitation speed (rpm), pH and dissolved oxygen (DO) (mg/L) of the culture medium over the fermentation time (hour) elapsed in one embodiment. In one embodiment, the fermentation is ended when the DO level of the culture medium sharply rose and reached about 15 mg/L. After ending the fermentation, L-glutamic acid is collected from the culture medium.

[0164] Methods of collecting the product of interest (L-glutamic acid) accumulated in the culture medium are not particularly restricted. For example, a method may be employed which includes removing microorganism cells from the culture medium by, for example, centrifugal separation, and thereafter separating the product of interest using a usual

separation method such as crystallization, ion exchange or membrane separation under conditions suitable for L-glutamic acid.

[0165] The L-amino acid may be collected from the culture liquid after completion of the culture according to a known collection method. For example, the collection may be carried out by a method in which concentration crystallization is carried out after removal of bacterial cells from a culture medium, or by ion-exchange chromatography. In a case in which the culture was carried out under conditions that allow precipitation of L-glutamic acid in a culture medium, the L-glutamic acid precipitated into the culture medium can be collected by centrifugal separation, filtration, or the like. In such cases, L-glutamic acid remaining dissolved in the culture medium may be crystallized, and the crystallized L-glutamic acid may be isolated together.

EXAMPLES

[0166] Hereinbelow, examples of the present disclosure is described in detail. However, the present disclosure is by no means limited to these examples.

[0167] [Example 1]

<Preparation of Plasmid pCASET>

[0168] PCR amplification was carried out using pHSG298 (Takara) as a template, and CGCCTCGAGTGACTCATACCAGGCCTG (SEQ ID NO: 1) and CGCCTCGAGGCAACACCTTCTTCACGAG (SEQ ID NO: 2) as primers, and the obtained DNA fragment was digested with the restriction enzyme *Xho*I, followed by ligation using ligase. Competent cells of the *Escherichia coli* DH5 α strain (Toyobo Co., Ltd., DNA-903) were transformed with the resulting ligation product, and transformants growing on an LB agar plate supplemented with 25 μ g/mL kanamycin were obtained. From the obtained bacterial cells, a plasmid having a recognition site of *Xho*I inserted in pHSG298 was recovered. The plasmid was designated pHSG298-*Xho*I.

[0169] In order to obtain the tac promoter, amplification by PCR was carried out using pKK223-3 (Pharmacia) as a template, and ATCATCCAGCTGTCAGGCAGCCATCGGAAG (SEQ ID NO: 3) and ATCCCCGGGAATTCTGTT (SEQ ID NO: 4) as primers, and the obtained DNA fragment was digested with the restriction enzymes *Pvu*II and *Sma*I. As a result, a DNA fragment of about 0.2 kbp encoding the tac promoter was obtained. The obtained DNA fragment was mixed with the DNA fragment of about 2.4 kbp prepared by digesting the plasmid pHSG298-*Xho*I with the restriction enzyme *Pvu*II and subjecting the resulting product to treatment with alkaline phosphatase, and the fragments were ligated

together using ligase. Competent cells of the *Escherichia coli* DH5 α strain (Toyobo Co., Ltd., DNA-903) were transformed with the resulting ligation product, and transformants growing on an LB agar plate supplemented with 25 μ g/mL kanamycin were obtained. From the obtained bacterial cells, a plasmid was recovered, to obtain the plasmid pHSGT1 in which the lac promoter of pHSG298-XhoI is replaced with the tac promoter and the direction of the tac promoter is the same as the direction of the lac promoter that was originally contained.

[0170] In order to link the multicloning site of pHSG298 to the downstream of the tac promoter of pHSGT1, pHSG298 was digested with the restriction enzymes *Eco*RI and *Cla*I to obtain a DNA fragment of about 1.0 kbp containing the multicloning site of pHSG298. The obtained DNA fragment was mixed with the DNA fragment of about 1.7 kbp prepared by digesting the plasmid pHSGT1 with the restriction enzymes *Eco*RI and *Cla*I and subjecting the resulting product to alkaline phosphatase treatment, and the fragments were ligated together using ligase. Competent cells of the *Escherichia coli* DH5 α strain (Toyobo Co., Ltd., DNA-903) were transformed with the resulting ligation product, and transformants growing on an LB agar plate supplemented with 25 μ g/mL kanamycin were obtained. From the obtained bacterial cells, a plasmid was recovered, to obtain the plasmid pHSGT2 wherein the multicloning site of pHSG298 is linked to the downstream of the tac promoter.

