

NOVEL O-SUCCINYL HOMOSERINE TRANSFERASE MUTANT AND METHOD FOR PRODUCING O-SUCCINYL HOMOSERINE USING SAME

TECHNICAL FIELD

[0001] The present disclosure relates to an O-succinyl homoserine transferase mutant, a polynucleotide encoding the same, a microorganism including the mutant, and a method for producing O-succinyl homoserine using the microorganism.

BACKGROUND ART

[0002] O-succinyl homoserine acts as a precursor of methionine, one of the essential amino acids of human body. Methionine has been used as a synthetic raw material for medical solutions and medical supplies as well as feed and food additives.

[0003] Methionine is produced by chemical or biological synthesis. Meanwhile, a two-step process of producing L-methionine by an enzyme conversion reaction from an L-methionine precursor produced by fermentation has been reported (International Publication No. WO/2008/013432).

[0004] In the two-step process, O-succinyl homoserine and O-acetyl homoserine are used as methionine precursors, and it is important to produce O-succinyl homoserine with a high yield for economical mass production of methionine.

[0005] *MetA* gene is a gene encoding homoserine O-succinyl transferase (MetA), as an enzyme involved in the synthesis of O-succinyl homoserine, by binding a succinyl group of succinyl-coA to homoserine. The *MetA* gene is one of the most important genes in development of O-succinyl homoserine-producing strains.

[0006] Strains in which O-succinyl homoserine is accumulated may be prepared by deleting *metB* gene that encodes cystathionine gamma synthase in a methionine biosynthesis pathway. However, O-succinyl homoserine-producing strains have a requirement for L-methionine. For this reason, the activity of homoserine O-succinyl transferase is weakened due to feedback inhibition by methionine added to a culture medium, and finally O-succinyl homoserine cannot be obtained at a high concentration.

[0007] Therefore, many of previous patents have been focused on how to free the feedback inhibition of *metA* from its feedback regulation system. However, homoserine O-succinyl

transferase encoded by the *metA* gene has a low stability even as a wild-type protein, and stability thereof may further deteriorate by introducing a variation to free feedback. There is a need to remove feedback inhibition of the *metA* gene and improve enzymatic stability to develop strains having high O-succinyl homoserine-producing capability.

[0008] Most microorganisms in nature use O-succinyl homoserine or O-acetyl homoserine as an intermediate for biosynthesis of methionine. In general, MetA produces O-succinyl homoserine, and homoserine O-acetyltransferase (MetX) produces O-acetyl homoserine. In addition, unlike MetA, MetX is not affected by feedback inhibition and has a high enzymatic stability.

SUMMARY

[0008a] In one aspect, the present disclosure refers to a polypeptide having O-succinyl homoserine transferase activity, the polypeptide comprises SEQ ID NO: 1, wherein the amino acid at position 313 is substituted to arginine, and the amino acid at position 176 is substituted to asparagine, tryptophan, histidine or glycine.

[0008b] In another aspect, the present disclosure refers to a polynucleotide encoding the polypeptide having the O-succinyl homoserine transferase activity as described herein.

[0008c] In another aspect, the present disclosure refers to a microorganism of the genus *Corynebacterium* producing O-succinyl homoserine, wherein the microorganism comprises the polypeptide as described herein having the O-succinyl homoserine transferase activity or wherein, in the microorganism, the polypeptide as described herein having the O-succinyl homoserine transferase activity is overexpressed.

[0008d] In another aspect, the present disclosure refers to a method of producing O-succinyl homoserine, the method comprising: culturing a microorganism of the genus *Corynebacterium* producing O-succinyl homoserine as described herein in a culture medium; and separating or recovering O-succinyl homoserine from the microorganism cultured in the culturing step or the culture medium.

[0008e] In another aspect, the present disclosure refers to a method of producing L-methionine, the method comprising: a) culturing a microorganism of the genus *Corynebacterium* producing O-succinyl homoserine as described herein in a culture medium; and (b) reacting the O-succinyl homoserine with a sulfide.

DESCRIPTION OF EMBODIMENTS

TECHNICAL PROBLEM

[0009] As a result of intensive efforts to increase production of O-succinyl homoserine, the present inventors have found a protein having O-succinyl homoserine-synthesizing activity, thereby completing the present disclosure.

SOLUTION TO PROBLEM

[0010] An object of the present disclosure is to provide a polypeptide having O-succinyl homoserine transferase activity and including a substitution of arginine for an amino acid at position 313 and a substitution of an amino acid other than glutamine for an amino acid at position 176 in an amino acid sequence of SEQ ID NO: 1.

[0011] Another object of the present disclosure is to provide a polynucleotide encoding the polypeptide.

[0012] Another object of the present disclosure is to provide a microorganism of the genus *Corynebacterium* producing O-succinyl homoserine and including a polypeptide having the O-succinyl homoserine transferase activity.

[0013] Another object of the present disclosure is to provide a method of producing O-succinyl homoserine including culturing the microorganism in a culture medium, and separating or recovering O-succinyl homoserine from the microorganism cultured in the culturing step or the culture medium.

[0014] Another object of the present disclosure is to provide a method of producing

L-methionine including culturing the microorganism in a culture medium, and reacting the O-succinyl homoserine with a sulfide.

ADVANTAGEOUS EFFECTS OF DISCLOSURE

[0015] Since the mutated O-succinyl homoserine transferase protein according to the present disclosure has an increased O-succinyl homoserine conversion activity compared to wild-type proteins, it may be used widely and effectively in mass production of O-succinyl homoserine as an alternative to conventional chemical synthesis pathways.

BEST MODE

[0016] Hereinafter, the present disclosure will be described in more detail.

[0017] Meanwhile, each description and embodiment disclosed in the present disclosure may be applied herein to describe different descriptions and embodiments. In other words, all combinations of various components disclosed in the present disclosure are included within the scope of the present disclosure. Furthermore, the scope of the present disclosure should not be limited by the detailed description provided below.

[0018] Those skilled in the art will recognize or be able to ascertain, using no more than routine experimentation, many equivalents to specific embodiments of the present disclosure. Such equivalents are intended to be encompassed in the scope of the following claims.

[0019] An aspect of the present disclosure to achieve the above objects is to provide a novel polypeptide having O-succinyl homoserine transferase activity. The novel variant polypeptide may be a polypeptide having O-succinyl homoserine transferase activity in which an amino acid at position 313 is substituted with arginine and an amino acid at position 176 is substituted with an amino acid other than glutamine in an amino acid sequence derived from *Corynebacterium glutamicum*, specifically, an amino acid sequence of SEQ ID NO: 1. In addition, the polypeptide may have the O-succinyl homoserine transferase activity and include a substitution of arginine for the amino acid at position 313 and a substitution of an amino acid other than glutamine for the amino acid at position 176 in the amino acid sequence of SEQ ID NO: 1. More specifically, the polypeptide may be a polypeptide having the O-succinyl homoserine transferase activity in which the amino acid at position 176 is substituted with asparagine, tryptophan, histidine, or glycine in the amino acid

sequence of SEQ ID NO: 1, without being limited thereto.

[0020] The above-described variant polypeptide has enhanced O-succinyl homoserine transferase activity compared to that of a polypeptide of SEQ ID NO: 1 having O-succinyl homoserine transferase activity.

[0021] As used herein, the term "O-succinyl homoserine transferase activity" refers to activity that converts homoserine into O-succinyl homoserine. The O-succinyl homoserine transferase is a generic name of enzymes capable of converting succinyl CoA and L-homoserine, as substrates, into CoA and O-succinyl homoserine.

[0022] [Reaction Scheme]

[0023] Succinyl CoA + L-homoserine $\rightleftharpoons$ CoA + O-succinyl homoserine

[0024] In the present disclosure, O-succinyl homoserine transferase refers to a modified MetX protein that is O-acetyl homoserine transferase via modification of a part of an amino acid sequence thereof with other amino acids, thereby having the activity of O-succinyl homoserine transferase. The MetX protein may be MetX derived from the genus *Corynebacterium*, more specifically MetX having an amino acid sequence of SEQ ID NO: 1 derived from *Corynebacterium glutamicum*, but is not limited thereto. The MetX protein may be obtained from known GenBank database of The National Center for Biotechnology Information (NCBI).

