

MICROORGANISM PRODUCING O-ACETYL HOMOSERINE AND THE METHOD OF PRODUCING O-ACETYL HOMOSERINE USING THE SAME

TECHNICAL FIELD

The present invention relates to a microorganism of *Escherichia* sp. producing *O*-acetyl homoserine, and a method of producing *O*-acetyl homoserine in high yield using the microorganism.

BACKGROUND ART

O-Acetyl homoserine acts as a precursor of methionine, which is one of the essential amino acids in the body. Methionine has been widely used as a component of medical infusion solutions and raw materials for medicinal products as well as an animal feed and food additive.

Methionine can be biologically or chemically synthesized. Recently, a two-step process, in which an L-methionine precursor produced by fermentation is converted to L-methionine by an enzyme reaction, was disclosed (International Publication No. WO 2008/013432). In the above two-step process, *O*-succinyl homoserine and *O*-acetyl homoserine may be used as the methionine precursor, and it is important that *O*-acetyl homoserine be produced in high yield for large-scale cost-effective production of methionine.

DISCLOSURE

Technical Problem

The present inventors, while endeavoring to improve the production of *O*-acetyl homoserine, discovered that the reduction of the expression or activity of citrate synthase protein can significantly increase the production capability of *O*-acetyl homoserine, thereby completing the present invention.

Technical Solution

An object of the present invention is to provide an *O*-acetyl homoserine-producing microorganism with improved *O*-acetyl homoserine production capability.

Another object of the present invention is to provide a method for producing *O*-acetyl homoserine using the microorganism.

Advantageous Effects

The use of the microorganism with *O*-acetyl homoserine production capability according

to the present invention can produce *O*-acetyl homoserine in a higher yield and in a more environmentally friendly manner than chemical synthesis. Additionally, the thus produced *O*-acetyl homoserine can be used as a precursor for the synthesis of methionine and acetic acid by *O*-acetyl homoserine sulfhydrylase, thereby enabling bioconversion of L-methionine, and the thus converted L-methionine can be widely used in the production of foods or food additives for humans as well as animal feeds or animal feed additives.

DESCRIPTION OF DRAWINGS

FIG. 1 is an expression cassette design for the construction of a microorganism with an attenuated activity of citrate synthase.

FIG. 2 is a restriction map of *pBAD24*-citrate synthase antisense RNA (asRNA) vector.

BEST MODE

In an aspect, the present invention provides a microorganism of *Escherichia* sp. producing *O*-acetyl homoserine, in which the activity of endogenous citrate synthase protein is attenuated or inactivated.

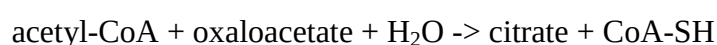
As used herein, the term “*O*-acetyl homoserine”, being a specific intermediate material in a methionine biosynthesis pathway of a microorganism, refers to an acetyl-derivative of L-homoserine. *O*-acetyl homoserine can be produced by an enzyme activity of transferring an acetyl group from acetyl-CoA to homoserine using homoserine and acetyl-CoA as substrates.

As used herein, the term “a microorganism producing *O*-acetyl homoserine” includes a microorganism, which, being a eukaryotic or prokaryotic microorganism producing *O*-acetyl homoserine within a living organism, is provided with *O*-acetyl homoserine-producing capability to its parent microorganism without *O*-acetyl homoserine-producing capability, or a microorganism which is endogenously provided with the *O*-acetyl homoserine-producing capability.

O-Acetyl homoserine-producing capability may be provided or promoted by improvement of species. The microorganisms having the *O*-acetyl homoserine-producing capability may include microorganism belonging to *Escherichia* sp., *Erwinia* sp., *Serratia* sp., *Providencia* sp., *Corynebacteria* sp., *Pseudomonas* sp., *Leptospira* sp., *Salmonella* sp., *Brevibacteria* sp., *Hypomononas* sp., *Chromobacterium* sp., and *Norcardia* sp., or fungi, or yeasts; specifically, microorganism belonging to *Escherichia* sp., *Corynebacteria* sp., *Leptospira* sp., and yeasts; and more specifically, microorganism belonging to *Escherichia* sp., as a specific example, *Escherichia coli*. The microorganisms having the *O*-acetyl homoserine-producing

capability may be microorganisms producing L-lysine, L-threonine, L-isoleucine, or L-methionine, or derivatives thereof, but are not limited thereto.