[0171] A DNA fragment (SEQ ID NO: 5) containing the origin of replication, repA and repB of pCASE1 (Appl Microbiol Biotechnol (2009) 81:1107-1115) isolated from *Corynebacterium casei* JCM 12072 was prepared by DNA synthesis. The sequence of the fragment is shown below.

[0172] The DNA fragment obtained by digesting the prepared DNA fragment with the restriction enzyme *Xho*I was mixed with the DNA fragment prepared by digesting the plasmid pHSGT2 with the restriction enzyme *Xho*I and subjecting the resulting product to alkaline phosphatase treatment, and the fragments were ligated together using ligase. Competent cells of the *Escherichia coli* DH5 α strain (Toyobo Co., Ltd., DNA-903) were transformed with the resulting ligation product, and transformants growing on an LB agar plate supplemented with 25 μ g/mL kanamycin were obtained. From the obtained bacterial cells, a plasmid was recovered, to obtain a plasmid wherein the DNA fragment containing the origin of replication, repA and repB of pCASE1 is inserted at the *Xho*I recognition site of pHSGT2. The plasmid was designated pCASET. In the recovered pCASET, the direction of the repA derived from pCASE1 was opposite to the direction of the tac promoter.

<Construction of Plasmids for Expression of Malate Thiokinase Derived from *Methylococcus*

capsulatus ATCC 33009>

[0173] Genomic DNA of *Methylococcus capsulatus* ATCC 33009D-5 was purchased from ATCC.

[0174] PCR was carried out using the chromosomal DNA of *Methylococcus capsulatus* as a template, and GGAATTCATATGGCTGTAAAAATCGTCTAC (SEQ ID NO: 6) and GCTCTAGATCAGAATCTGATTCCGTGTTC (SEQ ID NO: 7) as primers, to obtain an mcl-mtk fragment of *Methylococcus*. The fragment was digested with *Nde*I and *Xba*I and ligated to the plasmid obtained by digesting the plasmid pMWGKC or pMWGC with *Nde*I and *Xba*I. The construction of plasmids pMWGKC and pMWGC is the same as described in EP2738247. The thus obtained plasmid was designated pMWGKC_mcl(Mc)_mtk(Mc) or pMWGC_mcl(Mc)_mtk(Mc).

[0175] The pMWGKC_mcl(Mc)_mtk(Mc) or pMWGC_mcl(Mc)_mtk(Mc) has the *mcl* gene sequence (SEQ ID NO: 8), *mtkA* gene (SEQ ID NO: 9) and *mtkB* gene (SEQ ID NO: 10) derived from *Methylococcus capsulatus*.

[0176] [Example 2]

<Construction of Plasmid for Expression of mtk and mcl Derived from *Methylococcus capsulatus*>

[0177] PCR was carried out using pMWGKC_mcl(Mc)_mtk(Mc) as a template, and the primer pair of GGAATTCACAAAAAGGATAAAACAATGGCTGTCAAGAACCGTCTAC (SEQ ID NO: 11) and CGAATTCTCAGAATCTGATTCCGTGTTCCTG (SEQ ID NO: 12), to obtain a DNA fragment containing mcl-mtk of *Methylococcus*. Each of the primers of SEQ ID NO: 11 and SEQ ID NO: 12 has an *Eco*RI recognition site in the 5'-end side. This DNA fragment and the plasmid pCASET were digested with *Eco*RI, and the resulting fragments were ligated together. By DNA sequencing, insertion of the mcl-mtk fragment in the direction appropriate for expression with the promoter of the plasmid was confirmed, and this plasmid was designated pCASET_mcl(Mc)_mtk(Mc) and pCASEL_mcl(Mc)_mtk(Mc).