[0025] In the present disclosure, O-succinyl homoserine transferase may be obtained by various methods well known in the art. Examples of the methods include a gene synthesis technique including codon optimization such that an enzyme is obtained with a high yield in a microorganism of the genus *Corynebacterium* that has been widely used in expression of enzymes and a method of screening useful enzyme resources using a bioinformatic method based on a large amount of genome information of microorganisms, but are not limited thereto.

[0026] In the present disclosure, the term "O-succinyl homoserine transferase mutant" may be interchangeably used with the terms "mutated O-succinyl homoserine transferase" or "variant O-succinyl homoserine transferase". Meanwhile, this mutant may be a non-naturally occurring mutant.

[0027] Specifically, the mutated O-succinyl homoserine transferase according to the present disclosure may include an amino acid sequence in which a 313th amino acid residue from the N-terminal of MetX derived from *Corynebacterium sp.* having an amino acid sequence of

SEQ ID NO: 1 is substituted with arginine and a 176th amino acid thereof is substituted with an amino acid other than glutamine. Specifically, the 176th glutamine amino acid residue may be substituted with asparagine, tryptophan, histidine, or glycine, without being limited thereto. The mutated O-succinyl homoserine transferase according to the present disclosure may include a polypeptide having a variation at position 313 and/or position 176 from the N-terminal of the amino acid sequence set forth in SEQ ID NO: 1, wherein the variation at position 313 includes an amino acid substitution with arginine and/or the variation at position 176 includes an amino acid substitution with asparagine, tryptophan, histidine, or glycine, and the polypeptide has a homology or identity of at least 85% with SEQ ID NO: 1, without being limited thereto.

[0028] In addition, the polypeptide having the O-succinyl homoserine transferase activity according to the present disclosure may consist of at least one amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOS: 63, 75, 95, and 97. Specifically, these amino acid sequences may be amino acid sequences of polypeptides having mutated O-succinyl homoserine transferase activity in which the 313th amino acid from the N-terminal of the amino acid sequence of SEQ ID NO: 1 is substituted with arginine, and the 176th amino acid thereof is substituted with asparagine, tryptophan, histidine, or glycine, but the present disclosure is not limited thereto. Any polypeptides having a homology or identity of at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, or 99% with the sequences described above may be used without limitation so long as the polypeptides have the variations and better O-succinyl homoserine conversion activity than the wild-type.

[0029] Also, MetX of the present disclosure may be a MetX protein having the amino acid sequence of SEQ ID NO: 1 or an amino acid sequence having a homology or identity of at least 80% therewith, but is not limited thereto. Specifically, the MetX protein according to the present disclosure may include proteins having the amino acid sequence of SEQ ID NO: 1 and an amino acid sequence having a homology or identity of 80% or more, 85% or more, specifically 90% or more, more specifically 95% or more, even more specifically 99% or more with SEQ ID NO: 1.

[0030] As used herein, the term a "variant" of a polypeptide refers to a polypeptide having an amino acid sequence different from the recited sequence by conservative substitutions and/or modifications such that functions and properties of the polypeptide are retained. Variant polypeptides differ from a sequence identified by substitution, deletion, or addition of several

amino acids. Such variants may generally be identified by modifying one of the above polypeptide sequences and evaluating properties of the modified polypeptide. That is, the ability of the variant may be enhanced, unchanged, or diminished relative to a native protein. Such variants may generally be identified by modifying one of the above polypeptide sequences and evaluating the reactivity of the modified polypeptide. In addition, some variants may include those in which one or more portions, such as an N-terminal leader sequence or transmembrane domain, have been removed. Other variants may include those in which a portion has been removed from the N- and/or C-terminal of a mature protein. The term "variant" may be interchangeably used with terms such as mutant, modification, mutated protein, variant polypeptide, modified protein, modified polypeptide, mutein, divergent, and the like, without limitation, as long as the terms are used to indicate variation. Specifically, the variant includes variants having an effectively enhanced activity of O-succinyl homoserine transferase compared to wild-type by variation of amino acids of O-succinyl homoserine transferase derived from *Corynebacterium glutamicum*.

[0031] As used herein, the term "conservative substitution" refers to one amino acid substituted with another amino acid having a similar structural and/or chemical property. For example, the variant may have at least one conservative substitution while retaining at least one biological activity. Such amino acid substitution may generally occur based on similarity of polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or amphipathic nature of a residue. For example, positively charged (basic) amino acids include arginine, lysine, and histidine; negatively charged (acidic) amino acids include glutamic acid and aspartic acid; aromatic amino acids include phenylalanine, tryptophan, and tyrosine; and hydrophobic amino acids include alanine, valine, isoleucine, leucine, methionine, phenylalanine, tyrosine, and tryptophan. In general, conservative substitution has little or no influence on the activity of a produced polypeptide.

[0032] Variants may also include other modifications including deletion or addition of amino acids that have minimal influence on properties and a secondary structure of the polypeptide. For example, the polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal of a protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated with another sequence or linker to identify, purify, or synthesize the polypeptide. In other words, although it is disclosed as 'a protein or polypeptide having an amino acid sequence set forth in a given SEQ ID NO:', it will be obvious to those skilled in the art that any protein having an amino acid sequence including a

deletion, a modification, a substitution, a conservative substitution, or an addition of one or several amino acids may also be used in the present disclosure as long as the protein has homologous or identical activity to that of the polypeptide having the same SEQ ID NO:.. For example, it is obvious to those skilled in the art that any protein having an addition of a sequence not changing functions of the protein, a naturally occurring mutation or a silent mutation thereof, or a conservative substitution in the forward or reverse direction is not excluded as long as the protein has homologous or identical activity to with that of the variant polypeptide, and any protein having such addition of a sequence or mutation may also be within the scope of the present disclosure.

[0033] Also, it is obvious that the polynucleotide that is translated into the protein comprising at least one amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOS: 63, 75, 95, and 97 or proteins having a homology or identity therewith by codon degeneracy may also be used. Or, any probe prepared from known gene sequences, e.g., a sequence which are hybridized, under stringent conditions, with a sequence totally or partially complementary to a polynucleotide sequence and encodes the protein having the O-succinyl homoserine transferase activity may also be used, without limitation. The term "stringent conditions" refers to conditions which permit specific hybridization between polynucleotides. Such conditions are disclosed in detail in known documents (e.g., J. Sambrook et al., *supra*, 9.50-9.51, 11.7-11.8). For example, the stringent conditions may include performing hybridization between genes having a high homology or identity, a homology or identity of 80% or more, 85% or more, specifically 90% or more, more specifically 95% or more, even more specifically 97% or more, and most specifically 99% or more, without performing hybridization between genes having a homolog or identity lower than the above homologies or identities, or performing washing once, specifically twice or three times, under conventional washing conditions for Southern hybridization of 60°C, 1×SSC, and 0.1% SDS, specifically at a salt concentration and a temperature of 60°C, 0.1×SSC, and 0.1% SDS, and more specifically 68°C, 0.1×SSC, and 0.1% SDS.

[0034] Hybridization requires that two nucleic acids have complementary sequences, although mismatch between bases according to the degree of stringency of hybridization is possible. The term "complementary" is used to describe the relationship between nucleotide bases capable of hybridizing with each other. For example, with respect to DNA, adenosine is complementary to thymine, and cytosine is complementary to guanine. Thus, the present disclosure may include not only a substantially similar nucleic acid sequence but also a

nucleic acid fragment isolated but complementary to the entire sequence.

[0035] Specifically, the polynucleotide having homology or identity may be detected using the above-described hybridization conditions including a hybridization process at a T_m value of 55°C. Also, the T_m value may be, but is not limited to, 60°C, 63°C, or 65°C and may be appropriately adjusted by those skilled in the art according to the purpose.

[0036] An appropriate degree of stringency for hybridization of polynucleotides may depend on lengths of the polynucleotides and a degree of complementarity and parameters thereof are well known in the art (Sambrook et al., *supra*, 9.50-9.51, 11.7-11.8).

[0037] The homology or identity refers to a degree of relevance between two amino acid sequences or nucleotide sequences and may be expressed as a percentage.

[0038] The terms homology and identity may often be used interchangeably.

[0039] Sequence homology or identity of conserved polynucleotides or polypeptides may be determined by standard alignment algorithm and default gap penalties established by a used program may be used together therewith. Substantially, homologous or identical sequences may hybridize to each other along at least about 50%, 60%, 70%, 80%, or 90% of the entire sequence or the entire length under moderate or highly stringent conditions. In hybridized polynucleotides, polynucleotides including degenerated codon instead of codon may also be considered.

