

[DESCRIPTION]

[Invention Title]

MICROORGANISM PRODUCING O-ACETYL-HOMOSERINE, AND METHOD FOR PRODUCING O-ACETYL-HOMOSERINE BY USING SAME

[Technical Field]

The present disclosure relates to a protein having the activity of exporting *O*-acetylhomoserine and a novel modified protein thereof, a microorganism capable of producing *O*-acetylhomoserine with enhanced expression of the protein, and a method for producing *O*-acetylhomoserine using the microorganism.

[Background Art]

Methionine, which can be produced by chemical and biological synthesis, is used as a raw material for the synthesis of infusions and medicines as well as for the synthesis of feed and food additives. Recently, a two-step process for producing L-methionine from an L-methionine precursor, produced via fermentation, by an enzyme conversion reaction was disclosed (International Patent Publication No. WO 2008/013432). International Patent Publication No. WO 2008/013432 discloses that *O*-succinylhomoserine and *O*-acetylhomoserine can be used as a methionine precursor in the two-step process, and it is very important to produce methionine precursors in high yield for economical large-scale production of methionine.

LeuE is known as a leucine export protein. As one of the proteins belonging to homoserine/homoserine lactone efflux protein (RhtB) family, LeuE is a protein present in the inner membrane and is known to have the role of exporting leucine and its analogues as a putative uncharacterized transport protein.

In the prior art relating to LeuE, it is known that a purine nucleoside or purine nucleotide can be produced by enhancing an amino acid sequence of leuE (yeaS) gene or a modified amino acid sequence thereof, and the amount of amino acid production can be improved. Additionally, a modified leuE having the activity of exporting cysteine is known.

[Disclosure]

[Technical Problem]

The inventors of the present disclosure have made many efforts to improve the production of *O*-acetylhomoserine, and as a result, they have discovered a protein which has the activity of exporting *O*-acetylhomoserine and a modified protein thereof, thereby completing the present disclosure.

[Technical Solution]

An object of the present disclosure is to provide a polypeptide having the activity of exporting *O*-acetylhomoserine.

Another object of the present disclosure is to provide a polynucleotide which encodes the polypeptide.

Still another object of the present disclosure is to provide a microorganism of the genus *Escherichia* producing *O*-acetylhomoserine, in which a polypeptide having the activity of exporting *O*-acetylhomoserine is included or overexpressed.

Still another object of the present disclosure is to provide a method for producing *O*-acetylhomoserine, which includes: culturing a microorganism of the genus *Escherichia* producing *O*-acetylhomoserine in a medium; and recovering *O*-acetylhomoserine from the cultured microorganism or the cultured medium.

Still another object of the present disclosure is to provide a method for producing L-methionine, which includes: culturing the microorganism of the genus *Escherichia* producing *O*-acetylhomoserine in a medium; and converting the *O*-acetylhomoserine to L-methionine by treating the cultured microorganism or the cultured medium or the *O*-acetylhomoserine recovered from the cultured microorganism or the cultured medium with methyl mercaptan and a methionine-converting enzyme.

[Advantageous Effects of the Invention]

The microorganism of the present disclosure including a modified LeuE or LeuE, which are inner membrane proteins, has enhanced activity of exporting *O*-acetylhomoserine, and thus the production efficiency of *O*-acetylhomoserine can be enhanced. Accordingly, the microorganism of the present disclosure can be used for efficient production of *O*-acetylhomoserine. Additionally, *O*-acetylhomoserine produced with high efficiency may be used for economical large-scale production of L-methionine.

[Best Mode]

To achieve the above objects, an aspect of the present disclosure provides a polypeptide having the activity of exporting *O*-acetylhomoserine, in which at least one amino acid selected from the group consisting of valine at position 1, leucine at position 30, phenylalanine at position 95, and phenylalanine at position 165 in the amino acid sequence of SEQ ID NO: 1 is substituted with another amino acid.

As used herein, the term “*O*-acetylhomoserine”, which is a specific intermediate material in the methionine biosynthesis pathway of microorganisms, refers to an acetyl derivative of L-homoserine. *O*-acetylhomoserine is known to be produced by reacting homoserine and acetyl-CoA catalyzed by homoserine acetyltransferase, and it has the formula of C₆H₁₁NO₄.

As used herein, the term “peptide having the activity of exporting *O*-acetylhomoserine” refers to a polypeptide having the function of exporting *O*-acetylhomoserine in a cell of a microorganism to the outside of the cell. Specifically, the peptide may refer to a LeuE protein having the activity of exporting *O*-acetylhomoserine and a modified protein thereof, but the peptide is not particularly limited thereto as long as it has the activity of exporting *O*-acetylhomoserine.

As used herein, with regard to amino acid transporters, the term “LeuE”, which is a protein belonging to the homoserine/homoserine lactone efflux protein (RhtB) family, refers to a protein present in the inner membrane, but its exact function is not known. In this regard, the inventors of the present disclosure first confirmed that LeuE specifically exports *O*-acetylhomoserine.

The LeuE may be a protein derived from a microorganism of the genus *Escherichia*, and specifically LeuE derived from *E. coli*, but any LeuE having the activity of exporting *O*-acetylhomoserine can be included to the scope of the present disclosure without limitation with regard to the origin of the microorganism.

Specifically, the peptide having the activity of exporting *O*-acetylhomoserine may be a protein having the amino acid sequence of SEQ ID NO: 1. Additionally, the peptide may be a protein which has an amino acid sequence having the activity of exporting *O*-acetylhomoserine substantially the same as or equivalent to that of the amino acid sequence of SEQ ID NO: 1, while having a homology of at least 70%, specifically at least 80%, and more specifically at least

90% to the amino acid sequence of SEQ ID NO: 1. Alternatively, the peptide may be an amino acid sequence having such homology where there is deletion, modification, substitution, or addition in part of the amino acid sequence having the activity of exporting *O*-acetylhomoserine substantially the same as or equivalent to that of the amino acid sequence of SEQ ID NO: 1, and it is obvious that this peptide also belongs to the scope of the present disclosure.

As used herein, the term “modified polypeptide” of the polypeptide having the activity of exporting *O*-acetylhomoserine refers to a polypeptide that has enhanced activity of exporting *O*-acetylhomoserine compared to that of native wild-type polypeptide or unmodified polypeptide. Specifically, the modified polypeptide is a peptide which has enhanced activity of exporting *O*-acetylhomoserine compared to that of the polypeptide which has the amino acid sequence of SEQ ID NO: 1 due to a modification of at least one amino acid in the amino acid sequence of SEQ ID NO: 1.

For example, the modified polypeptide may be a polypeptide in which at least one amino acid selected from the group consisting of valine at position 1, leucine at position 30, phenylalanine at position 95, and phenylalanine at position 165 in the amino acid sequence of SEQ ID NO: 1 is substituted with another amino acid. Specifically, the modified polypeptide may be a polypeptide in which valine at position 1 in the amino acid sequence of SEQ ID NO: 1 is substituted with methionine; phenylalanine at position 30 in the amino acid sequence of SEQ ID NO: 1 is substituted with any one selected from the group consisting of alanine, tryptophan, leucine, valine, glycine, serine, asparagine, aspartic acid, histidine, isoleucine, proline, tyrosine, glutamine, lysine, glutamic acid, cysteine, threonine, and arginine; leucine at position 95 in the amino acid sequence of SEQ ID NO: 1 is substituted with any one selected from the group consisting of valine, phenylalanine, alanine, glycine, threonine, asparagine, aspartic acid, histidine, isoleucine, serine, proline, tyrosine, glutamine, lysine, glutamic acid, cysteine, tryptophan, and arginine; or phenylalanine at position 165 in the amino acid sequence of SEQ ID NO: 1 is substituted with any one selected from the group consisting of alanine, tryptophan, leucine, valine, glycine, serine, asparagine, aspartic acid, histidine, isoleucine, proline, tyrosine, glutamine, lysine, glutamic acid, cysteine, threonine, and arginine. More specifically, the modified polypeptide may be a polypeptide in which valine at position 1 in the amino acid sequence of SEQ ID NO: 1 is substituted with methionine; phenylalanine at position 30 in the amino acid sequence of SEQ ID NO: 1 is substituted with any one selected from the group consisting of alanine, tryptophan, leucine, valine, glycine, serine, asparagine, aspartic acid, and