As used herein, the term “citrate synthase (E.C. 2.3.3.1)” refers to an enzyme in the first step of the TCA cycle that mediates the reaction between oxaloacetate and acetyl-CoA. Specifically, citrate synthase mediates the condensation reaction between an acetate residue having two carbon atoms, which is in acetyl-CoA, and oxaloacetate having four carbon atoms, thereby generating a citrate having six carbon atoms. In *Escherichia coli*, citrate synthase is designated GltA, and citrate synthase and GltA are interchangeably used in the present invention.



Specifically, citrate synthase may be one derived from *Escherichia* sp., and more specifically, GltA derived from *Escherichia coli*. Citrate synthase may be a protein comprising an amino acid sequence represented by SEQ ID NO: 4 or those having a homology of 70% or higher with amino acid sequence of SEQ ID NO: 4, specifically 80% or higher, or more specifically, 90% or higher. Additionally, as a sequence having a homology, if the amino acid sequence is one having the same or corresponding activity of citrate synthase with that of SEQ ID NO: 4, it is obvious that amino acid sequences with a deletion, a modification, a substitution, or an addition, in part of the sequences should also be included in the scope of the present invention. Additionally, based on the genetic code degeneracy, polynucleotide sequences encoding the same amino acid sequence and variants thereof should also be included in the scope of the present invention.

As used herein, the term “endogenous” activity refers to a natural state of a protein in a microorganism or an activity state of the corresponding protein provided in the microorganism before modification.

The “the attenuation or inactivation of a protein activity compared to its endogenous activity” refers to a reduction or elimination of the protein activity when compared with that possessed in its natural state. The attenuation is a concept referring to a case when the activity of a protein is reduced compared with that originally possessed by the microorganism due to a modification in the protein-encoding gene, a case when the level of overall protein expression is lower than that of the natural type strain of the microorganism, or a combination thereof, but is not limited thereto. The inactivation includes a case when the gene encoding the protein is not expressed at all compared to that of the natural type strain, and a case when the gene is expressed but exhibits no activity.

The attenuation or inactivation of a protein activity may be achieved by various methods well known in the art. Examples of the methods may include a method of replacing the gene encoding the protein on the chromosome with a gene modified so that the enzyme activity can be reduced including the case when the protein activity is removed; a method of introducing a modification on the expression-regulating sequence of the gene encoding the protein on the chromosome; a method of replacing the expression-regulating sequence of the gene encoding the protein with a sequence having a weak activity or no activity; a method of deleting a part of or the entire gene encoding the protein on the chromosome; a method of introducing an antisense oligonucleotide (e.g., antisense RNA), which inhibits the translation from the mRNA into a protein via a complementary binding to the transcript of the gene on the chromosome; a method of making the attachment of ribosome impossible by forming a secondary structure by artificially adding a Shine-Dalgarno (SD) sequence and its complementary sequence on the front end of the SD sequence of the gene encoding the protein; a method of reverse transcription engineering (RTE), which adds a promoter so as to be reversely transcribed on the 3' terminus of the open reading frame (ORF) of the corresponding sequence, etc., and also include a combination thereof, but are not limited thereto.

Specifically, the method of deleting a part of or the entire gene encoding a protein may be performed by replacing the polynucleotide, encoding the endogenous target protein within the chromosome via a vector for inserting chromosome into a microorganism, with a polynucleotide or a marker where part of the polynucleotide sequence is deleted. For example, a method of gene deletion via homologous recombination may be used, but is not limited thereto. Additionally, as used herein the term "part", although it may vary depending on the kinds of polynucleotide, 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, but is not limited thereto.