[0178] [Example 3]

<Construction of Plasmid for Expression of mtk, mcl, gcl and glxR in *Corynebacterium*>

[0179] From NBRC (Biological Resource Center, Biotechnology Field, National Institute of Technology and Evaluation), *Rhodococcus jostii* NBRC16295 was purchased. NBRC16295 was cultured in medium number 802 of NBRC, and genomic DNA was obtained using DNeasy Blood & Tissue Kit (QIAGEN). PCR was carried out using this genomic DNA as a template, and

CGAGCTCAAGCTTACAAAAAGGATAAAACAATGAGCACCATTGCATTTCATCGG

(SEQ ID NO: 13) and CGGGATCCCTAGTCCAGCAGCATGAGAG (SEQ ID NO: 14) as primers, to obtain a glxR-gcl fragment of *Rhodococcus* (SEQ ID NO: 15). The fragment was digested with *SacI* and *BamHI* and ligated to the plasmid obtained by digesting pCASET_mcl(Mc)_mtk(Mc) with *SacI* and *BamHI*. The resulting plasmid was designated pCASET_mcl(Mc)_mtk(Mc)_glxR(Rj)_gcl(Rj).

[0180] [Example 4]

<Construction of Plasmid pHSG-P1-MMGG>

[0181] The Tac promoter of plasmid pCASET was replaced by promoter P1 (5' TTGACAATTTTCTTAAATTGTGTTACAATGGGTT 3') by PCR amplification. The resulting PCR product was digested with *MfeI* and self-ligated to obtain plasmid pHSG-P1 that harbors promoter P1 for expression of genes in *Corynebacterium*. The mcl(Mc)_mtk(Mc)_genes were cloned into the *EcoRI* site of pHSG-P1 to obtain plasmid pHSG-P1-MM. The glxR(Rj)_gcl(Rj) genes were cloned downstream at *SacI* and *XbaI* site of pHSG-P1-MM to obtain plasmid pHSG-P1-MMGG.

[0182]

[Example 5]

< Method for constructing bacterial strains>

As a model Glu-producing strain, 2256ΔldhAΔsucA yggB* strain was constructed by the following method using the *C. glutamicum* 2256 strain (ATCC 13869) as the parent strain. The primers used are shown in Table 1.

[0183]

[Table 1]

Primer	Nucleotide Sequence (5'→3')	SEQ ID NO
1	cactgcacggccctgcgaac	16
2	cgccaactaggcgcaaaaattcctgattccctaaccggac	17
3	tgtgggccttcggcgaggac	18
4	gtccggtagggaaatcaggaattttggcgctagtgtggcg	19
5	gagtcgaccgcacccattttcata	20
6	tggtcgacgtgaatgctcgcgggatcc	21
7	ccaggcactcgtcctcggtt	22
8	aggctagtgcaggactataaagaccagttctcctaaaaataacgtgtc	23
9	tccatcgtggccaccgatcc	24
10	gacacgttatttttaggagaactggtctttatagtcctgcactagcct	25
11	cgggatccccaccggcgctactcgtg	26
12	ccacggatcctccaatgctattggtg	27
13	agcttgcctgcctgcagaggaggattataatggtcacccaatca	28

[0184] First, DNA fragments for deleting the *ldhA* gene were amplified by using the chromosomal DNA of the 2256 strain as the template, and the pair of the primers 1 and 2 and the pair of the primers 3 and 4, individually. Then, PCR was performed by using a mixture of equivalent amounts of both the amplified fragments as the template as well as the primers 5 and 6 to obtain a DNA fragment consisting of both the fragments fused together. The obtained DNA fragment was treated with *SalI*, and introduced into the plasmid pBS4S (WO2005/113745) at the *SalI* site to construct a plasmid for deleting *ldhA*. This plasmid for deleting *ldhA* was inserted into the chromosome of the 2256 strain, and then cured therefrom, so as to delete the *ldhA* gene.