[0040] Whether any two polynucleotides or polypeptide sequences have homology, similarity, or identity may be determined using computer algorithms known in the art, e.g., "FASTA" program using default parameters introduced by Pearson et al (1988) [Proc. Natl. Acad. Sci. USA 85]: 2444. Alternatively, Needleman-Wunsch algorithm (1970, J. Mol. Biol. 48: 443-453) performed in a Needleman program of The European Molecular Biology Open Software Suite (EMBOSS) package (Rice et al., 2000, Trends Genet. 16: 276-277) (version 5.0.0 or later) may be used to determine the same (including GCG program package (Devereux, J., et al, Nucleic Acids Research 12: 387 (1984)), BLASTP, BLASTN, FASTA (Atschul, [S.] [F.,] [ET AL, J MOLEC BIOL 215]: 403 (1990); Guide to Huge Computers, Martin J. Bishop, [ED.,] Academic Press, San Diego, 1994, and [CARILLO ETA/.](1988) SIAM J Applied Math 48: 1073). For example, the homology, similarity, or identity may be determined using BLAST, from the National Center for Biotechnology Information database, or ClustalW.

[0041] The homology, similarity, or identity between polynucleotides or polypeptides may be determined by comparing sequence information using a GAP computer program, such as a

program introduced by Needleman et al., (1970), J Mol Biol. 48: 443 as disclosed in Smith and Waterman, Adv. Appl. Math (1981) 2:482. In brief, the GAP program defines homology, similarity, or identity as the number of aligned symbols (i.e., nucleotides or amino acids) which are similar, divided by the total number of symbols in a shorter of two sequences. Default parameters for the GAP program may include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identifies) and the weighted comparison matrix of Gribskov, et al., (1986) Nucl. Acids Res. 14: 6745 as described by Schwartz and Dayhoff, eds., Atlas Of Protein Sequence And Structure, National Biomedical Research Foundation, pp. 353-358 (1979) (or EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap (or a gap open penalty of 10 and a gap extension penalty of 0.5); and (3) no penalty for end gaps. Thus, as used herein, the term "homology" or "identity" refers to relevance between sequences.

[0042] Another aspect of the present disclosure is to provide a polynucleotide encoding the polypeptide having the O-succinyl homoserine transferase activity.

[0043] As used herein, the term "polynucleotide" refers to a polymer of nucleotides, wherein nucleotide monomers are connected in a long chain-like manner by covalent bonds, generally indicating a DNA or RNA strand having a certain minimum length, more specifically a polynucleotide fragment encoding the variant polypeptide.

[0044] In the present disclosure, a gene encoding the amino acid sequence of O-succinyl homoserine transferase is a variant O-succinyl homoserine transferase gene, specifically derived from *Corynebacterium glutamicum*. Based on genetic code degeneracy, a nucleotide sequence encoding the same amino acid sequence and mutants thereof are also included in the present disclosure, and examples thereof may be set forth in SEQ ID NOs: 64, 76, 96, or 98, without being limited thereto.

[0045] In addition, in the case of the variant polynucleotide, a nucleotide sequence encoding the same amino acid sequence and mutants thereof are also included in the present disclosure based on genetic code degeneracy.

[0046] Another aspect of the present disclosure is to provide a host cell including a polynucleotide encoding the variant polypeptide and a microorganism transformed by the vector including a polynucleotide encoding the variant polypeptide. Specifically, the introduction may be performed by transformation, but is not limited thereto.

[0047] Specifically, since a microorganism including a variant O-succinyl homoserine

transferase polypeptide has enhanced O-succinyl homoserine-producing capability without inhibiting the growth of the host cell when compared with a microorganism including a wild-type O-succinyl homoserine transferase polypeptide, O-succinyl homoserine may be obtained from these microorganisms with a high yield.

[0048] As used herein, the term "vector" refers to a DNA construct containing the nucleotide sequence of a target protein-encoding polynucleotide operably linked to a suitable regulatory sequence so as to be able to express the target protein in a suitable host cell. The regulatory sequence may include a promoter capable of initiating transcription, any operator sequence for regulating the transcription, a sequence encoding a suitable mRNA ribosome binding site, and a sequence for regulating termination of transcription and translation. Once transformed into a suitable host cell, the vector may replicate or function independently from the host genome, or may integrate into genome thereof.

[0049] The vector used in the present disclosure is not particularly limited as long as it may replicate in a host and may be any vector known in the art. Examples of the commonly used vectors may include natural or recombinant plasmids, cosmids, viruses, and bacteriophages. For example, pWE15, M13, MBL3, MBL4, IXII, ASHII, APII, t10, t11, Charon4A, and Charon21A may be used as a phage vector or cosmid vector, and pBR-based, pUC-based, pBluescriptII-based, pGEM-based, pTZ-based, pCL-based, and pET-based vectors may be used as a plasmid vector. Specifically, pDZ, pACYC177, pACYC184, pCL, pECCG117, pUC19, pBR322, pMW118, and pCC1BAC vectors may be used, without being limited thereto.

[0050] Vectors available in the present disclosure are not particularly limited and any known expression vectors may be used. In addition, a polynucleotide encoding a target protein may be inserted into a chromosome using a vector for chromosomal insertion into cells. The insertion of the polynucleotide into the chromosome may be performed by any method known in the art, for example, homologous recombination, without being limited thereto. The polynucleotide may further include a selection marker to confirm chromosomal insertion. The selection marker is used to select cells that are transformed by the vector, that is, to confirm insertion of a desired nucleic acid molecule, and the selection marker may include markers providing selectable phenotypes, such as drug resistance, nutrient requirement, resistance to cytotoxic agents, or surface protein expression. Only cells expressing the selection marker are able to survive or to show different phenotypes under the environment treated with the selective agent, and thus the transformed cells may be selected.

[0051] As used herein, the term "transformation" means the introduction of a vector including a polynucleotide encoding a target protein into a host cell in such a way that the protein encoded by the polynucleotide is expressed in the host cell. As long as the transformed polynucleotide may be expressed in the host cell, it may be inserted into and located in the chromosome of the host cell or exist extrachromosomally. In addition, the polynucleotide includes DNA and RNA encoding the target protein. The polynucleotide may be introduced in any form, as long as it is able to be introduced into the host cell and expressed therein. For example, the polynucleotide may be introduced into the host cell in the form of an expression cassette, which is a gene construct including all elements required for autonomous expression thereof. Typically, the expression cassette includes a promoter operably linked to the polynucleotide, a transcriptional termination signal, a ribosome binding site, and a translation termination signal. The expression cassette may be in the form of a self-replicable expression vector. Also, the polynucleotide as it is may be introduced into the host cell and operably linked to a sequence required for expression in the host cell, without being limited thereto. Methods for transformation include any methods for introducing a nucleic acid into cells, and may be performed by suitable standard techniques known in the art. For instance, transformation methods may include electroporation, calcium phosphate ($\text{Ca}(\text{H}_2\text{PO}_4)_2$, CaHPO_4 , or $\text{Ca}_3(\text{PO}_4)_2$) precipitation, calcium chloride (CaCl_2) precipitation, microinjection, polyethylene glycol (PEG) method, DEAE-dextran method, cationic liposome method, and Lithium acetate dihydrate-DMSO method, without being limited thereto.

[0052] In addition, as used herein, the term "operably linked" means a functional linkage between a promoter sequence which initiates and mediates transcription of the polynucleotide encoding the target protein of the present disclosure and the polynucleotide sequence. An operable linkage may be performed by a genetic recombination technique known in the art, and site-specific DNA cleavage and ligation may be performed using a restriction enzyme, a ligase, and the like, known in the art, without being limited thereto.

[0053] As used herein, the "microorganism producing O-succinyl homoserine" refers a microorganism naturally having O-succinyl homoserine-producing capability or a microorganism prepared by providing the O-succinyl homoserine-producing capability to a parent strain unable to produce O-succinyl homoserine.