Additionally, the method of modifying the expression regulation sequence may be performed by inducing a variation on the expression regulation sequence of the polynucleotide sequence via deletion, insertion, conservative substitution, non-conservative substitution, or a combination thereof so as to further attenuate the activity of the expression regulation sequence; or by replacing the polynucleotide sequence with a polynucleotide sequence with a weaker activity. The polynucleotide sequence may include a promoter, an operator sequence, a sequence encoding a ribosome-binding domain, and a sequence for regulating termination of transcription and translation, but is not limited thereto.

Additionally, the method of modifying the gene sequence on the chromosome may be

performed by inducing a variation in the sequence via deletion, insertion, conservative substitution, non-conservative substitution, or a combination thereof so as to further attenuate the activity of the expression regulation sequence; or by replacing the sequence with a gene sequence improved to have a weaker activity or a gene sequence improved to have no activity, but is not limited thereto.

Specifically, for the attenuation of the activity of citrate synthase protein, part of an amino acid(s) in the amino acid sequence of the citrate synthase protein may be substituted with other amino acid(s). More specifically, a citrate synthase having an amino acid sequence, in which the 145th amino acid or the 167th amino acid in the amino acid sequence of the citrate synthase protein is substituted from tyrosine (Y) or lysine (K) to other amino acid(s) may be included. Even more specifically, the citrate synthase may be one having a gene sequence encoding a modified polypeptide, in which the 145th amino acid in the amino acid sequence of the citrate synthase protein is substituted from tyrosine (Y) to alanine (A), and the 167th amino acid is substituted from lysine (K) to alanine (A). In particular, the amino acid residue number was determined in sequential order after setting the amino acid positioned next to the methionine, which is encoded by the initiation codon, as the 1st amino acid. The polypeptide may respectively have an amino acid sequence represented by SEQ ID NO: 1 or 2. Additionally, if the activity of the citrate synthase is weaker than that of a wild-type, the citrate synthase may include amino acid sequences having a homology of 80% or higher with the amino acid sequence of SEQ ID NO: 1 or 2, specifically 90% or higher, more specifically 95% or higher, and even more specifically 97% or higher. As a sequence having a homology, if the amino acid sequence is one which has substantially the same or corresponding biological activity of a protein of SEQ ID NO: 1 or 2, it is obvious that amino acid sequences with a deletion, a modification, a substitution, or an addition in part of the sequences should also be included in the scope of the present invention.

As used herein, the term “homology” refers to a percentage in identity between two polynucleotides or polypeptide moieties. The homology between sequences from a moiety to another moiety may be determined by a technology known in the art. For example, the homology may be determined by directly arranging the sequence information between two different polynucleotide molecules or two different polypeptides using a computer program arranging and easily obtaining the sequence information. The computer program may include BLAST (NCBI), CLC Main Workbench (CLC bio), MegAlign™ (DNASTAR Inc), etc. Additionally, the homology between polynucleotides may be determined by hybridizing the

polynucleotides under the condition of forming a stable double strand between homologous regions, decomposing with a single-strand-specific nuclease, and determining the decomposed fragments.

As used herein, the term “homology” refers to a relationship between proteins having “common evolutionary origin” including homologous proteins derived from superfamily proteins in all grammatical forms or with spelling variations, and those derived from different species. These proteins (and the genes encoding the same) have sequence homologies reflected by high levels of sequence similarities. However, the term “homology”, for its general use and the use in the present invention, would refer to a sequence similarity when modified by an adjective such as “very high”, instead of referring to common evolutionary origin.

In an exemplary embodiment of the present invention, the microorganism may be one in which the activity of cystathionine gamma synthase (EC 2.5.1.48), homoserine kinase (EC 2.7.1.39), or the activities of both are weaker than their endogenous activities, or inactivated.

As used herein, the term “cystathionine gamma synthase” refers to an enzyme which can synthesize cystathionine by a chemical reaction described below, using *O*-succinyl homoserine and L-cysteine as substrates. In the present invention, the cystathionine gamma synthase from *E. coli*, is designated as “MetB”.