[0185] Then, DNA fragments for deleting the *sucA* gene were amplified by using the chromosomal DNA of the 2256 strain as the template, and the pair of the primers 7 and 8 and the pair of the primers 9 and 10, individually. Then, PCR was performed by using a mixture of equivalent amounts of both the amplified fragments as the template as well as the primers 11 and 12 to obtain a DNA fragment consisting of both the fragments fused together. The obtained DNA fragment was treated with *BamHI*, and introduced into the plasmid pBS3 (WO2006/070944) at the *BamHI* site to construct a plasmid for deleting *sucA*. This plasmid for deleting *sucA* was inserted into the chromosome of the 2256 Δ *ldhA* strain, and then cured therefrom, so as to delete the *sucA* gene. Several variants of the obtained *sucA* deficient strain were cultured under a biotin-sufficient condition, and a strain having Glu-producing ability was selected. As a result, a Glu-producing strain having an IS mutation (V419::IS) in the *yggB* gene was obtained. The nucleotide sequence of the *yggB* gene having the IS mutation (V419::IS) and the amino acid sequence of the *YggB* protein encoded by this gene are shown in SEQ ID NOS: 30 and 31, respectively. The obtained Glu-producing strain was designated 2256 Δ *ldhA* Δ *sucA* *yggB** strain referred to as *Corynebacterium glutamicum* AJ3 in the current disclosure.

[0186]

[Example 6]

<Construction of *Corynebacterium glutamicum* AJ3/pHSG-P1-MMGG >

<Composition of CM2GU (liquid/solid) Medium>

[0187] Glucose: 5 g/L

Polypeptone: 10 g/L

Yeast extract: 10 g/L

NaCl : 5 g/L

Urea: 3 g/L

pH (set with KOH): 7.0

Agar (for solid medium): 20 g/L

Kanamycin: 50 µg/ml

[0188] *Corynebacterium glutamicum* AJ3 strain was transformed by electroporation with pHSG-P1-MMGG so that the malyl-CoA lyase, the malate thiokinase, the glyoxylate carboligase, and/or the tartronate semialdehyde reductase can be made in *Corynebacterium glutamicum* AJ3. The resultant was applied to CM2GU agar medium supplemented with 50 µg/ml kanamycin, and the cells were cultured at 30°C for 1 to 4 days. The transformed *Corynebacterium glutamicum* AJ3 with the pHSG-P1-MMGG is referred in the present disclosure as AJ3/pHSG-P1-MMGG.

[0189] For confirmation of transformation, the plasmids stability in *Corynebacterium glutamicum* AJ3/pHSG-P1-MMGG was evaluated by plating cultured cells on CM2GU agar medium without kanamycin. After growth of colonies at 31.5 °C for 2-3 days, twenty colonies were evaluated for their ability to grow on CM2GU agar medium with 50 µg/ml kanamycin. All 20 colonies demonstrated the ability to grow in the presence of kanamycin which confirmed 100 % plasmid stability of pHSG-P1-MMGG in *Corynebacterium glutamicum* AJ3/pHSG-P1-MMGG. The integrity of pHSG-P1-MMGG produced in *Corynebacterium glutamicum* AJ3/pHSG-P1-MMGG was also confirmed by DNA sequencing of the plasmid.

< Measurement of Activity of mtk mcl in *Corynebacterium glutamicum* AJ3/pHSG-P1-MMGG >

[0190] *Corynebacterium glutamicum* AJ3/pHSG-P1-MMGG was cultured in CM2GU liquid medium supplemented with 50 µg/ml kanamycin at 31.5 °C for 16 hours and bacterial cells were collected by centrifugation. The bacterial cells were suspended in 100 mM Tris buffer (pH 7.5), and homogenized using 0.1 mm glass beads and Beads Shocker (Yasui Kikai Corporation, MB5000). Thereafter, the supernatant obtained by centrifugation (14,000 rpm at 4°C for 15 minutes) as crude enzyme extract was subjected to measurement of the activity in the bacterial cells.