[0054] The microorganism producing O-succinyl homoserine may be a cell or microorganism including a polynucleotide encoding a variant polypeptide or a cell or microorganism

transformed by a vector including a polynucleotide encoding the variant polypeptide to have the ability to express the variant polypeptide. For the purpose of the present disclosure, the host cell or microorganism may be any microorganisms capable of producing O-succinyl homoserine by including a variant MetX polypeptide. Examples of the microorganism may include microorganisms belonging to the genus *Escherichia*, the genus *Serratia*, the genus *Erwinia*, the genus *Enterobacteria*, the genus *Salmonella*, the genus *Streptomyces*, the genus *Pseudomonas*, the genus *Brevibacterium*, or the genus *Corynebacterium*, specifically microorganisms of the genus *Corynebacterium*, and more specifically *Corynebacterium glutamicum*, without being limited thereto.

[0055] As used herein, the term "a microorganism of the genus *Corynebacterium* producing O-succinyl homoserine" refers to a microorganism belonging to the genus *Corynebacterium* naturally having O-succinyl homoserine-producing capability or having the same by mutation. It is well known in the art that cultures of the microorganism of the genus *Corynebacterium* contain O-succinyl homoserine. However, the O-succinyl homoserine-producing capability is considerably low and a gene or mechanism affecting a production mechanism thereof has not been discovered yet. Thus, the microorganism of the genus *Corynebacterium* having the O-succinyl homoserine-producing capability according to the present disclosure refers to a wild-type microorganism of the genus *Corynebacterium*, a microorganism of the genus *Corynebacterium* into which an external gene related to the O-succinyl homoserine-producing mechanism is inserted or a microorganism of the genus *Corynebacterium* modified to have enhanced O-succinyl homoserine-producing capability by enhancing intrinsic activity of the gene or inactivating it.

[0056] In the present disclosure, the term "microorganism of the genus *Corynebacterium*" refers specifically to *Corynebacterium glutamicum*, *Corynebacterium ammoniagenes*, *Brevibacterium lactofermentum*, *Brevibacterium flavum*, *Corynebacterium thermoaminogenes*, *Corynebacterium efficiens*, or the like, without being limited thereto. More specifically, the microorganism of the genus *Corynebacterium* according to the present disclosure may be *Corynebacterium glutamicum*, the growth and survival of which are less affected even when exposed to a high concentration of O-succinyl homoserine.

[0057] In the microorganism, at least one protein selected from the group consisting of cystathionine synthase, O-acetyl homoserine (thiol)-lyase, and homoserine kinase may be inactivated. That is, one, two, or three proteins selected therefrom may be inactivated.

[0058] As used herein, the term "inactivation" of a protein activity means that the activity of

the protein is weakened compared to the intrinsic activity or the protein has no activity.

[0059] The inactivation of the protein activity may be achieved by various methods well known in the art. Examples of the methods may include: a method of deleting a part or the entirety of a gene encoding the protein on the chromosome including removing the activity of the protein; a method of replacing the gene encoding the protein on the chromosome with a mutated gene to reduce the enzymatic activity; a method of introducing a variation into an expression regulatory sequence of a gene encoding the protein on the chromosome; a method of replacing the expression regulatory sequence of the gene encoding the protein with a sequence having a weak activity or no activity (e.g., a method of replacing a promoter of the gene with a promoter weaker than an endogenous promoter); a method of deleting a part or the entirety of the gene encoding the protein on the chromosome; a method of introducing an antisense oligonucleotide (e.g., antisense RNA), which inhibits translation from an mRNA into the protein via a complementary binding to a transcript of the gene on the chromosome; a method of making the attachment of a ribosome impossible by forming a secondary structure by artificially adding a complementary sequence to the Shine-Dalgarno (SD) sequence on the frontend of the SD sequence of the gene encoding the protein; and a reverse transcription engineering (RTE) method, which adds a promoter for reverse transcription to the 3' terminal of the open reading frame (ORF) of the corresponding sequence, and a combination thereof, but are not particularly limited thereto.

[0060] Specifically, the method of deleting a part or the entirety of the gene encoding the protein may be executed by replacing the polynucleotide encoding the endogenous target protein within the chromosome with a polynucleotide or a marker gene having a partially deleted nucleic acid sequence using a vector for chromosomal insertion into microorganisms. For example, a method of deleting a gene by homologous recombination may be used, without being limited thereto. Also, as used herein, the term "part" may specifically refer to 1 nucleotide to 300 nucleotides, more specifically 1 nucleotide to 100 nucleotides, and even more specifically 1 nucleotide to 50 nucleotides although it may vary depending on the kinds of polynucleotide, and those skilled in the art may decide it appropriately. However, the part is not particularly limited thereto.

[0061] Additionally, the method of modifying the expression regulatory sequence may be performed by inducing a variation of nucleic acid sequence in the expression regulatory sequence via deletion, insertion, conservative substitution, non-conservative substitution, or any combination thereof so as to further weaken the activity of the expression regulatory

sequence; or by replacing the sequence with a nucleic acid sequence having a weaker activity. The expression regulatory sequence may include a promoter, an operator sequence, a sequence encoding a ribosome-binding site, and a sequence for regulating transcription and translation, but is not limited thereto.

[0062] In addition, the method of modifying the gene sequence on the chromosome may be performed by inducing a variation in the gene sequence via deletion, insertion, conservative substitution, non-conservative substitution, or any combination thereof so as to further weaken the activity of the protein; or by replacing the sequence with a gene sequence modified to have a weaker activity or a gene sequence modified to have no activity at all, but is not limited thereto. Specifically, in the microorganism, at least one gene selected from the group consisting of *metB* gene encoding cystathionine gamma synthase, *metY* gene encoding O-acetyl homoserine (thiol)-lyase used in a degradation pathway of O-succinyl homoserine, and gene *thrB* encoding homoserine kinase may further be deleted or weakened.

[0063] As used herein, the term "deletion" refers to a type of removal, within the chromosome, of a nucleotide sequence corresponding to from a start codon to a stop codon of a target gene, or a part or the entirety of a nucleotide sequence of a regulatory region thereof.

[0064] As used herein, the term "weakening" refers to removal or reduction of intracellular activity of at least one enzyme encoded by a corresponding DNA in a microorganism strain. For example, expression of a protein may be weakened by modifying a promoter region and a nucleotide sequence of 5'-UTR region, or the activity of the protein may be weakened by introducing a mutation into the ORF region of the corresponding gene. Specifically, in the microorganism, at least one gene selected from the group consisting of *metB* gene encoding cystathionine gamma synthase, *metY* gene encoding O-acetyl homoserine (thiol)-lyase in the degradation pathway of O-succinyl homoserine, and gene *thrB* encoding homoserine kinase may further be deleted or weakened.

[0065] In addition, the microorganism of the genus *Corynebacterium* may be a microorganism of the genus *Corynebacterium* producing O-succinyl homoserine with enhanced aspartokinase activity compared to non-mutated microorganisms.

[0066] As used herein, the term "enhancement" of protein activity means that the activity of the protein is introduced or increased compared to intrinsic activity thereof. The "introduction" of the activity means that a microorganism acquires activity of a particular polypeptide which has not been naturally or artificially possessed by the microorganism.

[0067] As used herein, the term "increase" in the activity of the protein relative to the intrinsic

activity means that the activity of the protein included in the microorganism is enhanced compared to the intrinsic activity of the protein or the activity before modification. The term "intrinsic activity" refers to activity of a particular protein originally possessed by a parent strain or non-modified microorganism before transformation when the microorganism is transformed by genetic variation caused by a natural or artificial factor. The intrinsic activity may also be interchangeably used with activity before modification.

[0068] Specifically, the increase in activity according to the present disclosure may be achieved by one of the following methods:

[0069] (1) a method of increasing copy number of a polynucleotide encoding the protein,

[0070] (2) a method of modifying an expression regulatory sequence to increase expression of the polynucleotide,

[0071] (3) a method of modifying a polynucleotide sequence on a chromosome to enhance the activity of the protein,

[0072] (4) a method of introducing a foreign polynucleotide having the activity of the protein or a codon optimized variant polynucleotide of the polynucleotide, or

[0073] (5) a method of enhancing the activity by any combination thereof, but the methods are not limited thereto.

[0074] The increase in the copy number of polynucleotide described in (1) above is not particularly limited, but may be performed in a form operably linked to a vector or in an integrated form into a chromosome of a host cell. Specifically, this method may be performed by introducing a vector, which may replicate and function irrespective of a host, operably linked to a polynucleotide encoding the protein of the present disclosure, into a host cell; or by introducing a vector, which may insert the polynucleotide into the chromosome of the host cell, operably linked to the polynucleotide into a host cell, thereby increasing the copy number of the polynucleotide in the chromosome of the host cell.