O-succinyl-L-homoserine + L-cysteine → L-cystathionine + succinate

Specifically, the cystathionine gamma synthase from *E. coli*, although not particularly limited thereto, may be a protein comprising an amino acid sequence represented by SEQ ID NO: 9 or those having a homology of 70% or higher with the amino acid sequence of SEQ ID NO: 9, specifically 80% or higher, and more specifically 90% or higher. Additionally, as a sequence having a homology, if the amino acid sequence is one having the same or corresponding activity of homoserine kinase with the amino acid sequence of SEQ ID NO: 9, it is obvious that amino acid sequences with a deletion, a modification, a substitution, or an addition in part of the sequences should also be included in the scope of the present invention. Additionally, based on the genetic code degeneracy, polynucleotide sequences encoding the same amino acid sequence and variants thereof should also be included in the scope of the present invention.

The method for attenuation and inactivation of the cystathionine gamma synthase activity may be performed according to the method described above.

As used herein, the term “homoserine kinase” refers to an enzyme causing the phosphorylation of homoserine, which performs the chemical reaction described below. In the present invention, the homoserine kinase from *E. coli*, is designated as “ThrB”.



Specifically, homoserine kinase from *Escherichia* sp., although not particularly limited thereto, may be a protein comprising an amino acid sequence represented by SEQ ID NO: 11 or those having a homology of 70% or higher with the amino sequence of SEQ ID NO: 11, specifically 80% or higher, or more specifically, 90% or higher. Additionally, as a sequence having a homology, if the amino acid sequence is one having the same or corresponding activity of homoserine kinase with the amino sequence of SEQ ID NO: 11, it is obvious that amino acid sequences with a deletion, a modification, a substitution, or an addition in part of the sequences should also be included in the scope of the present invention. Additionally, based on the genetic code degeneracy, polynucleotide sequences encoding the same amino acid sequence and variants thereof should also be included in the scope of the present invention.

The method for attenuation and inactivation of the homoserine kinase activity may be performed according to the method described above.

In a specific aspect of the present invention, the microorganism may be one, in which the activity of homoserine *O*-acetyltransferase is further introduced or enhanced, or the endogenous homoserine *O*-succinyltransferase is further modified to have the activity of homoserine *O*-acetyltransferase.

As used herein, the term “homoserine *O*-acetyltransferase (EC 2.3.1.31)” refers to an enzyme having an activity of transferring an acetyl group from acetyl-CoA to homoserine.

Specifically, the microorganism according to the present invention may be introduced with the activity of homoserine *O*-acetyltransferase. The homoserine *O*-acetyltransferase may be derived from various microorganism species, for example, from a microorganism selected from *Corynebacteria* sp., *Leptospira* sp., *Deinococcus* sp., *Deinococcus* sp., *Pseudomonas* sp., and *Mycobacterium* sp. Specifically, the homoserine *O*-acetyltransferase may be those which include the amino acid sequences represented by SEQ ID NO: 13 (*Leptospira meyeri*), SEQ ID NO: 14 (*Corynebacterium glutamicum*), or SEQ ID NO: 15 (*Deinococcus radiodurans*), but is

not limited thereto. Additionally, the homoserine *O*-acetyltransferase may be a protein comprising the above amino acid sequences or those having a homology of 70% or higher with the above amino acid sequences, specifically 80% or higher, or more specifically, 90% or higher. Additionally, based on the genetic code degeneracy, polynucleotide sequences encoding the same amino acid sequence and variants thereof should also be included in the scope of the present invention.

Examples of the homoserine *O*-acetyltransferase to be used in the present invention are disclosed in Korean Patent Application Publication No. 10-2011-0023703 and European Patent Application Publication No. EP 2290051, and the entire specifications of these patent documents may be included as references in the present invention.

Additionally, the protein, in which the endogenous homoserine *O*-succinyltransferase (EC 2.3.1.46) is modified to have the activity of homoserine *O*-acetyltransferase, refers to a polypeptide, in which the substrate specificity of the polypeptide having the homoserine *O*-succinyltransferase activity is changed from succinyl-CoA to acetyl-CoA. Additionally, the modified protein, although not particularly limited thereto, may be a polypeptide having homoserine *O*-acetyltransferase activity, unlike its wild-type, by replacing part of the amino acid sequence of the polypeptide having homoserine *O*-succinyltransferase activity.