histidine; leucine at position 95 in the amino acid sequence of SEQ ID NO: 1 is substituted with any one selected from the group consisting of valine, phenylalanine, alanine, glycine, threonine, asparagine, aspartic acid, and histidine; or phenylalanine at position 165 in the amino acid sequence of SEQ ID NO: 1 is substituted with any one selected from the group consisting of alanine, tryptophan, leucine, valine, glycine, serine, asparagine, aspartic acid, and histidine. Even more specifically, the modified polypeptide may be a polypeptide in which valine at position 1 in the amino acid sequence of SEQ ID NO: 1 is substituted with methionine; and phenylalanine at position 30, leucine at position 95, and phenylalanine at position 165 in the amino acid sequence of SEQ ID NO: 1 is substituted with another amino acid. Even more specifically, the modified polypeptide may be a polypeptide consisting of an amino acid sequence of SEQ ID NO: 2, 133, 134, 137, 138, 141, or 142. Specifically, the modified polypeptide may be a protein which has an amino acid sequence having enhanced activity of exporting *O*-acetylhomoserine substantially the same as or equivalent to that of the amino acid sequence of the modified polypeptide, while having a homology of at least 70%, specifically at least 80%, and more specifically at least 90% to the above amino acid sequences. Alternatively, in an amino acid sequence having such homology and having enhanced activity of exporting *O*-acetylhomoserine substantially the same as or equivalent to that of the amino acid sequence of the modified polypeptide, the amino acid sequence may be one where there is deletion, modification, substitution, or addition in part of the amino acid sequence. The polypeptide is an example of a modified polypeptide of the polypeptide with enhanced activity of exporting *O*-acetylhomoserine compared to that of the native wild-type polypeptide or unmodified polypeptide, but the polypeptide is not limited thereto. As used herein, the term “natural native state or unmodified state” refers to a state where the introduction of the corresponding polypeptide or the introduction of modification of activity in the present disclosure has not been achieved.

As used herein, the term “homology” refers to the degree of identity between nucleotides or amino acid residues of two amino acid sequences or nucleic acid sequences of a protein-encoding gene determined after aligning them to maximally match with each other for a particular comparison region. When the homology is sufficiently high, the expression products of the corresponding gene may have the same or similar activity. The percentage of the sequence identity can be determined using a known sequence comparison program (*e.g.*, BLAST (NCBI), CLC Main Workbench (CLC bio), MegAlign (DNASTAR Inc), *etc.*).

An aspect of the present disclosure provides a polynucleotide which encodes the polypeptide having the activity of exporting *O*-acetylhomoserine. In the present disclosure, the polypeptide having the activity of exporting *O*-acetylhomoserine is the same as explained above.

For example, the polynucleotide may be one in which the initiation codon is substituted with ATG and may be the nucleotide sequence of SEQ ID NO: 4, 135, 136, 139, 140, 143, or 144 but the nucleotide sequence is not limited thereto. Additionally, with regard to the polynucleotide, the nucleotide sequence and modified nucleotide sequences thereof encoding the same amino acid sequence are also included in the present disclosure based on codon degeneracy. For example, the nucleotide sequence may be modified to have an optimum codon depending on the microorganism being used.

Specifically, the nucleotide sequence may be one which encodes an amino acid sequence having the activity of exporting *O*-acetylhomoserine substantially the same as or equivalent to that of the above nucleotide sequences, while having a homology of at least 70%, specifically at least 80%, and more specifically at least 90% to the above amino acid sequences. Alternatively, the nucleotide sequence may be a sequence capable of hybridizing with a probe, which can be prepared from a known gene sequence (*e.g.*, a sequence complementary to all or part of the above nucleotide sequences), under stringent conditions to encode a protein having the activity of exporting *O*-acetylhomoserine. As used herein, the term “stringent condition” refers to a condition in which so-called a specific hybrid is formed while a non-specific hybrid is not formed. For example, the stringent condition may include a condition in which genes having a high homology (*e.g.*, 80% or more, specifically 90% or more, more specifically 95% or more, even more specifically 97% or more, and even more specifically 99% or more) can hybridize between them, whereas genes having a lower homology thereof cannot hybridize with each other; or conditions for conventional southern hybridization (*i.e.*, conditions for washing once, and specifically two or three times under a salt concentration and temperature corresponding to 60°C, 1× SSC, and 0.1% SDS; specifically under 60°C, 0.1× SSC, and 0.1% SDS, and more specifically under 68°C, 0.1×SSC, and 0.1% SDS) (Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2001)). The probe used for the hybridization may optionally be a part of the nucleotide sequence complementary to the above nucleotide sequences. Such a probe can be prepared by PCR using an oligonucleotide prepared based on a known sequence as a primer and a gene

fragment containing such a nucleotide sequence as a template. For example, as the probe, a gene fragment of about 300 bp may be used. More specifically, when a gene fragment of about 300 bp is used as a probe, the conditions of 50°C, 2× SSC, and 0.1% SDS are listed as washing conditions for the hybridization.

The genes used in the present disclosure, the protein sequences and the promoter sequences they encode can be obtained from a known database (*e.g.*, GenBank of NCBI), but are not limited thereto.

An aspect of the present disclosure relates to a microorganism in which the polypeptide having the activity of exporting *O*-acetylhomoserine or a modified polypeptide thereof is included or overexpressed. Specifically, the microorganism may be a microorganism producing *O*-acetylhomoserine or a modified polypeptide thereof, in which a polypeptide consisting of the amino acid sequence of SEQ ID NO: 1 is included or overexpressed.

The polypeptide having the activity of exporting *O*-acetylhomoserine and the modified polypeptide thereof are the same as explained above.

As used herein, the term “microorganism producing *O*-acetylhomoserine” refers to a microorganism capable of producing *O*-acetylhomoserine in the microorganism and exporting it to a medium. The activity of producing *O*-acetylhomoserine can be provided or enhanced by natural or artificial mutations or species improvement. Specifically, those microorganisms which produce *O*-acetylhomoserine can be included regardless of their microbial origin, as long as they can produce *O*-acetylhomoserine. In an embodiment, the microorganism may be one belonging to the genus *Escherichia*, and more specifically, *Escherichia coli*.

Meanwhile, in the present disclosure, the microorganisms producing *O*-acetylhomoserine may be a modified microorganism in which a known modification is additionally introduced with regard to related mechanisms such as homoserine biosynthesis-related pathways and mechanisms related to exporting *O*-acetylhomoserine, *etc.* so as to enhance the productivity of *O*-acetylhomoserine apart from the LeuE.

Another specific embodiment of the present disclosure may relate to the microorganism producing *O*-acetylhomoserine in which, additionally, the activity of cystathionine synthase is

inactivated. Specifically, the microorganism may be one in which the gene encoding cystathionine synthase (*metB*) is deletion or its expression is weakened compared to that of an unmodified microorganism, but is not limited thereto. The amino acid sequence of the *metB* gene can be obtained from a known database and any amino acid sequence having the activity of cystathionine synthase can be included without limitation (*e.g.*, a protein having the amino acid sequence of SEQ ID NO: 5). –The protein having the amino acid sequence of SEQ ID NO: 5 may be a protein encoded by the nucleotide sequence of SEQ ID NO: 6, but is not limited thereto.

Additionally, still another specific embodiment of the present disclosure may relate to the microorganism producing *O*-acetylhomoserine in which, additionally, the activity of homoserine kinase is inactivated. Specifically, the microorganism may be one in which the activity of homoserine kinase is reduced compared to its endogenous activity of an unmodified microorganism or is removed. For example, the microorganism may be one in which the gene (*thrB*) encoding homoserine kinase is linked to a weaker promoter compared to a native promoter, or is modified or deletion to have weak activity, but the promoter is not limited thereto. The amino acid sequence of the *thrB* gene can be obtained from a known database and any amino acid sequence having the activity of homoserine kinase can be included without limitation (*e.g.*, a protein having the amino acid sequence of SEQ ID NO: 7). The protein having the amino acid sequence of SEQ ID NO: 7 may be a protein encoded by the nucleotide sequence of SEQ ID NO: 8, but is not limited thereto.

As used herein, the term “inactivation” of the protein refers to a case where the activity of the protein of a microorganism is reduced compared to the enzyme activity possessed by the microorganism in a native wild-type protein or unmodified protein; a case where the protein is not expressed at all; or a case where the protein is expressed but exhibits no activity. The inactivation is a concept including a case where the activity of the enzyme itself is reduced or removed compared to the activity of the enzyme originally possessed by the microorganism due to the modification, *etc.* of the gene encoding the enzyme; a case where the entire activity level of the enzyme in a cell is reduced or removed compared to the activity of the enzyme originally possessed by the wild-type strain of the microorganism due to the inhibition of the expression or translation of the gene encoding the enzyme; a case where part or the entirety of the gene is deleted; and a combination thereof; but the inactivation is not limited thereto.