[0075] Next, the modification of the expression regulatory sequence to increase the expression of the polynucleotide described in (2) above may be performed by inducing a variation in the nucleic acid sequence by deletion, insertion, conservative substitution, non-conservative substitution, or any combination thereof to further enhance the activity of the expression regulatory sequence, or by replacing with a nucleic acid sequence having a stronger activity, without being limited thereto. The expression regulatory sequence may include a promoter, an operator sequence, a sequence encoding a ribosome-binding site, and a sequence for regulating of termination of transcription and translation, without being limited

thereto.

[0076] A strong heterologous promoter instead of the original promoter may be linked upstream of the polynucleotide expression unit, and examples of the strong promoter may include a CJ7 promoter (Korean Patent No. 0620092 and International Publication No. WO2006/065095), a lysCP1 promoter (International Publication No. WO2009/096689), an EF-Tu promoter, a groEL promoter, or an aceA, or aceB promoter, without being limited thereto. In addition, the modification of the polynucleotide sequence on the chromosome described in (3) above, may be performed by inducing a variation in the expression regulatory sequence by deletion, insertion, conservative substitution, non-conservative substitution, or any combination thereof to further enhance the activity of the polynucleotide sequence, or by replacing with a polynucleotide sequence modified to having a stronger activity, without being limited thereto.

[0077] In addition, the introduction of the foreign polynucleotide sequence described in (4) above may be performed by introducing a foreign polynucleotide encoding a protein having activity identical/similar to that of the protein, or a codon optimized variant polynucleotide thereto into the host cell. The foreign polynucleotide may be any polynucleotides having activity identical/similar to that of the protein without limitation. In addition, an optimized codon thereof may be introduced into the host cell to perform optimized transcription and translation of the introduced foreign polynucleotide in the host cell. The introduction may be performed by any known transformation method suitably selected by those of ordinary skill in the art. When the introduced polynucleotide is expressed in the host cell, the protein is produced and the activity thereof may be increased.

[0078] Finally, the method of enhancing the activity by any combination of the methods (1) to (4) described in (5) above may be performed by combining at least one of the methods of increasing the copy number of polynucleotide encoding the protein, modifying the expression regulatory sequence to increase expression thereof, modifying the polynucleotide sequence on the chromosome, and modifying the foreign polynucleotide having the activity of the protein or a codon optimized variant polynucleotide thereof.

[0079] In the present disclosure, the sequences of the genes or polynucleotides above may be obtained from database of The National Center for Biotechnology Information (NCBI), and the like.

[0080] Another aspect of the present disclosure is to provide a method of producing O-succinyl homoserine, the method including culturing the above-described microorganism,

and recovering O-succinyl homoserine from the cultured microorganism or a culture medium.

[0081] Another aspect of the present disclosure is to provide a method of producing L-methionine, the method including culturing the above-described microorganism, and reacting the cultured microorganism or O-succinyl homoserine with a sulfide.

[0082] Specifically, the reaction with the sulfide refers to a process of generating L-methionine from O-succinyl homoserine using any known method. For example, L-methionine may be produced by reacting O-succinyl homoserine with methyl mercaptan, as a sulfide, or by a step-like reaction after producing cystathionine via reaction with cysteine, as a sulfide. In addition, a catalyst or an enzyme may be added or reaction may be performed in a microorganism including an enzyme to improve reaction rates and yields.

[0083] The 'O-succinyl homoserine' may be a fermentation liquid or purified form containing O-succinyl homoserine produced by the microorganism according to the present disclosure. In addition, the 'sulfide' may be, for example, methyl mercaptan, and the methyl mercaptan may mean any methyl mercaptan derivatives capable of providing sulfur atoms such as dimethylsulfide (DMS) disclosed in International Publication No. WO2010/098629 as well as sodium methyl mercaptan ($\text{CH}_3\text{S-Na}$) in a liquid phase and methyl mercaptan (CH_3SH) in a gaseous or liquid state.

[0084] The method of producing L-methionine may be easily determined by those of ordinary skill in the art based on optimized culture conditions and enzymatic activity conditions well known in the art. Detailed descriptions of the culturing method and culture medium are given above.

[0085] In addition, the method of producing L-methionine may further include separating or recovering O-succinyl homoserine from the microorganism cultured in the culturing process or the medium.

[0086] It will be obvious that the "O-succinyl homoserine" of the present disclosure may include salt forms of O-succinyl homoserine as well as O-succinyl homoserine itself.

[0087] In the method, the step of culturing the microorganism may be performed by, but is not limited to, batch culture, continuous culture, and fed-batch culture known in the art. In this regard, the culture conditions are not particularly limited, but an optimal pH (e.g., pH 5 to 9, preferably pH 6 to 8, and most preferably pH 6.8) may be adjusted by using a basic compound (e.g., sodium hydroxide, potassium hydroxide, or ammonia) or an acidic compound (e.g., phosphoric acid or sulfuric acid). Also, an aerobic condition may be maintained by adding oxygen or an oxygen-containing gas mixture to a cell culture. The

culture temperature may be maintained at 20°C to 45°C, and preferably 25°C to 40°C, and the cultivation may be performed for about 10 hours to 160 hours, without being limited thereto. O-succinyl homoserine produced during the cultivation may be exported into the medium or remain in the cells.

[0088] Examples of a carbon source to be contained in the culture medium may include saccharides and carbohydrates (e.g., glucose, sucrose, lactose, fructose, maltose, molasse, starch, and cellulose), oils and fats (e.g., soybean oil, sunflower oil, peanut oil, and coconut oil), fatty acids (e.g., palmitic acid, stearic acid, and linoleic acid), alcohols (e.g., glycerol and ethanol), and organic acids (e.g., acetic acid). These carbon sources may be used alone or in combination, but are not limited thereto. Examples of a nitrogen source may include a nitrogen-containing organic compound (e.g., peptone, yeast extract, meat gravy, malt extract, corn steep liquor, bean flour, and urea), and an inorganic compound (e.g., ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate, and ammonium nitrate). These nitrogen sources may be used alone or in combination, but are not limited thereto. As a phosphorous source, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and sodium-containing salts corresponding thereto may be used alone or in combination without being limited thereto. In addition, the medium may include essential growth-promoting materials such as a metal salt (e.g., magnesium sulfate or iron sulfate), amino acids, and vitamins.

[0089] The O-succinyl homoserine or L-methionine produced in the culturing step in the present disclosure may be recovered from the culture media using any known method of collecting desired amino acids suitably selected according to the culturing method. For example, centrifugation, filtration, anion exchange chromatograph, crystallization, and HPLC may be used, and desired O-succinyl homoserine or L-methionine may be recovered from the media or microorganism using any suitable method well known in the art.

MODE OF DISCLOSURE

[0090] Hereinafter, the present disclosure will be described in more detail with reference to the following examples. However, these examples are for illustrative purposes only and are not intended to limit the scope of the present disclosure.

[0091] **Example 1: Preparation of metX Plasmid Having O-acetyl Homoserine Transferase Activity**

[0092] In order to amplify a gene encoding O-acetyl homoserine transferase (MetX), a BamHI restriction enzyme site was inserted into both ends of each of primers (SEQ ID NOs: 5 and 6) for amplification from a promoter region (located about 300 bp upstream from a start codon) to a terminator region (located about 100 bp downstream from a stop codon) based on a reported sequence derived from a wild-type (WT).

[0093] Table 1

SEQ ID NO:	Primer	Sequence (5'-3')
5	Primer 1	GGATCCCCTCGTTGTTACCCAGCAACC
6	Primer 2	GGATCCCAAAGTCACAACACTTATGTTAG

[0094] PCR was performed under the following conditions. After denaturation at 95°C for 5 minutes, cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and polymerization at 72°C for 90 seconds were repeated 30 times, and then polymerization was performed at 72°C for 7 minutes. As a result, a DNA fragment of 1546 bp was obtained as a coding region of a *metX* gene. A pECCG117 vector (Korean Patent No. 10-0057684) and the *metX* DNA fragment were treated with a restriction enzyme BamHI, ligated using a DNA ligase, and cloned to obtain a plasmid which was named pECCG117-*metX* WT.

[0095] **Example 2: Preparation of Variant *metX* Plasmid having O-succinyl Homoserine Transferase Activity**

[0096] New *metX* mutation sites were selected, and amino acids at position 176 and 313 of the amino acid sequence of SEQ ID NO: 1 were substituted with another amino acid, respectively.