[0191] The enzymatic activity in the solution was determined by the following procedure. In a microwell, 200 mM MOPS buffer (pH 7.5), 5 mM phenylhydrazine, 10 mM MgCl₂, 10 mM ATP, 0.5 mM CoA and 10% volume of crude enzyme liquid were mixed, and the resulting mixture was incubated at room temperature for 20 minutes. Thereafter, changes in OD 324 nm with time were measured with a UV plate reader to obtain the background, and

sodium malate solution (pH 7.5) was added to a final concentration of 5 mM, followed by measuring OD 324 nm with time for 20-60 mins. In terms of a calibration curve, glyoxylic acid, which is the product of mtk mcl reaction, was added to the buffer and the resulting mixture was left to stand for 5 minutes at room temperature, followed by measuring OD 324 nm, to prepare a calibration curve for glyoxylic acid. In terms of the value of enzymatic activity, the slope of the background was subtracted from the slope of OD 324 nm after the addition of sodium malate, and the obtained value was converted into the rate of consumption of glyoxylic acid based on the calibration curve for glyoxylic acid.

[0192] The enzymatic mtk mcl activity for crude extracts from *Corynebacterium glutamicum* AJ3/pHSG-P1-MMGG was determined to be 40.6 units/ml thus confirming the expression of the introduced CO₂ fixation enzymes.

[0193] According to the present disclosure, AJ3/pHSG-P1-MMGG having an acetyl-CoA production cycle efficiently synthesizes acetyl-CoA using CO₂. Further, according to the present disclosure, AJ3/pHSG-P1-MMGG efficiently produces substances such as L-glutamic acid via the acetyl-CoA.

[Example 7]

<Selection of Complex Supplement>

[0194] AJ3/pHSG-P1-MMGG was cultured in a jar fermentation system (batch fermentation) using a culture medium with various complex supplements to select a supplement that can improve the biomass of AJ3/pHSG-P1-MMGG and/or L-glutamic acid yield of AJ3/pHSG-P1-MMGG. The composition of the culture medium is as follows.

<Composition of Culture Medium>

[0195] Glucose: 60 g/L

(NH₄)₂SO₄: 15 g/L

KH₂PO₄: 1.0 g/L

MgSO₄·7H₂O: 0.9 g/L

FeSO₄·7H₂O: 0.01 g/L

MnSO₄·H₂O: 0.01 g/L

Vitamin B₁. HCl (Thiamine.HCl): 23 mg/L

Biotin: 3 mg/L

Soybean Acid hydrolysate(Sigma Aldrich) : 9.4 g/L

Adekanol LG126 (Adeka Corporation): 1 ml/L

Complex supplements: 3 g/L

Antibiotics: 50 µg/ml kanamycin

Balance: water to 500 ml medium in 1 L capacity jar fermentor

<Various Complex Supplements>

[0196] Different complex supplements were tested as a candidate to improve the growth and L-glutamic acid production of *Corynebacterium glutamicum* AJ3/pHSG-P1-MMGG. Tested complex supplements included soybean oil (Sigma Aldrich, Bacto soytone (BD), Bacto tryptone (BD), Bacto peptone (BD), Bacto malt extract (BD), soybean acid hydrolysate (Sigma Aldrich), casein hydrolysate (Fluka), Bacto casamino acids (BD), beef extract (Fluka), corn steep liquor (Sigma Aldrich), and yeast extract (BD).

[0197] *Corynebacterium glutamicum* AJ3/pHSG-P1-MMGG was cultured in the culture medium with one of the various complex supplements in the following conditions for 20-40 hours, respectively.