The inactivation of an enzyme may be achieved by applying various methods well known

in the art. Examples of the methods may include a method of substituting the gene encoding the enzyme on the chromosome with a gene modified to reduce the activity of the enzyme, including the case when the enzyme activity is removed; a method of introducing a modification in the expression control sequence of the gene encoding the enzyme on the chromosome; a method of substituting the expression control sequence of the gene encoding the enzyme with a sequence having weak or no activity; a method of deleting part or the entirety of the gene encoding the enzyme on the chromosome; a method of introducing an antisense oligonucleotide (*e.g.*, antisense RNA) which binds complementary to a transcript of the gene on the chromosome, thereby inhibiting the translation from the mRNA into the enzyme; a method of artificially incorporating a sequence complementary to the SD sequence into the upstream of the SD sequence of the gene encoding the enzyme, forming a secondary structure, thereby making the attachment of ribosome thereto impossible; a method of incorporating a promoter to the 3' terminus of the open reading frame (ORF) to induce a reverse transcription (reverse transcription engineering (RTE)), *etc.*, and also a combination thereof, but the methods are not particularly limited thereto.

The method of modifying the expression control sequence may be performed by inducing a modification of the expression control sequence by deletion, insertion, non-conservative or conservative substitution, or a combination thereof in the nucleic acid sequence of the expression control sequence so as to further weaken the activity of the expression control sequence; or by substituting with a nucleic acid having weaker activity. The expression control sequence may include a promoter, an operator sequence, a sequence encoding a ribosome-binding region, and sequences controlling the termination of transcription and translation, but is not limited thereto.

Furthermore, the gene sequence on the chromosome may be modified by inducing a modification in the sequence by deletion, insertion, non-conservative or conservative substitution, or a combination thereof in the gene sequence for further weakening the enzyme activity; or by substituting with a gene sequence which was improved to have weaker activity or a gene sequence which was improved to have no activity, but the method is not limited thereto.

Additionally, the method of deleting part or the entirety of a gene encoding an enzyme may be performed by substituting the polynucleotide encoding the endogenous target protein within the chromosome with a polynucleotide or marker gene having a partial deletion in the nucleic acid sequence using a vector for chromosomal insertion within a bacterial strain. In an

exemplary embodiment of the method of deleting part or the entirety of a gene, a method for deleting a gene by homologous recombination may be used, but the method is not limited thereto.

As used herein, the term “part” may vary depending on the kinds of polynucleotides, and it may specifically refer to 1 to 300, more specifically 1 to 100, and even more specifically 1 to 50, but is not particularly limited thereto.

As used herein, the term “homologous recombination” refers to genetic recombination that occurs via crossover at genetic chain loci having a mutual homology.

Furthermore, still another specific embodiment of the present disclosure may relate to the microorganism producing *O*-acetylhomoserine in which, additionally, the activity of homoserine acetyltransferase is enhanced compared to that of an unmodified microorganism. Specifically, the microorganism may be one in which the activity of homoserine acetyltransferase is enhanced compared to that of an unmodified microorganism, and particularly, may be one in which a modified *metA* gene encoding homoserine acetyltransferase with enhanced activity is introduced. The modified *metA* gene may be a gene encoding one in which the 111th amino acid of homoserine acetyltransferase is substituted with glutamic acid and the 112th amino acid of homoserine acetyltransferase is substituted with histidine, but is not limited thereto. The modified *metA* gene may include without limitation any amino acid sequence having enhanced activity of homoserine acetyltransferase compared to that of its wild-type, and for example, may be a protein having the amino acid sequence of SEQ ID NO: 10. Embodiments of the preparation of the modified *metA* gene and use thereof, a strain having enhanced activity of homoserine acetyltransferase, *etc.* are disclosed in Korean Patent No. 10-1335841, and the entire specification of the patent may be incorporated herein as a reference for the present disclosure.

Additionally, still another specific embodiment of the present disclosure may relate to a microorganism producing *O*-acetylhomoserine belonging to the genus *Escherichia* in which, additionally, the activity of aspartate semialdehyde dehydrogenase, pyridine nucleotide transhydrogenase, or a combination thereof is enhanced compared to that of an unmodified microorganism.

Additionally, still another specific embodiment of the present disclosure may relate to a microorganism producing *O*-acetylhomoserine in which, additionally, the activity of

phosphoenolpyruvate carboxylase, aspartate aminotransferase, or a combination thereof is enhanced compared to that of an unmodified microorganism. As used herein, the term “enhancement” refers to enhancing the activity level of a protein possessed by a microorganism. Enhancement of the activity of a protein is not limited as long as it can enhance the activity of each protein compared to that of the native wild-type protein or unmodified protein, as in the enhancement of the activity of a target protein. The enhancement may be performed by a method selected from the group consisting of i) a method of increasing the copy number of a polynucleotide encoding each protein, ii) a method of introducing a modification in the expression control sequence for increasing the expression of the polynucleotide, iii) a method of modifying the polynucleotide sequence on the chromosome for enhancing the activity of each protein, and iv) a combination thereof. Specifically, the enhancement may be performed by a method selected from the group consisting of a method of inserting a polynucleotide including a nucleotide sequence encoding each protein into the chromosome, a method of introducing the polynucleotide into a microorganism after introducing it into a vector system, a method of introducing a promoter with enhanced activity into an upstream region of the nucleotide sequence encoding each protein or introducing each protein with a modification on its promoter, a method of modifying the nucleotide sequence in the 5'-UTR region, and a method of introducing a modified nucleotide sequence of the nucleotide sequence encoding each protein, but the methods of enhancement are not limited thereto.

Still another aspect of the present disclosure relates to a method for producing *O*-acetylhomoserine including culturing the microorganism of the genus *Escherichia* producing *O*-acetylhomoserine in a medium.

Specifically, the above method relates to a method for producing *O*-acetylhomoserine including culturing the microorganism of the genus *Escherichia* producing *O*-acetylhomoserine in a medium, and recovering *O*-acetylhomoserine from the cultured microorganism or the cultured medium.

As used herein, the term “culture” refers to growing a microorganism in an appropriately-adjusted environment. In the present disclosure, the culture process may be performed using an appropriate medium and culture conditions well known in the art. The culture process may be easily adjusted for use by one of ordinary skill in the art according to the strain being selected. The culture may be performed in a batch process, continuous culture,

fetch-batch culture, *etc.* known in the art, but is not particularly limited thereto. The medium and other culture conditions used for culturing the microorganism of the present disclosure may not be particularly limited, but any medium conventionally used for culturing microorganisms of the genus *Escherichia* may be used. Specifically, the microorganism of the present disclosure may be cultured under an aerobic condition in a common medium containing an appropriate carbon, nitrogen, and phosphorus sources, inorganic compounds, amino acids, and/or vitamins, *etc.*, while adjusting temperature, pH, *etc.*

In the present disclosure, the carbon sources may include carbohydrates such as glucose, fructose, sucrose, maltose, mannitol, sorbitol, *etc.*; alcohols such as sugar alcohol, glycerol, *etc.*; organic acids such as pyruvic acid, lactic acid, citric acid, *etc.*; amino acids such as glutamic acid, methionine, lysine, *etc.*, but the carbon sources are not limited thereto. Additionally, natural organic nutrients such as starch hydrolysate, molasses, blackstrap molasses, rice bran, cassava, sugar cane bagasse, corn steep liquor, *etc.* may be used. Specifically, carbohydrates such as glucose and sterilized pretreated molasses (*i.e.*, molasses converted to reducing sugar) may be used, and additionally, various other carbon sources in an appropriate amount may be used without limitation. These carbon sources may be used alone or in a combination of at least two kinds.

Examples of the nitrogen sources may include inorganic nitrogen sources (*e.g.*, ammonia, ammonium sulfate, ammonium chloride, ammonium acetate, ammonium phosphate, ammonium carbonate, ammonium nitrate, *etc.*); amino acids (glutamic acid, methionine, glutamine, *etc.*); and organic nitrogen sources (*e.g.*, peptone, N-Z amine, meat extract, yeast extract, malt extract, corn steep liquor, casein hydrolysate, fish or decomposition product thereof, defatted soybean cake or decomposition product thereof, *etc.*). These nitrogen sources may be used alone or in a combination of at least two kinds, but are not limited thereto.