[0097] More specifically, Q176N and L313R mutation was performed. A primer pair for mutation at position 176 (SEQ ID NOs: 7 and 8) and a primer pair for mutation at position 313 (SEQ ID NOS: 9 and 10) were designed to prepare a mutation vector to substitute the 176th amino acid of O-acetyl homoserine transferase with another amino acid and substitute the 313th amino acid thereof with arginine using the pECCG117-*metX* WT plasmid prepared in Example 1 as a template.

[0098] Table 2

SEQ ID NO:	Primer	Sequence (5'-3')
7	Primer 3	ACGCGCCAGCGCCTGGAACATCGGCATTCAATCCG

8	Primer 4	CGGATTGAATGCCGATGTTCCAGGCGCTGGCGCGT
9	Primer 5	GTAGATACCGATATTCGGTACCCCTACCACCAG
10	Primer 6	CTGGTGGTAGGGGTACCGAATATCGGTATCTAC

[0099] A mutated *metX* gene was prepared using the primers and a site-directed mutagenesis kit (Stratagene, USA). Mutated L313R plasmid based on the existing wild-type (WT) plasmid was named WT_L313R, and mutated Q176N and L313R plasmid was named WT_Q176N_L313R.

[00100] Example 3: Comparison Test of Substrate Specificity and Activity of Variant metX having O-succinyl Homoserine Transferase Activity

[00101] For comparisons of activities of mutated metX that produce excessive amounts of O-succinyl homoserine, strains in which homoserine is accumulated and utilization of produced O-succinyl homoserine was deleted were prepared. Prepared were strains in which a *metB* gene encoding cystathionine gamma synthase in a degradation pathway of O-succinyl homoserine was deleted and a *metY* gene encoding O-acetyl homoserine (thiol)-lyase in a degradation pathway of O-succinyl homoserine was deleted. First, for deletion of the *metB* gene, a primer pair (SEQ ID NOs: 11 and 12) for amplification of 5' upstream region of the *metB* gene and a primer pair (SEQ ID NOs: 13 and 14) for amplification of 3' downstream region of the *metB* gene were designed based on nucleotide sequence information of the WT-derived *metB* gene. An XbaI restriction enzyme site (underlined) was inserted to ends of each of the primers of SEQ ID NOs: 11 and 14.

[00102] Table 3

SEQ ID NO:	Primer	Sequence (5'-3')
11	Primer 7	<u>TCTAGAT</u> GCCTGATTATCTCACC
12	Primer 8	ACTGGTGGGTCATGGTTGCATATGAGATCAACTC CTGTAA
13	Primer 9	TTACAGGAGTTGATCTCATATGCAACCATGACCC ACCAGT
14	Primer 10	<u>TCTAGAC</u> CTTGAAGTTCTTGACTG

[00103] PCR was performed using a WT chromosome as a template and using the primers of

SEQ ID NOs: 11 and 12 and SEQ ID NOs: 13 and 14. PCR was performed under the following conditions. After denaturation at 95°C for 5 minutes, cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and polymerization at 72°C for 90 seconds were repeated 30 times, and then polymerization was performed at 72°C for 7 minutes. As a result, a DNA fragment of 450 bp of 5' upstream region of the *metB* gene and a DNA fragment of 467 bp of 3' downstream region of the *metB* gene were obtained.

[00104] PCR was performed using the two amplified DNA fragments as templates and the primers of SEQ ID NOs: 11 and 14. PCR was performed under the following conditions. After denaturation at 95°C for 5 minutes, cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and polymerization at 72°C for 3 minutes were repeated 30 times, and then polymerization was performed at 72°C for 7 minutes. As a result, a DNA fragment of 917 bp including only upstream and downstream ends of the *metB* gene with a deleted central region thereof was amplified.

[00105] A pDZ vector and the DNA fragment of 917 bp were treated with the restriction enzyme XbaI, ligated using a DNA ligase, and cloned to obtain a plasmid which was named pDZ- Δ *metB*.

[00106] The pDZ- Δ *metB* vector was introduced into WT strains by an electric-pulse method and transformed strains were obtained from a medium for selection including 25 mg/L of kanamycin. The selected strains were subjected to a secondary recombination process of cross-over to obtain the WT Δ *metB* strain in which the *metB* gene was deleted by the DNA fragment inserted into the chromosome.

[00107] For deletion of the *metY* gene in another degradation pathway of O-succinyl homoserine, a primer pair (SEQ ID NOs: 15 and 16) for amplification of 5' upstream region of the *metY* gene and a primer pair (SEQ ID NOs: 17 and 18) for amplification of 3' downstream region of the *metY* gene were designed based on nucleotide sequence information of WT-derived *metY* gene. An XbaI restriction enzyme site (underlined) was inserted into ends of each of the primers of SEQ ID NOs: 15 and 18.

[00108] Table 4

SEQ ID NO:	Primer	Sequence (5'-3')
15	Primer 11	<u>TCTAGA</u> AAGTAGCGTTGCTGTACAC

16	Primer 12	ATCAATGGTCTCGATGCCCATATGGCATTGAG GTCCTTAAG
17	Primer 13	CTTAAGGACCTCCAAATGCCATATGGGCATCGAG ACCATTGAT
18	Primer 14	<u>TCTAGAT</u> GGGAACCGTTGCAACCAC

[00109] PCR was performed using the WT chromosome as a template and using the primers of SEQ ID NOs: 15 and 16 and SEQ ID NOs: 17 and 18. PCR was performed under the following conditions. After denaturation at 95°C for 5 minutes, cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and polymerization at 72°C for 90 seconds were repeated 30 times, and then polymerization was performed at 72°C for 7 minutes. As a result, a DNA fragment of 512 bp of 5' upstream region of the *metY* gene and a DNA fragment of 520 bp of 3' downstream region of the *metY* gene were obtained.

[00110] PCR was performed using the two amplified DNA fragments as templates and the primers of SEQ ID NOs: 15 and 18. PCR was performed under the following conditions. After denaturation at 95°C for 5 minutes, cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and polymerization at 72°C for 3 minutes were repeated 30 times, and then polymerization was performed at 72°C for 7 minutes. As a result, a DNA fragment of 1032 bp including only upstream and downstream ends of the *metY* gene with a deleted central region thereof was amplified.

[00111] A pDZ vector and the DNA fragment of 1032 bp were treated with the restriction enzyme XbaI, ligated using a DNA ligase, and cloned to obtain a plasmid which was named pDZ- Δ *metY*.

[00112] The pDZ- Δ *metY* vector was introduced into the prepared WT Δ *metB* strain by an electric-pulse method and transformed strains were obtained from a medium for selection including 25 mg/L of kanamycin. The selected strains were subjected to a secondary recombination process of cross-over to obtain WT Δ *metB* Δ *metY* strain in which the *metY* gene was deleted by the DNA fragment inserted into the chromosome.

[00113] In order to prepare a vector for introduction of a mutation into a *lysC* gene (SEQ ID NO: 20) encoding a WT-derived aspartokinase (SEQ ID NO: 19) to maximize production of O-succinyl homoserine, a primer pair (SEQ ID NOs: 21 and 22) for amplification of 5'

upstream region of a mutation site and a primer pair (SEQ ID NOs: 23 and 24) for amplification of 3' downstream region of the mutation site were designed. An XbaI restriction enzyme site (underlined) was inserted to ends of each of the primers of SEQ ID NOs: 21 and 24, and the primers of SEQ ID NOs: 22 and 23 were arranged to place nucleotide substitution (underlined) at sites designed to cross over each other.

[00114] Table 5

SEQ ID NO:	Primer	Sequence (5'-3')
21	Primer 15	tcct <u>ctaga</u> GCTGCGCAGTGTTGAATACG
22	Primer 16	CACCGACATCA <u>T</u> CTTCACCTGCC
23	Primer 17	GGCAGGTGAAG <u>A</u> TGATGTCGGTG
24	Primer 18	gact <u>ctaga</u> GTTACCTCAGAGACGATTA

[00115] PCR was performed using a WT chromosome as a template and using the primers of SEQ ID NOs: 21 and 22 and SEQ ID NOs: 23 and 24. PCR was performed under the following conditions. After denaturation at 95°C for 5 minutes, cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and polymerization at 72°C for 30 seconds were repeated 30 times, and then polymerization was performed at 72°C for 7 minutes. As a result, a DNA fragment of 509 bp of 5' upstream region of the mutation of the *lysC* gene and a DNA fragment of 520 bp of 3' downstream region of the mutation of the *lysC* gene were obtained.