Jar fermentor capacity: 1 L

Medium working volume: 500 ml

Temperature: 31.5 °C

Aeration: 0.6 vvm

Dissolved Oxygen (DO): Controlled to maintain DO at 4 mg/L by variable agitation

Agitation: 500-1,000 rpm to control DO value at 4 mg/L

pH: 7.3 ± 0.02 (20 volume % ammonia solution was used to control the pH of the culture medium)

Inoculation method: *Corynebacterium glutamicum* AJ3/pHSG-P1-MMGG cultured on CM2GU agar plate at 31.5 °C for 2 days was harvested and inoculated to the culture medium in the jar

[0198] Fig 3. Shows a comparison graph of relative biomass (%) difference and the time of fermentation (hour) between the various supplements evaluated. As a control, *Corynebacterium glutamicum* AJ3/pHSG-P1-MMGG was also cultured in the culture medium with only soybean acid hydrolysate added at final concentration of 12.5 g/L and excluding any additional complex supplements. As shown in Fig. 3, the relative biomass was the highest in the culture medium with yeast extract followed by corn steep liquor and casamino acids. Advantageously, the fermentation duration was also the shortest for the case of yeast extract followed by corn steep liquor. For yeast extract addition, the combined effect of improved biomass and shorter fermentation time can benefit to improve the productivity of L-glutamic acid production process.

<Measurement of L-glutamic acid>

[0199] Fermentation was ended when the DO level sharply rose and reached about 15 mg/L

as shown in the fermentation profile in Fig. 6. When the fermentation ended, the bacterial cells were separated from the culture medium by centrifugal separation (4000 rpm for 5 minutes). Thereafter, the supernatant obtained by the centrifugal separation was used to measure L-glutamic acid yield and 2-ketoglutaric acid yield. 2-ketoglutaric acid is a precursor for L-glutamic acid in *Corynebacterium glutamicum*. It can be assumed that 2-ketoglutaric acid can be converted to L-glutamic acid during the fermentation.

[0200] The amount of L-glutamic acid in the supernatant was measured using a HPLC (2695, Waters) equipped with an NN-814 column (Showa Denko K.K.) and a UV/Vis detector (2489, Waters). The amounts of 2-ketoglutaric acid and other products in the supernatant were measured using a HPLC (2695, Waters) equipped with an ULTRON PS-80H column (Shinwa Chemical Industries Ltd.) and an RI detector (2414, Waters). The amount of by-product acetic acid was also measured under the same HPLC conditions.

[0201] In Fig. 4, the L-glutamic acid carbon yield % means the ratio % of carbon in L-glutamic acid produced from carbon provided as glucose. The L-glutamic acid+2-ketoglutaric acid (2-KG; precursor for L-glutamic acid; assumed to be converted to L-glutamic acid during fermentation) carbon yield % means the ratio % of carbon in L-glutamic acid + 2-ketoglutaric acid produced from carbon provided as glucose. Acetic acid carbon yield % means the ratio % of carbon in byproduct acetic acid produced from carbon provided as glucose.

[0202] The results are shown in Fig. 4. As shown in Fig. 4, the relative L-glutamic acid+2-ketoglutaric acid carbon yield was the highest in the supernatant cultured with 3 g/L yeast extract. The L-glutamic acid carbon yield was also the highest in the supernatant cultured with yeast extract. The relative amount of the by-product acetic acid was the lowest in the supernatant cultured with yeast extract. In the culture medium with yeast extract, the fermentation completed in 25.4 hours which was the second shortest fermentation duration as compared to other added complex supplements, and the productivity of L-glutamic acid per hour in the culture medium with the yeast extract was the highest among all the added complex supplements. With these results, the culture medium with yeast extract resulted in the best relative L-glutamic acid yield as compared to other complex supplements. Therefore, a suitable amount of yeast extract in the culture medium was further optimized.

[Example 8]

<Optimization of yeast extract content>

[0203] *Corynebacterium glutamicum* AJ3/pHSG-P1-MMGG, was cultured with the culture medium as Example 7 except that the culture medium included various concentrations of

yeast extract as a complex supplement. The L-glutamic acid yields in the supernatant were measured by the same method as Example 7. The yeast extract was added to the culture medium at 1 g/L, 3 g/L, 4 g/L, 6 g/L, and 8 g/L of the culture medium, respectively. As a control, *Corynebacterium glutamicum* AJ3/pHSG-P1-MMGG was also cultured in the culture medium with no yeast extract added *i.e.* containing 12.5 g/L soybean acid hydrolysate. Each sample was cultured under the same conditions as Example 7.