Examples of the phosphorus sources may include monopotassium phosphate, dipotassium phosphate, and sodium-containing salts corresponding thereto. Examples of inorganic compounds to be used may include sodium chloride, calcium chloride, iron chloride, magnesium sulfate, iron sulfate, manganese sulfate, calcium carbonate, *etc.* Additionally, amino acids, vitamins, and/or appropriate precursors may be included, but are not limited thereto. These media or precursors may be added in a batch culture process or continuous culture process to a culture, but are not limited thereto.

During the culture period in the present disclosure, the pH of a culture may be adjusted by adding a compound such as ammonium hydroxide, potassium hydroxide, ammonia, phosphoric acid, sulfuric acid, *etc.* to the culture in an appropriate manner. Additionally, during the culture period, an antifoaming agent, such as fatty acid polyglycol ester, may be added to prevent foam generation. Additionally, for maintaining the aerobic state of the culture, oxygen or an oxygen-containing gas may be injected into the culture, while for maintaining the anaerobic and microaerobic states of the culture, nitrogen, hydrogen, or carbon dioxide gas may be injected without the injection of air.

The culture temperature may normally be from 27°C to 37°C, and specifically from 30°C to 35°C, but the culture temperature is not limited thereto. Additionally, the culture may be continued until the production of desired material(s) can be obtained, and specifically for 10 hours to 100 hours, but is not limited thereto.

The recovery of *O*-acetylhomoserine may be performed using the method of culturing a microorganism of the present disclosure. For example, the target *O*-acetylhomoserine can be recovered from a culture using an appropriate method known in the art (*e.g.*, a batch-type culture, continuous culture, or fed-batch culture, *etc.*). For example, methods such as centrifugation, filtration, anion exchange chromatography, crystallization, HPLC, *etc.* may be used, and additionally, a combined method of appropriate methods known in the art may be used.

The recovery process may include a separation process and/or a purification process.

An aspect of the present disclosure relates to a method for producing L-methionine, which includes culturing the microorganism of the genus *Escherichia* producing *O*-acetylhomoserine in a medium; and converting the *O*-acetylhomoserine to L-methionine by treating the cultured microorganism or the cultured medium or the *O*-acetylhomoserine recovered from the cultured microorganism or the cultured medium with methyl mercaptan and a methionine-converting enzyme.

For example, methionine can be produced from *O*-acetylhomoserine, which is recovered from a culture of a microorganism of the genus *Escherichia* producing *O*-acetylhomoserine in a medium, by a two-step process (Korean Patent No. 10-0905381).

The two-step process includes a process of producing L-methionine and an organic acid by an enzyme reaction using an enzyme having the activity of converting *O*-acetylhomoserine to methionine using *O*-acetylhomoserine and methyl mercaptan as substrates or a strain containing

the enzyme.

The methionine-converting enzyme includes all of the enzymes that convert *O*-acetylhomoserine to methionine, and particularly *O*-acetylhomoserine sulfhydrylase, but is not limited thereto.

Specifically, the *O*-acetylhomoserine sulfhydrylase to be used may be one derived from microbial strains belonging to the genus *Leptospira*, the genus *Chromobacterium*, and the genus *Hyphomonas*, and more specifically, one derived from microbial strains belonging to the genus *Leptospira meyeri*, *Pseudomonas aurogenosa*, *Hyphomonas neptunium*, and *Chromobacterium violaceum*.

The above reaction is shown below:



Such additional process of producing methionine is disclosed in Korean Patent No. 10-0905381, and the entire specification of the patent may be included as a reference for the present disclosure.

[DETAILED DESCRIPTION OF THE INVENTION]

Hereinafter, the present disclosure will be described in detail through exemplary embodiments. However, these exemplary embodiments are provided for the purpose of illustration only and are not intended to limit the scope of the present disclosure.

Reference Example 1: Preparation of strains producing *O*-acetylhomoserine

1-1. Deletion of *metB* gene in wild-type *E. coli*

To produce strains producing *O*-acetylhomoserine, *E. coli*, which is a representative microorganism among the microorganisms of the genus *Escherichia*, was used. For this purpose, *E. coli* K12 W3110 (ATCC 27325), a wild-type *E. coli*, was obtained from the American Type Culture Collection (ATCC) and used. A strain which has defects in the *metB* gene (SEQ ID NO: 6) encoding cystathionine gamma synthase and the *thrB* gene (SEQ ID NO: 8) encoding homoserine kinase in *E. coli* K12 W3110 strain was prepared. The thus-prepared

strain producing *O*-acetylhomoserine was named *W3-BT*. An embodiment with regard to the deletion of *metB* and *thrB* genes deletion strains is disclosed in Korean Patent No. 10-0905381 or International Patent Publication WO 2008/013432 (see particularly, Examples 1-1 and 1-2 of Korean Patent No. 10-0905381), and the entire specification of the patent may be included herein as a reference for the present disclosure.

1-2. Preparation of strain introduced with modified *metA* gene having activity of homoserine acetyltransferase

To enhance the activity of homoserine acetyltransferase in the strain obtained in Reference Example 1-1, it was attempted to introduce the modified *metA* gene (SEQ ID NO: 10) encoding homoserine acetyltransferase having enhanced activity into the strain. In an attempt to prepare such a strain, a *pCL_Pcjl_metA* (EH) plasmid was prepared by the method described in Examples 1 and 3 of Korean Patent No. 10-1335841.

Then, to prepare a replacement cassette as a way to substitute the above-prepared modified *metA* gene by introducing it into the strain, PCR was performed using the *pKD3* vector as a template along with primers of SEQ ID NO: 23 and SEQ ID NO: 24. Specifically, PCR was repeatedly performed for a total of 30 cycles, in which denaturation was performed at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 2 minutes.

For the *metA* (EH) portion of the replacement cassette, PCR was performed using *pCL-Pcjl-metA* (EH) as the template along with primers of SEQ ID NO: 19 and SEQ ID NO: 20, whereas, for the *metA* wild-type portion, primers of SEQ ID NO: 21 and SEQ ID NO: 22 were used, and thereby the respective PCR products were obtained. Based on 3 PCR products, the *metA* (EH) replacement cassette containing a chloramphenicol marker was prepared using the primers of SEQ ID NO: 19 and SEQ ID NO: 22, and introduced by electroporation into the *W3-BT* strain, which was transformed with the *pKD46* vector, prepared in Reference Example 1-1.

The strains which were confirmed to have been introduced by the above process were again transformed with the *pCP20* vector and cultured in LB medium. The strain in which the chloramphenicol marker was removed and the *metA* gene was replaced with *metA* (EH) was named as *W3-BTA*.

An embodiment with regard to the strain with enhanced activity of homoserine acetyltransferase, *etc.* is disclosed in Korean Patent No. 10-1335841 or International Patent

Publication WO 2012/087039, and the entire specification of the patent may be included herein as a reference for the present disclosure.

1-3. Preparation of strain including 2 copies of *ppc*, *aspC*, and *asd* genes

To increase the productivity of *O*-acetylhomoserine of the *W3-BTA* strain prepared in Reference Example 1-2, a known strategy of enhancing the biosynthetic pathway was introduced. An attempt was made to prepare strains in which the genes, which are associated with phosphoenolpyruvate carboxylase involved in the biosynthesis of oxaloacetate from phosphoenolpyruvate, aspartate aminotransferase involved in the biosynthesis of aspartate from oxaloacetate, and aspartate-semialdehyde dehydrogenase involved in the biosynthesis of homoserine from β -aspartyl phosphate were amplified to 2 copies, that is, *ppc*, *aspC*, and *asd* genes, were amplified to 2 copies.

For the preparation of the strains, *pSG-2ppc*, *pSG-2aspC*, and *pSG-2asd* plasmids were prepared by the method disclosed in Examples 1-1 to 1-3 of Korean Patent No. 10-1117012, the above plasmids were introduced into the *W3-BTA* strain, and the strain in which the 3 different genes were sequentially amplified to 2 copies was prepared by the method described in Example 1-5 of the Korean patent. The thus-prepared strain was named as *W3-BTA2PCD* (= *WCJM*).