[00116] PCR was performed using the two amplified DNA fragments as templates and the primers of SEQ ID NOs: 21 and 24. PCR was performed under the following conditions. After denaturation at 95°C for 5 minutes, cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and polymerization at 72°C for 60 seconds were repeated 30 times, and then polymerization was performed at 72°C for 7 minutes. As a result, a DNA fragment of 1011 bp including a mutated *lysC* gene (SEQ ID NO: 26) encoding an aspartokinase mutant (SEQ ID NO: 25) in which threonine at position 311 was substituted with isoleucine was amplified.

[00117] A pDZ vector (Korean Patent No. 0924065) unable to replicate in *Corynebacterium glutamicum* and the DNA fragment of 1011 bp were treated with the restriction enzyme XbaI, ligated using a DNA ligase, and cloned to obtain a plasmid which was named pDZ-*lysC*(T311I).

[00118] The pDZ-*lysC*(T311I) vector was introduced into the WT Δ *metB* Δ *metY* by an electric-pulse method (Appl. Microbiol. Biotechnol. (1999) 52:541-545) and transformed strains were obtained from a medium for selection including 25 mg/L of kanamycin. The selected strains were subjected to a secondary recombination process of cross-over to obtain WT Δ *metB* Δ *metY*, *lysC*(T311I) strain in which the nucleotide mutation was introduced into the *lysC* gene by the DNA fragment inserted into the chromosome, and the strain was named *Corynebacterium glutamicum* WT Δ *metB* Δ *metY*, *lysC*(T311I).

[00119] The pECCG117-*metX* WT, the pECCG117-*metX* WT_L313R, and pECCG117-*metX* WT_Q176N_L313R vector prepared in Examples 1 and 2 were introduced into the prepared WT Δ *metB* Δ *metY* by an electric-pulse method and were smeared into a medium for selection including 25 mg/L of kanamycin to obtain transformed strains.

[00120] For comparison of O-acetyl homoserine (O-AH)-producing capabilities and O-succinyl homoserine (O-SH)-producing capabilities of the prepared strains, the strains were cultured in the following method and concentrations of O-acetyl homoserine and O-succinyl homoserine in culture media were analyzed.

[00121] 1 platinum loop of each strain was inoculated onto a 250 ml corner-baffled flask containing 25 ml of the following medium and cultured while shaking at 37°C at 200 rpm for 20 hours. Concentrations of O-acetyl homoserine and O-succinyl homoserine were analyzed by high performance liquid chromatography (HPLC), and the analyzed concentrations are shown in Table 6.

[00122] <Composition of Culture Medium (pH 7.0)>

[00123] 100 g of glucose, 40 g of (NH₄)₂SO₄, 2.5 g of soybean protein, 5 g of corn steep solids, 3 g of urea, 1 g of KH₂PO₄, 0.5 g of MgSO₄·7H₂O, 100 µg of biotin, 1000 µg of thiamine HCl, 2000 µg of calcium-pantothenic acid, 3000 µg of nicotinamide, 30 g of CaCO₃, and 0.3 g of L-methionine (based on 1 L of distilled water).

[00124] Table 6

Strains	O-acetyl homoserine (g/L)			O-succinyl homoserine (g/L)		
	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3

WT $\Delta metB\Delta metY$, <i>lysC</i> (T311I)/ pECCG117- <i>metX</i> WT	2.0	2.2	2.1	0.01	0.03	0.01
WT $\Delta metB\Delta metY$, <i>lysC</i> (T311I)/ pECCG117- <i>metX</i> WT_L313R	0.05	0.06	0.04	1.2	1.1	1.0
WT $\Delta metB\Delta metY$, <i>lysC</i> (T311I)/ pECCG117- <i>metX</i> WT_Q176N_L313R	0.03	0.01	0.02	1.4	1.6	1.7

[00125] Referring to Table 6 above, it was confirmed that while O-acetyl homoserine was produced by the strain into which the control *metX* WT plasmid was introduced, O-succinyl homoserine was produced by the both strains into which the *metX* mutated plasmids were introduced. Particularly, it was confirmed that production of O-succinyl homoserine was significantly increased in the case of *metX* WT_Q176N_L313R. That is, the strains into which the mutation was introduced had changed substrate specificity of transferase, thereby producing O-succinyl homoserine.

[00126] Example 4: Preparation of MetX Mutation by Saturated Mutagenesis and Evaluation of O-acetyl Homoserine-producing Capability

[00127] In order to prepare a mutant of MetX, which has high O-succinyl homoserine-producing capability, mutated by substituting an amino acid at position 176 with another amino acid, saturated mutagenesis was used. 18 types of mutants in which an amino acid at position 313 was substituted with arginine and an amino acid at position 176 was substituted with another amino acid were prepared using the plasmid prepared in Example 1 as a template. Variants, substituted amino acids, and sequence numbers of the primers used in the respective variants are shown in Table 7 below.

[00128] Table 7

Mutated plasmid	Amino acid substitution	Primer SEQ ID NO:
313 mutation	L313R	SEQ ID NO: 9,10
L313R & 176 mutation	Q176N,L313R	SEQ ID NO: 7,8
	Q176F,L313R	SEQ ID NO: 27,28
	Q176S,L313R	SEQ ID NO: 29,30
	Q176Y,L313R	SEQ ID NO: 31,32

	Q176C,L313R	SEQ ID NO: 33,34
	Q176P,L313R	SEQ ID NO: 35,36
	Q176H,L313R	SEQ ID NO: 37,38
	Q176L,L313R	SEQ ID NO: 39,40
	Q176I,L313R	SEQ ID NO: 41,42
	Q176T,L313R	SEQ ID NO: 43,44
	Q176R,L313R	SEQ ID NO: 45,46
	Q176K,L313R	SEQ ID NO: 47,48
	Q176V,L313R	SEQ ID NO: 49,50
	Q176A,L313R	SEQ ID NO: 51,52
	Q176D,L313R	SEQ ID NO: 53,54
	Q176E,L313R	SEQ ID NO: 55,56
	Q176G,L313R	SEQ ID NO: 57,58
	Q176W,L313R	SEQ ID NO: 59,60

[00129] Specifically, a variant *metX* gene was prepared using the primers shown in Table 2 and a site-directed mutagenesis kit (Stratagene, USA). The prepared mutated plasmid was introduced into WT Δ *metB* Δ *metY*, *lysC*(T311I) strains, and then flask evaluation was performed in the same manner as in Example 4. The results are shown in Table 8 below.

[00130] Table 8

Strains	Mutation site	O-acetyl homoserine (g/L)			O-succinyl homoserine (g/L)		
		Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3
WT Δ <i>metB</i> Δ <i>metY</i> , <i>lysC</i> (T311I)/ pECCG117- <i>metX</i> WT		2.0	2.2	2.1	0.01	0.03	0.01
WT Δ <i>metB</i> Δ <i>metY</i> , <i>lysC</i> (T311I)/ pECCG117- <i>metX</i> WT_L313R	L313R	0.05	0.06	0.04	1.2	1.1	1.0

SEQ ID NO: 61							
WT $\Delta metB\Delta metY$, <i>lysC</i> (T311I)/ pECCG117- <i>metX</i> WT_Q176N_L313R SEQ ID NO: 63	Q176N, L313R	0.03	0.01	0.02	1.4	1.6	1.7
WT $\Delta metB\Delta metY$, <i>lysC</i> (T311I)/ pECCG117- <i>metX</i> WT_Q176F_L313R SEQ ID NO: 65	Q176F, L313R	2.1	2.1	2.2	0.01	0.02	0.01
WT $\Delta metB\Delta metY$, <i>lysC</i> (T311I)/ pECCG117- <i>metX</i> WT_Q176S_L313R SEQ ID NO: 67	Q176S, L313R	2.0	2.0	2.2	0.02	0.03	0.02
WT $\Delta metB\Delta metY$, <i>lysC</i> (T311I)/ pECCG117- <i>metX</i> WT_Q176Y_L313R SEQ ID NO: 69	Q176Y, L313R	2.1	2.2	2.2	0.01	0.01	0.02
WT $\Delta metB\Delta metY$, <i>lysC</i> (T311I)/ pECCG117- <i>metX</i> WT_Q176C_L313R SEQ ID NO: 71	Q176C, L313R	2.0	2.1	1.9	0.04	0.01	0.03
WT $\Delta metB\Delta metY$, <i>lysC</i> (T311I)/ pECCG117- <i>metX</i>	Q176P, L313R	2.1	1.9	2.1	0.01	0.03	0.01