[0204] The results are shown in Fig. 5. The parameters described in Fig. 5 are the same as the parameters shown in Fig. 4. As shown in Fig. 5, the L-glutamic acid carbon yield was the highest in the supernatant cultured with 8 g/L yeast extract followed by 6 g/L, 4 g/L, 3 g/L, and 1 g/L of yeast extract. The L-glutamic acid+2-ketoglutaric acid (2-KG; precursor for L-glutamic acid; precursor for L-glutamic acid; assumed to be converted to L-glutamic acid during fermentation) carbon yield was proportional to the amount of the yeast extract added. The L-glutamic acid yield was the highest in the supernatant cultured with 8 g/L and 6 g/L yeast extract followed by 4 g/L, 3 g/L, and 1 g/L yeast extract in medium, respectively.

[0205] In the culture medium with 3g/L yeast extract, the fermentation completed in 29.9 hours which was the shortest fermentation duration as compared to other concentrations of added yeast extract. Therefore, the productivity of L-glutamic acid per hour in the culture medium with 3 g/L yeast extract was the highest. With these results, the culture medium with 3 g/L yeast extract added showed the highest productivity of L-glutamic acid followed by 6 g/L and 4 g/L.

[0206] The disclosure of US 9,822,387 which is a national phase of International Application No. PCT/JP2012/069247 filed on July 27, 2012 and the disclosure of US 9,506,094 are incorporated herein by reference in their entirety.

[0207] All publications, patent applications, and technical standards mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent application, or technical standard was specifically and individually indicated to be incorporated by reference.

CLAIMS

What is claimed is:

1. A method of producing L-glutamic acid comprising:
providing a culture medium comprising 1 g/L to 6 g/L of yeast extract;
culturing a L-glutamic acid producing coryneform bacterium and increasing an amount of the produced L-glutamic acid in the culture medium by the yeast extract,
wherein the L-glutamic acid producing coryneform bacterium includes an acetyl-CoA production cycle obtained by imparting enzymatic activity of malate thiokinase, and optionally imparting at least one type of enzymatic activity selected from the group consisting essentially of malyl-CoA lyase, glyoxylate carboligase, and 2-hydroxy-3-oxopropionate reductase to the L-glutamic acid producing coryneform bacterium that does not have any of:
 - (a) a carbon dioxide fixation cycle having an enzymatic reaction from malonyl-CoA to malonate semialdehyde or 3-hydroxypropionate;
 - (b) a carbon dioxide fixation cycle having an enzymatic reaction from acetyl-CoA and CO₂ to pyruvate;
 - (c) a carbon dioxide fixation cycle having an enzymatic reaction from crotonyl-CoA and CO₂ to ethylmalonyl-CoA or glutaconyl-CoA;
 - (d) a carbon dioxide fixation cycle having an enzymatic reaction from CO₂ to formate; or
 - (e) at least one selected from the group consisting of malate thiokinase and malyl-CoA lyase, wherein none of (a), (b), (c), or (d) is imparted to the bacterium, or the bacterium exhibits none of the functions of (a), (b), (c), and (d) even though at least one of (a), (b), (c), or (d) is imparted.
2. The method of claim 1, wherein the L-glutamic acid producing coryneform

bacterium is *Corynebacterium glutamicum*.

3. The method of claim 1 or 2, wherein the L-glutamic acid producing coryneform bacterium is modified by using a *yggB* gene so that L-glutamic acid-producing ability of the strain is enhanced as compared to a non-modified strain.
4. The method of claim 3, wherein said coryneform bacterium is modified by introducing a mutant *yggB* gene.
5. The method of any one of claims 1 to 4, wherein said coryneform bacterium is further modified so to decrease α -ketoglutarate dehydrogenase activity.
6. The method of any one of claims 1 to 5, wherein said coryneform bacterium is further modified so to decrease lactate dehydrogenase activity.
7. The method of claim 1, wherein the method comprises providing the culture medium comprising more than 1 g/L and less than 4 g/L of the yeast extract.