An embodiment with regard to the strain with enhanced activity of phosphoenolpyruvate carboxylase, aspartate aminotransferase, and aspartate-semialdehyde dehydrogenase, *etc.*, is disclosed in Korean Patent No. 10-0905381 or International Patent Publication WO 2008/013432, and the entire specification of the patent may be included herein as a reference for the present disclosure.

1-4. Flask culture experiment

To test the amount of *O*-acetylhomoserine production in the strains prepared in Reference Examples 1-2 and 1-3, Erlenmeyer flask culture was performed. *W3110*, *W3-BTA*, and *WCJM* strains were seeded in LB medium and cultured at 33°C overnight. Single colonies were seeded in 3 mL of LB medium and incubated at 33°C for 5 hours, diluted 200-fold in a 250 mL Erlenmeyer flask containing 25 mL of medium for producing *O*-acetylhomoserine, and incubated again at 33°C at 200 rpm for 30 hours, and the amount of *O*-acetylhomoserine production was confirmed by HPLC analysis. The composition of the medium used is summarized in Table 1 below.

[Table 1] Composition of flask medium producing *O*-acetylhomoserine

Composition	Concentration (per Liter)
Glucose	40 g
Ammonium Sulfate	17 g
KH ₂ PO ₄	1.0 g
MgSO ₄ ·7H ₂ O	0.5 g
FeSO ₄ ·7H ₂ O	5 mg
MnSO ₄ ·8H ₂ O	5 mg
ZnSO ₄	5 mg
Calcium Carbonate	30 g
Yeast Extract	2 g
Methionine	0.15 g
Threonine	0.15 g

The amount of *O*-acetylhomoserine production was confirmed by HPLC analysis after culturing for 30 hours using the above medium, and the results are summarized in Table 2 below.

[Table 2] *O*-Acetylhomoserine production by flask culture

	OD (562 nm)	Glucose Consumption (g/L)	O-AH (g/L)
<i>W3110</i>	14.2	40	0
<i>W3-BTA</i>	8.4	36	0.9
<i>WCJM</i>	9.6	35	1.2

As can be seen in Table 2 above, *O*-acetylhomoserine was not produced at all in the wild-type strain *W3110*, however, the *W3-BTA* strain produced *O*-acetylhomoserine (O-AH) at a concentration of 0.9 g/L in, and the *WCJM* strain with an enhanced biosynthetic pathway produced *O*-acetylhomoserine (O-AH) at a concentration of 1.2 g/L.

Example 1: Selection of membrane proteins increasing *O*-acetylhomoserine productivity

The inventors of the present disclosure made an attempt to apply LeuE (SEQ ID NO: 1) derived from *Escherichia coli*, which was disclosed as a membrane protein but has not been disclosed with regard to the activity of exporting *O*-acetylhomoserine and producing

O-acetylhomoserine, to *O*-acetylhomoserine production.

In order to enhance the *leuE* gene in the strain, the *leuE* gene was cloned using a *SmaI* restriction site of the *pCL* vector.

First, to prepare the *leuE* gene, PCR was performed for a total of 30 cycles using primers of SEQ ID NOS: 11 and 12, in which denaturation was performed at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 1 minute. The resulting PCR product was electrophoresed on a 1.0% agarose gel and DNA was purified from the 800 bp band. The purified DNA was treated with restriction enzyme *SmaI* at 37°C overnight, and after additional purification, *leuE* gene and the *pCL* vector were cloned using T4 ligase. After transforming *E. coli DH5* using the cloned plasmid, the transformed *E. coli DH5* were selected on LB plate medium containing spectinomycin (50 µg/mL) to obtain the plasmid. The thus-prepared plasmid was introduced into *W3-BTA* and *WCJM* strains, which are strains producing *O*-acetylhomoserine. They were named as *W3-BTA/pCL-leuE* and *WCJM/pCL-leuE*, respectively, and flask evaluation on their productivity of *O*-acetylhomoserine was performed.

Additionally, as the control groups, the empty vector *pCL1920* was introduced into *W3-BTA* and *WCJM* strains in the same method as described above, and named as *W3-BTA/pCL1920* and *WCJM pCL1920*, respectively, and flask evaluation on their productivity of *O*-acetylhomoserine was performed.

Specifically, each strain was plated on LB solid medium and cultured overnight in a 33°C incubator. A single colony of the strain cultured overnight in LB plate medium was seeded in 3 mL of LB medium and incubated at 33°C for 5 hours, diluted 200-fold in a 250 mL Erlenmeyer flask containing 25 mL of medium for producing *O*-acetylhomoserine, and incubated again at 33°C at 200 rpm for 30 hours, and the amount of *O*-acetylhomoserine production was confirmed by HPLC analysis. The results are summarized in Table 3 below.

[Table 3] Measurement of *O*-acetylhomoserine production by flask culture

	OD (562 nm)	Glucose Consumption (g/L)	O-AH (g/L)
<i>W3-BTA/pCL1920</i>	9.5	35	0.9
<i>W3-BTA/pCL-leuE</i>	8.2	36	1.0
<i>WCJM/pCL1920</i>	9.6	35	1.2

<i>WCJM/pCL-leuE</i>	8.4	36	1.5
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As can be seen in Table 3 above, the *WCJM* strain introduced with *leuE* plasmid showed a lower OD compared to that of the control strain introduced with the empty vector, and the *WCJM* strain also showed higher glucose consumption. However, the *WCJM* strain produced *O*-acetylhomoserine at a concentration of 1.5 g/L, and this could not confirm that the increase of *O*-acetylhomoserine production was due to the introduction of the wild-type *leuE*. Nevertheless, the results of being capable of controlling OD and increase of glucose consumption rate confirmed the potential exporting activity of the strain. Accordingly, an attempt was made to select modified strains having enhanced activity of exporting *O*-acetylhomoserine compared to that of the wild-type strain through structural modeling.

Example 2: Preparation of plasmid with modification of start codon of *leuE* and evaluation of *O*-acetyl homoserine productivity

The start codon of wild-type *leuE* is known to be gtg, which encodes valine, an amino acid. To confirm the enhanced effect of *leuE* protein by changing the start codon to atg (*i.e.*, a methionine-encoding codon), an experiment to change the start codon based on the plasmid prepared in Example 1 was performed. Specifically, the first amino acid in the amino acid sequence of SEQ ID NO: 1 was substituted with methionine to enhance the activity of exporting *O*-acetylhomoserine. More specifically, a *leuE(ATG)* modification was prepared. To prepare the *leuE(ATG)* modification, primers of SEQ ID NO: 145 and SEQ ID NO: 146 were used, and a modified *leuE(ATG)* gene was prepared by site-specific mutagenesis (site-directed mutagenesis kit, Stratagene, USA). The existing wild-type plasmid was named as WT, and the initiation codon variant plasmid was named *WT_ATG*, and the thus-prepared plasmid was introduced to the *WCJM* strain and the flask evaluation on its productivity of *O*-acetylhomoserine was performed.

Specifically, each strain was plated on LB solid medium and cultured overnight in a 33°C incubator. The strain cultured overnight in LB plate medium was seeded in 25 mL titer medium and incubated at 33°C at 200 rpm for 40 hours. The results are summarized in Table 4 below.

[Table 4] Measurement of *O*-acetylhomoserine production by flask culture

	OD (562 nm)	Glucose	O-AH (g/L)
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		Consumption (g/L)	
<i>WCJM/pCL-leuE WT</i>	8.4	36	1.5
<i>WCJM/pCL-leuE WT(ATG)</i>	7.6	39	2.6

As can be seen in Table 4 above, the strain introduced with the *pCL-leuE WT(ATG)* plasmid having the start-codon modification showed a lower OD compared to that of the wild-type strain but showed more rapid glucose consumption. The strain introduced with the *pCL-leuE WT(ATG)* plasmid having the start-codon modification produced *O*-acetylhomoserine at a concentration of 2.6 g/L, which is an increase of productivity as much as 173% compared to that of the wild-type strain.

Example 3: Preparation of *leuE*-modified plasmid and evaluation of productivity of *O*-acetylhomoserine

3-1. Preparation of *leuE*-modified plasmid

Experiments to prepare each of the three modified polypeptides which were expected to have a stronger exporting activity compared to that of the wild-type *leuE* based on the two kinds of plasmids, *i.e.*, *plasmidpCL-leuE WT* and *pCL-leuE WT(ATG)* prepared in Examples 1 and 2, were performed. Specifically, the positions of *leuE* modification were selected via structure modeling to enhance the activity of exporting *O*-acetylhomoserine, and the amino acids at positions 30, 95, and 165 in the amino acid sequences of SEQ ID NOS: 1 and 2 were substituted with different amino acids, respectively.