WT_Q176P_L313R SEQ ID NO: 73							
WT $\Delta metB \Delta metY$, <i>lysC</i> (T311I)/ pECCG117- <i>metX</i> WT_Q176H_L313R SEQ ID NO: 75	Q176H, L313R	1.6	1.3	1.6	0.1	0.3	0.1
WT $\Delta metB \Delta metY$, <i>lysC</i> (T311I)/ pECCG117- <i>metX</i> WT_Q176L_L313R SEQ ID NO: 77	Q176L, L313R	2.2	2.1	1.9	0.01	0.02	0.05
WT $\Delta metB \Delta metY$, <i>lysC</i> (T311I)/ pECCG117- <i>metX</i> WT_Q176I_L313R SEQ ID NO: 79	Q176I, L313R	2.0	2.3	2.1	0.01	0.02	0.02
WT $\Delta metB \Delta metY$, <i>lysC</i> (T311I)/ pECCG117- <i>metX</i> WT_Q176T_L313R SEQ ID NO: 81	Q176T, L313R	2.2	1.7	2.1	0.01	0.04	0.01
WT $\Delta metB \Delta metY$, <i>lysC</i> (T311I)/ pECCG117- <i>metX</i> WT_Q176R_L313R SEQ ID NO: 83	Q176R, L313R	2.0	2.0	1.9	0.05	0.03	0.06
WT $\Delta metB \Delta metY$, <i>lysC</i> (T311I)/	Q176K, L313R	2.2	2.2	2.1	0.01	0.00	0.01

pECCG117- <i>metX</i> WT_Q176K_L313R SEQ ID NO: 85							
WT Δ <i>metB</i> Δ <i>metY</i> , <i>lysC</i> (T311I)/ pECCG117- <i>metX</i> WT_Q176V_L313R SEQ ID NO: 87	Q176V, L313R	2.0	2.2	1.9	0.05	0.01	0.02
WT Δ <i>metB</i> Δ <i>metY</i> , <i>lysC</i> (T311I)/ pECCG117- <i>metX</i> WT_Q176A_L313RSE Q ID NO: 89	Q176A, L313R	2.1	1.9	1.9	0.01	0.03	0.03
WT Δ <i>metB</i> Δ <i>metY</i> , <i>lysC</i> (T311I)/ pECCG117- <i>metX</i> WT_Q176D_L313RSE Q ID NO: 91	Q176D, L313R	2.0	1.8	2.1	0.04	0.06	0.08
WT Δ <i>metB</i> Δ <i>metY</i> , <i>lysC</i> (T311I)/ pECCG117- <i>metX</i> WT_Q176E_L313R SEQ ID NO: 93	Q176E, L313R	1.9	1.9	2.0	0.07	0.02	0.05
WT Δ <i>metB</i> Δ <i>metY</i> , <i>lysC</i> (T311I)/ pECCG117- <i>metX</i> WT_Q176G_L313R SEQ ID NO: 95	Q176G, L313R	1.4	1.2	1.4	0.2	0.4	0.1
WT Δ <i>metB</i> Δ <i>metY</i> ,	Q176W, L313R	0.1	0.08	0.07	1.0	1.1	1.3

lysC(T311I)/ pECCG117- <i>metX</i> WT_Q176W_L313R SEQ ID NO: 97							
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[00131] Referring to Table 8, it was confirmed that while most of the mutants were unable to produce O-succinyl homoserine, mutated *metX* (L313R, Q176N), (L313R, Q176W), (L313R, Q176H), or (L313R, Q176G) produced O-succinyl homoserine with a high level compared to wild type, respectively. That is, it was confirmed that when the amino acid at position 313 of the amino acid sequence of SEQ ID NO: 1 is substituted with arginine, and the amino acid at position 176 thereof is substituted with asparagine, tryptophan, histidine, or glycine, substrate specificity to succinyl CoA is provided to the transferase, thereby producing O-succinyl homoserine.

[00132] The above-described results show that the mutant according to the present disclosure may increase production of O-succinyl homoserine.

[00133] In addition, the prepared WT Δ *metB* Δ *metY*,*lysC*(T311I)/pECCG117-*metX* WT_Q176N_L313R strains and WT Δ *metB* Δ *metY*, *lysC*(T311I)/pECCG117-*metX* WT_Q176W_L313R strains are designated as CA05-5136 and CA05-5137, respectively and deposited under the Budapest Treaty to the Korean Culture Center of Microorganisms (KCCM) on May 11, 2017, with Accession Nos. KCCM12024P and KCCM12025P.

[00134] The above description of the present invention is provided for the purpose of illustration, and it would be understood by those skilled in the art that various changes and modifications may be made without changing technical conception and essential features of the present invention. Thus, it is clear that the above-described embodiments are illustrative in all aspects and do not limit the present invention. The various embodiments disclosed herein are not intended to be limiting, with the true scope and spirit being indicated by the following claims. The present invention is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled.

[00135] Deposition Number

[00136] Depositary Authority: Korea Culture Center of Microorganisms (KCCM)

[00137] Accession number: KCCM12024P

[00138] Date of deposit: May 11, 2017

[00139] Depository Authority: Korea Culture Center of Microorganisms (KCCM)

[00140] Accession number: KCCM12025P

[00141] Date of deposit: May 11, 2017

Claims

1. A polypeptide having O-succinyl homoserine transferase activity, the polypeptide comprises SEQ ID NO: 1, wherein the amino acid at position 313 is substituted to arginine, and the amino acid at position 176 is substituted to asparagine, tryptophan, histidine or glycine.
2. The polypeptide of claim 1, wherein the polypeptide comprises at least one amino acid sequence selected from the group consisting of amino acid sequences of SEQ ID NOs: 63, 75, 95, and 97.
3. A polynucleotide encoding the polypeptide of claim 1 having the O-succinyl homoserine transferase activity.
4. The polynucleotide of claim 3, wherein the polynucleotide comprises at least one nucleic acid sequence selected from the group consisting of nucleic acid sequences of SEQ ID NOs: 64, 76, 96, and 98.
5. A microorganism of the genus *Corynebacterium* producing O-succinyl homoserine, wherein the microorganism comprises the polypeptide of claim 1 having the O-succinyl homoserine transferase activity or wherein, in the microorganism, the polypeptide of claim 1 having the O-succinyl homoserine transferase activity is overexpressed.
6. The microorganism of claim 5, wherein the microorganism of the genus *Corynebacterium* has further enhanced aspartokinase activity compared to endogenous activity of this enzyme in non-mutated microorganisms.
7. The microorganism of claim 5, wherein the microorganism of the genus *Corynebacterium* is *Corynebacterium glutamicum*.
8. The microorganism of claim 5, wherein activity of at least one enzyme selected from the group consisting of cystathionine synthase, O-acetyl homoserine (thiol)-lyase, and homoserine kinase is inactivated in the microorganism of the genus *Corynebacterium*.
9. The microorganism of claim 5, wherein the microorganism of the genus *Corynebacterium* has further enhanced aspartokinase activity compared to non-mutated microorganisms and activity of at least one enzyme selected from the group consisting of cystathionine synthase, O-acetyl homoserine (thiol)-lyase, and homoserine kinase is inactivated.
10. A method of producing O-succinyl homoserine, the method comprising:
culturing a microorganism of the genus *Corynebacterium* producing O-succinyl

homoserine according to any one of claims 5 to 9 in a culture medium; and separating or recovering O-succinyl homoserine from the microorganism cultured in the culturing step or the culture medium.

11. A method of producing L-methionine, the method comprising:

- (a) culturing a microorganism of the genus *Corynebacterium* producing O-succinyl homoserine according to any one of claims 5 to 9 in a culture medium; and
- (b) reacting the O-succinyl homoserine with a sulfide.

12. The method of claim 11, further comprising separating or recovering O-succinyl homoserine from the cultured microorganism or the culture medium in step (a).

13. The method of claim 11, further comprising separating or recovering L-methionine produced by the reaction between O-succinyl homoserine and the sulfide in step (b).