More specifically, L95V, F30A, and F165A modifications were prepared. For the preparation of L95V modification, primers of SEQ ID NOS: 13 and 14 were used; for F30A modification, primers of SEQ ID NOS: 25 and 26 were used; and for F165A modification, primers of SEQ ID NOS: 27 and 28 were used. Modified *leuE* genes were prepared using site-directed mutagenesis kit (Stratagene, USA) along with each of the primer sets described above. Based on the existing wild-type plasmid *WT*, the modified plasmid *L95V* was named as *WT_M3*; the modified plasmid *F30A* as *WT_M4*, and the modified plasmid *F165A* as *WT_M6*, respectively. Additionally, based on the plasmid with a start codon modification (*i.e.*, *WT(ATG)*), the modified plasmid *L95V* was named as *WT(ATG)_M3*, the modified plasmid *F30A* as *WT(ATG)_M4*, and the modified plasmid *F165A* as *WT(ATG)_M6*, respectively. The

thus-prepared modified plasmids were introduced into the *WCJM* strain to evaluate the productivity of *O*-acetylhomoserine in a flask.

Specifically, each strain was plated on LB plate medium and cultured in a 33°C incubator overnight. The strain cultured overnight in LB solid medium was inoculated into a 25 mL of the titer medium, and then cultured in an incubator at 33°C incubator at 200 rpm for 40 hours. The results are shown in Table 5 below.

[Table 5] Measurement of *O*-acetylhomoserine production by flask culture

	OD (562 nm)	Glucose Consumption (g/L)	O-AH (g/L)
<i>WCJM/pCL1920</i>	9.6	35	1.3
<i>WCJM/pCL-leuE WT</i>	8.4	36	1.5
<i>WCJM/pCL-leuE WT_M3</i>	8.2	38	2.3
<i>WCJM/pCL-leuE WT_M4</i>	7.9	38	3.7
<i>WCJM/pCL-leuE WT_M6</i>	8.0	39	4.8
<i>WCJM/pCL-leuE WT(ATG)</i>	7.6	39	2.6
<i>WCJM/pCL-leuE WT(ATG)_M3</i>	7.5	40	3.1
<i>WCJM/pCL-leuE WT(ATG)_M4</i>	7.3	39	3.6
<i>WCJM/pCL-leuE WT(ATG)_M6</i>	7.5	40	4.9

As can be seen in Table 5 above, all of the 3 strains introduced with the *leuE*-modified plasmid showed a decrease in OD compared to that of the wild-type, but all of the 3 strains showed more rapid glucose consumption compared to that of the wild-type strain, and in particular, the *WT(ATG)_M6* strain was shown to produce *O*-acetylhomoserine at a concentration of 4.9 g/L, thus showing the highest productivity of *O*-acetylhomoserine. Accordingly, it was confirmed that all of the 3 modified strains of the present disclosure exhibited enhanced

productivity of *O*-acetylhomoserine. Additionally, it was confirmed that when the amount of protein expression was increased by modifying the start codon of *leuE*, the productivity of *O*-acetylhomoserine was further enhanced.

3-2. Preparation of biosynthesis pathway genes and modified plasmids

To maximize the productivity of *O*-acetylhomoserine, a plasmid capable of enhancing the biosynthetic pathway to homoserine was prepared. For the cloning of aspartate semialdehyde dehydrogenase, pyridine nucleotide transhydrogenase, and wild-type *LeuE* and modified *LeuE* into the *pCL* vector, *asd* and *pntAB* genes were first introduced into the *pCL* vector.

First, in obtaining the *asd* and *pntAB* genes, the PCR was performed for a total of 30 cycles, in which denaturation was performed at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 3 minutes, using primers of SEQ ID NOS: 15 and 16 for *asd* gene and primers of SEQ ID NOS: 17 and 18 for *pntAB* gene. The resulting PCR products were electrophoresed on a 1.0% agarose gel and the DNAs respectively obtained from 1.4 kb (*asd*) and 3 kb (*pntAB*) sized bands were purified.

The purified two genes were ligated using the sewing PCR (a technique in which the overlapping parts of two genes are ligated first without using any primer and then amplified using the primers at both ends). The conditions for the sewing PCR were performing the PCR described above for 10 cycles and then performing PCR for 20 cycles after adding primers of SEQ ID NOS: 15 and 18. As a result, combined fragments of *asd-pntAB* genes were prepared, and purified by electrophoresis. The purified fragments and the *pCL* vector were treated with *SmaI* at 37°C overnight, purified further, and the *pCL-asd-pntAB* plasmid was prepared using T4 ligase.

The *leuE* gene was cloned into the thus-prepared plasmid. In cloning, specifically, to obtain the *leuE* gene, PCR was performed for a total of 30 cycles, in which denaturation was performed at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 1 minute, using primers of SEQ ID NOS: 29 and 30.

The resulting PCR product was electrophoresed on a 1.0% agarose gel and the DNA obtained from 800 bp was purified. The purified DNA and the *pCL* vector were treated with *KpnI* at 37°C overnight, purified further, and the *leuE* gene and *pCL-asd-pntAB* vector were cloned. The cloned plasmids were transformed into *E. coli DH5α*, and the transformed *E. coli*

DH5α was selected in LB plate medium containing spectinomycin (50 µg/mL) and the plasmids were obtained therefrom. The thus-prepared plasmids were introduced into the *WCJM* strain, which is a strain producing *O*-acetylhomoserine, and a flask evaluation was performed with regard to its productivity of *O*-acetylhomoserine. The thus-prepared plasmids were a total of 4 kinds and the wild-type and 3 modified strains prepared in Example 2-1 were used. The 4 kinds of plasmids were introduced into the *WCJM* strain by electroporation and a flask evaluation was performed in the same manner as in Example 3-1. The results are shown in Table 6-1 below.

[Table 6] Measurement of *O*-acetylhomoserine production by flask culture

	OD (562 nm)	Glucose Consumption (g/L)	O-AH (g/L)
<i>WCJM/pCL-asd-pntAB</i>	9.8	36	1.8
<i>WCJM/pCL-asd-pntAB-leuE WT</i>	9.5	37	2.0
<i>WCJM/pCL-asd-pntAB-leuE WT_M3</i>	8.2	38	3.0
<i>WCJM/pCL-asd-pntAB-leuE WT_M4</i>	7.5	38	4.2
<i>WCJM/pCL-asd-pntAB-leuE WT_M6</i>	7.8	38	5.9

As can be seen in Table 6 above, as a result of simultaneously enhancing the biosynthesis pathway and the *leuE* modification, the productivity of *O*-acetylhomoserine was further improved. In particular, in the case of the strain in which the *pCL-asd-pntAB-leuE WT_M6* plasmid was introduced, the OD was decreased compared to that of the wild-type strain, but the strain showed more rapid glucose consumption and produced *O*-acetylhomoserine at a concentration of 5.9 g/L, the highest among the strains.

Example 4: Preparation of *leuE* modification by saturated mutagenesis and evaluation of productivity of *O*-acetylhomoserine

4-1. Preparation of strains with *leuE* modification by saturated mutagenesis and evaluation thereof

Modifications were prepared by saturated mutagenesis to produce different types of amino acid substitutions of the 3 *leuE* variants, which had shown high productivity of *O*-acetylhomoserine. The substituted amino acids were prepared using 17 kinds of *M3* mutation, *M4* mutation, and *M6* mutation, respectively, using the plasmids prepared in Example 2 as the templates. The details are shown in Table 7 below.

[Table 7]

Modified Plasmid	Amino Acid Substituted	SEQ ID NO of Primers
<i>M3</i>	L95F	SEQ ID NOS: 31, 32
	L95A	SEQ ID NOS: 33, 34
	L95G	SEQ ID NOS: 35, 36
	L95T	SEQ ID NOS: 37, 38
	L95N	SEQ ID NOS: 39, 40
	L95D	SEQ ID NOS: 41, 42
	L95H	SEQ ID NOS: 43, 44
	L95I	SEQ ID NOS: 45, 46
	L95S	SEQ ID NOS: 47, 48
	L95P	SEQ ID NOS: 49, 50
	L95Y	SEQ ID NOS: 51, 52
	L95Q	SEQ ID NOS: 53, 54
	L95K	SEQ ID NOS: 55, 56
	L95E	SEQ ID NOS: 57, 58
	L95C	SEQ ID NOS: 59, 60
	L95W	SEQ ID NOS: 61, 62
	L95R	SEQ ID NOS: 63, 64
<i>M4</i>	F30W	SEQ ID NOS: 65, 66
	F30L	SEQ ID NOS: 67, 68
	F30V	SEQ ID NOS: 69, 70
	F30G	SEQ ID NOS: 71, 72
	F30S	SEQ ID NOS: 73, 74
	F30N	SEQ ID NOS: 75, 76

	F30D	SEQ ID NOS: 77, 78
	F30H	SEQ ID NOS: 79, 80
	F30I	SEQ ID NOS: 81, 82
	F30P	SEQ ID NOS: 83, 84
	F30Y	SEQ ID NOS: 85, 86
	F30Q	SEQ ID NOS: 87, 88
	F30K	SEQ ID NOS: 89, 90
	F30E	SEQ ID NOS: 91, 92
	F30C	SEQ ID NOS: 93, 94
	F30T	SEQ ID NOS: 95, 96
	F30R	SEQ ID NOS: 97, 98
<i>M6</i>	F165W	SEQ ID NOS: 99, 100
	F165L	SEQ ID NOS: 101, 102
	F165V	SEQ ID NOS: 103, 104
	F165G	SEQ ID NOS: 105, 106
	F165S	SEQ ID NOS: 107, 108
	F165N	SEQ ID NOS: 109, 110
	F165D	SEQ ID NOS: 111, 112
	F165H	SEQ ID NOS: 113, 114
	F165I	SEQ ID NOS: 115, 116
	F165P	SEQ ID NOS: 117, 118
	F165Y	SEQ ID NOS: 119, 120
	F165Q	SEQ ID NOS: 121, 122
	F165K	SEQ ID NOS: 123, 124
	F165E	SEQ ID NOS: 125, 126
	F165C	SEQ ID NOS: 127, 128
	F165T	SEQ ID NOS: 129, 130
	F165R	SEQ ID NOS: 131, 132

Specifically, *leuE*-modified genes were prepared by performing a site-directed mutagenesis kit (Stratagene, USA) using the primers shown in Table 7 above. The plasmid was introduced into the *WCJM* strain and the flask was evaluated in the same manner as Example 3-1. The results are shown in Table 8 below.

[Table 8] Measurement of *O*-acetylhomoserine production by flask culture

Strain	Plasmid	Location of Modification	OD (562 nm)	Glucose Consumption (g/L)	O-AH (g/L)
<i>WCJM</i>	<i>pCL1920</i>		9.6	35	1.3
	<i>pCL-leuE WT</i>		8.4	36	1.5
	<i>pCL-leuE WT_M3</i>	L95V	8.2	38	2.3
	<i>pCL-leuE WT_M4</i>	F30A	7.9	38	3.7
	<i>pCL-leuE WT_M6</i>	F165A	8.0	39	4.8
	<i>M3 Modification</i>	L95F	8.6	38	2.3
		L95A	8.3	38	2.2
		L95G	9.2	37	2.1
		L95T	9.4	37.5	2.3
		L95N	8.8	38	2.4
		L95D	8.7	36	2.2
		L95H	9.5	35	2.3
		L95I	9.5	37.5	2.2
		L95S	9.3	37	2.5
		L95P	9.2	36	2.5
		L95Y	8.9	35	2.2
		L95Q	9.4	38	3.1
		L95K	9.2	38.5	2.2
		L95E	8.6	37	2.6
		L95C	8.9	37.5	2.4
		L95W	9.9	38	2.1
		L95R	9.3	38	2.3
	<i>M4 Modification</i>	F30W	7.5	38	3.2
		F30L	7.2	36	3.1
		F30V	7.3	35	2.6
		F30G	8.3	36	3.4

		F30S	7.9	35	3.6
		F30N	8.2	37	3.5
		F30D	8.6	38	3.0
		F30H	8.8	34	2.9
		F30I	8.3	35	3.5
		F30P	8.6	35.5	3.1
		F30Y	7.9	34	2.9
		F30Q	8.6	34	2.8
		F30K	8.8	35	3.1
		F30E	7.6	35.5	2.5
		F30C	7.9	35	2.4
		F30T	8.9	36	3.0
		F30R	8.6	38.5	2.9
	M6 Modification	F165W	8.2	39	4.2
		F165L	8.3	38	4.5
		F165V	8.4	38	4.1
		F165G	8.0	39	4.6
		F165S	7.9	37	4.7
		F165N	8.8	39	4.7
		F165D	7.8	38	4.5
		F165H	7.9	38	4.5
		F165I	7.8	37	4.1
		F165P	7.7	37.5	4.2
		F165Y	8.2	38	4.6
		F165Q	8.4	38	3.9
		F165K	7.6	39	4.0
		F165E	7.7	36.5	4.2
		F165C	7.6	36.5	4.3
		F165T	8.5	34	3.7
		F165R	8.3	38	3.9

As can be seen in Table 8 above, as a result of evaluating each of the modified strains,

there was a slight difference in OD and glucose consumption rate. However, all of the above modified strains were found to have an enhanced amount of *O*-acetylhomoserine production compared to the *WCJM/pCL1920* and *WCJM/pCL-leuE WT* strains used as the control group.

4-2. Preparation of strain with enhanced *leuE*-modification in strain with high-yield of *O*-acetylhomoserine and evaluation of its productivity of *O*-acetylhomoserine

A method for producing a strain capable of producing *O*-acetylhomoserine by using a strain capable of producing threonine via NTG mutation derived from wild-type *W3110* is disclosed (International Patent Publication No. WO 2012/087039). In particular, the thus-prepared strain producing *O*-acetylhomoserine with high yield was deposited with the Korean Microorganism Conservation Center under the Accession No. *KCCM11146P*.

An attempt was made whether the productivity of *O*-acetylhomoserine can be further enhanced by introducing the *leuE* gene and modified strains thereof based on the above strain.

Specifically, the *leuE* gene and 3 modified strains thereof were introduced by electroporation. The strains introduced were named as *KCCM11146P/pCL1920*, *KCCM11146P/pCL-leuE WT*, *KCCM11146P/pCL-leuE M3*, *KCCM11146P/pCL-leuE M4*, and *KCCM11146P/pCL-leuE M6*, respectively. To measure the productivity of *O*-acetylhomoserine of the *leuE* gene and 3 modified strains thereof, flask culture evaluation was performed. Specifically, LB medium was inoculated with 4 kinds of the above strains and incubated overnight at 33°C. Then, single colonies were inoculated into 3 mL of LB medium and cultured again at 33°C for 5 hours, diluted 200-fold in a 250 mL Erlenmeyer flask containing 25 mL of medium for producing *O*-acetylhomoserine, and incubated again at 33°C at 200 rpm for 30 hours, and the amount of *O*-acetylhomoserine production was confirmed by HPLC analysis. The results of the experiment are summarized in Table 9 below.

[Table 9] Measurement of *O*-acetylhomoserine production by flask culture

	OD (562 nm)	Glucose Consumption (g/L)	O-AH (g/L)
<i>KCCM11146P/pCL1920</i>	18.3	40	14.2
<i>KCCM11146P/pCL-leuE WT</i>	17.9	40	16.3
<i>KCCM11146P/pCL-leuE</i>	17.5	40	16.9

<i>M3</i>			
<i>KCCM11146P/pCL-leuE</i> <i>M4</i>	16.8	40	19.2
<i>KCCM11146P/pCL-leuE</i> <i>M6</i>	17.2	40	18.8

As can be seen in Table 9 above, it was confirmed that the strain which was prepared by introducing only the *pCL1920* into the *KCCM11146P* strain produced 14.2 g/L of *O*-acetylhomoserine, and the *leuE WT* strain also showed an increase in the amount of *O*-acetylhomoserine production compared to the original strain. Additionally, all of the 3 modified strains showed a decrease of OD, whereas the *M4* strain showed the highest yield of *O*-acetylhomoserine production (19.2 g/L). The *M4* and *M6* strains showed an increase in the amount of the *O*-acetylhomoserine production.

The inventors of the present disclosure confirmed that the *O*-acetylhomoserine production was increased in “*KCCM11146P/pCL-leuE M3, M4, and M6* strains”, which are 3 *leuE*-modified strains of *M3, M4, and M6* based on the *KCCM11146P* strain. As a result, they named the strains as “*CA05-4009*”, “*CA05-4010*”, and “*CA05-4011*”, and were deposited with the KCCM on December 15, 2014, under the Accession Nos. *KCCM11645P, KCCM11646P, and KCCM11647P*, respectively.

Example 5: Production of L-methionine using *O*-acetylhomoserine culture solution produced and transferase

An experiment for producing L-methionine by using the culture solution of *O*-acetylhomoserine obtained in Example 4 and *O*-acetylhomoserine sulfhydrylase, which is an enzyme converting *O*-acetylhomoserine to methionine, was performed.

O-Acetylhomoserine sulfhydrylase, a converting enzyme, was prepared by the method provided in Example 1-2 of Korean Patent No. 10-1250651, and the amount of L-methionine produced by a conversion reaction using the method provided in Example 3 of Korean Patent No. 10-1250651 was measured. For the *O*-acetylhomoserine used as the substrate, the culture solution of *KCCM11146P-pCL-leuE M4* (*O*-AH concentration; 19.2 g/L) obtained in Example 4 in the present disclosure was used, and the concentration of the L-methionine produced therefrom is shown in Table 10 below.

[Table 10]

		Time (min)				
		2	4	6	8	10
MetZ-rsp	Methionine (g/L)	3.21	4.34	4.52	4.78	5.04
	Conversion (%)	50%	68%	71%	75%	79%

As can be seen in Table 10 above, it was confirmed that the *O*-acetylhomoserine contained in the culture solution of the *KCCM11146P-pCL-leuE M4* strain obtained in Example 4 was converted to methionine at a conversion rate of 79% for 10 minutes. From this result, it was confirmed that methionine can be successfully produced using the strain of the present disclosure.

From the foregoing, a skilled person in the art to which the present disclosure pertains will be able to understand that the present disclosure may be embodied in other specific forms without modifying the technical concepts or essential characteristics of the present disclosure. In this regard, the exemplary embodiments disclosed herein are only for illustrative purposes and should not be construed as limiting the scope of the present disclosure. On the contrary, the present disclosure is intended to cover not only the exemplary embodiments but also various alternatives, modifications, equivalents, and other embodiments that may be included within the spirit and scope of the present disclosure as defined by the appended claims.



BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To: CJ CheilJedang Corporation
CJ CHEILJEDANG CENTER,
330, DONGHO-RO,
JUNG-GU, SEOUL 100-400,
REPUBLIC OF KOREA

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : <i>Escherichia coli</i> CA05-4009	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM11645P
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: ☐ a scientific description ☐ a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on December. 15. 2014. (date of the original deposit) ¹	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : Yurim B/D 45, Hongjense-2ga-gil Seodaemun-gu SEOUL 120-861 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: December. 15. 2014.

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired; where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

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KCCM 002-0001

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II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
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KOREAN CULTURE CENTER OF MICROORGANISMS

Korea's repository of culture microbe

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[CLAIMS]

[Claim 1]

A polypeptide having the activity of exporting *O*-acetylhomoserine, wherein at least one amino acid selected from the group consisting of valine at position 1, phenylalanine at position 30, leucine at position 95, and phenylalanine at position 165 in the amino acid sequence of SEQ ID NO: 1 is substituted with another amino acid.

[Claim 2]

The polypeptide according to claim 1, wherein valine at position 1 in the amino acid sequence of SEQ ID NO: 1 is substituted with methionine; phenylalanine at position 30 in the amino acid sequence of SEQ ID NO: 1 is substituted with any one selected from the group consisting of alanine, tryptophan, leucine, valine, glycine, serine, asparagine, aspartic acid, and histidine; leucine at position 95 in the amino acid sequence of SEQ ID NO: 1 is substituted with any one selected from the group consisting of valine, phenylalanine, alanine, glycine, threonine, asparagine, aspartic acid, and histidine; or phenylalanine at position 165 in the amino acid sequence of SEQ ID NO: 1 is substituted with any one selected from the group consisting of alanine, tryptophan, leucine, valine, glycine, serine, asparagine, aspartic acid, and histidine.

[Claim 3]

The polypeptide according to claim 1, wherein valine at position 1 in the amino acid sequence of SEQ ID NO: 1 is substituted with methionine; and phenylalanine at position 30, leucine at position 95, or phenylalanine at position 165 in the amino acid sequence of SEQ ID NO: 1 is substituted with another amino acid.

[Claim 4]

The polypeptide according to claim 1, wherein the polypeptide is selected from the group consisting of amino acid sequences of SEQ ID NOS: 2, 133, 134, 137, 138, 141, and 142.

[Claim 5]

A polynucleotide encoding the polypeptide having the activity of exporting *O*-acetylhomoserine of claim 1.

[Claim 6]

The polynucleotide according to claim 5, wherein the start codon of the polynucleotide is additionally substituted with ATG.

[Claim 7]

The polynucleotide according to claim 5, wherein the polynucleotide is selected from the group consisting of nucleic acid sequences of SEQ ID NOS: 4, 135, 136, 139, 140, 143, and 144.

[Claim 8]

A microorganism of the genus *Escherichia* producing *O*-acetylhomoserine, wherein a polypeptide consisting of the amino acid sequence of SEQ ID NO: 1 or a modified polypeptide thereof is comprised or overexpressed.

[Claim 9]

The microorganism according to claim 8, wherein, with regard to the modified polypeptide, at least one amino acid selected from the group consisting of valine at position 1, leucine at position 30, phenylalanine at position 95, and phenylalanine at position 165 in the amino acid sequence of SEQ ID NO: 1 is substituted with another amino acid.

[Claim 10]

The microorganism according to claim 8, wherein, with regard to the modified polypeptide, valine at position 1 in the amino acid sequence of SEQ ID NO: 1 is substituted with methionine; phenylalanine at position 30 in the amino acid sequence of SEQ ID NO: 1 is substituted with any one selected from the group consisting of alanine, tryptophan, leucine, valine, glycine, serine, asparagine, aspartic acid, and histidine; leucine at position 95 in the amino acid sequence of SEQ ID NO: 1 is substituted with any one selected from the group consisting of valine, phenylalanine, alanine, glycine, threonine, asparagine, aspartic acid, and histidine; or phenylalanine at position 165 in the amino acid sequence of SEQ ID NO: 1 is substituted with any one selected from the group consisting of alanine, tryptophan, leucine, valine, glycine, serine, asparagine, aspartic acid, and histidine.

[Claim 11]

The polypeptide according to claim 9, wherein, with regard to the modified polypeptide, valine at position 1 in the amino acid sequence of SEQ ID NO: 1 is substituted with methionine; and phenylalanine at position 30, leucine at position 95, or phenylalanine at position 165 in the amino acid sequence of SEQ ID NO: 1 is substituted with another amino acid.

[Claim 12]

The microorganism according to claim 8, wherein the microorganism of the genus *Escherichia* is *Escherichia coli*.

[Claim 13]

The microorganism according to claim 8, wherein, additionally, the activity of cystathionine synthase is inactivated.

[Claim 14]

The microorganism according to claim 8, wherein, additionally, the activity of homoserine kinase is inactivated.

[Claim 15]

The microorganism according to claim 8, wherein, additionally, the activity of homoserine acetyltransferase is enhanced compared to that of an unmodified microorganism.

[Claim 16]

The microorganism according to claim 8, wherein, additionally, the activities of aspartate semialdehyde dehydrogenase, pyridine nucleotide transhydrogenase, or a combination thereof is enhanced compared to that of an unmodified microorganism.

[Claim 17]

A method for producing *O*-acetylhomoserine, comprising:
culturing the microorganism of the genus *Escherichia* producing *O*-acetylhomoserine of any one of claims 8 to 16 in a medium; and
recovering *O*-acetylhomoserine from the cultured microorganism or the cultured medium.

[Claim 18]

A method for producing L-methionine, comprising:

culturing the microorganism of the genus *Escherichia* producing *O*-acetylhomoserine of any one of claims 8 to 16 in a medium; and

converting the *O*-acetylhomoserine to L-methionine by treating the cultured microorganism or the cultured medium or the *O*-acetylhomoserine recovered from the cultured microorganism or the cultured medium with methyl mercaptan and a methionine-converting enzyme.

[Claim 19]

The method according to claim 18, wherein the methionine-converting enzyme is *O*-acetylhomoserine sulfhydrylase.