Examples of the homoserine *O*-succinyltransferase may be a polypeptide from *Enterobacteria* sp., *Salmonella* sp., *Pseudomonas* sp., *Bacillus* sp., or *Escherichia* sp., specifically, a polypeptide from *Escherichia* sp. having the homoserine *O*-succinyltransferase activity, for example, a polypeptide having homoserine *O*-succinyltransferase activity from *E. coli*. More specifically, the homoserine *O*-succinyltransferase from *E. coli* may have the amino acid sequence represented SEQ ID NO: 16, but is not limited thereto. The homoserine *O*-succinyltransferase from *E. coli* is designated as "MetA".

The modified homoserine *O*-succinyltransferase may be a variant polypeptide, in which the 111th amino acid of the polypeptide represented by SEQ ID NO: 16 or polypeptides having a homology of 95% or above with the polynucleotide sequence of SEQ ID NO: 16 is substituted with glutamic acid, and additionally, the 112nd amino acid is substituted with threonine or histidine. Specifically, the variant polypeptide may be a polypeptide having the amino acid sequence of any of SEQ ID NOS: 17 to 19. Additionally, the variant polypeptide may be a protein comprising an amino acid sequence having a homology of 70% or higher with the above amino acid sequence, specifically 80% or higher, or more specifically 90% or higher. Additionally, based on the genetic code degeneracy, polynucleotide sequences encoding the

same amino acid sequence and variants thereof should also be included in the scope of the present invention. The information on the modified homoserine *O*-succinyltransferase can be obtained from Korean Patent Application Publication No. 10-2012-0070531 or International Publication No. WO2012/087039, and the entire specifications of these patent documents are included as references to the present invention.

As used herein, the term “introduction or enhancement of activity” refers to providing the activity of a particular protein to a microorganism which does not possess the protein; or enhancement of the intracellular activity of the protein in the microorganism which possesses the protein, and the like, and refers to the increase of the intracellular activity of the protein compared to the endogenous activity of the protein.

As used herein, the term “introduction or enhancement of protein activity” refers to not only the drawing of a higher effect than the original function due to the increase in the activity of the protein itself, but also the increase in the activity of the protein due to the increase in endogenous gene activity, endogenous gene amplification by the internal or external factors, the increase in copy number, gene introduction from outside, increase in enzyme activity due to the substitution, modification, or mutation, etc., but is not limited thereto.

In the above, the increase in gene copy number, although not particularly limited thereto, may be performed in a state operably connected to a vector, or by being inserted into the chromosome within a host cell. Specifically, the method may be executed by introducing a vector, which a polynucleotide encoding the protein of the present invention is operably connected to and can be replicated and function irrespective of a host, into a cell of the host; or introducing a vector, to which the polynucleotide is operably connected, inserting the polynucleotide into the chromosome of the host cell, into the host cell, thereby increasing the number of gene copies within the chromosome of the host cell.

The vector is a DNA construct including the polynucleotide sequence of the polynucleotide encoding the target protein, which is operably connected to a suitable regulation sequence so that the target protein can be expressed in an appropriate host, wherein the regulation sequence includes a promoter initiating transcription, a random operator sequence for regulation of the transcription, a sequence encoding a suitable mRNA ribosome-binding domain, and a sequence for regulation of transcription and translation. The vector, after being transformed into a suitable host cell, may be replicated or function irrespective of the host genome, or may be integrated into the host genome itself.

The vector used in the present invention may not be specifically limited as long as the

vector is replicable in the host cell, and any vector known in the art may be used. Examples of the vector may include natural or recombinant plasmids, cosmids, viruses, and bacteriophages. For example, as a phage vector or cosmid vector, *pWE15*, *M13*, *λMBL3*, *λMBL4*, *λIXII*, *λASHII*, *λAPII*, *λt10*, *λt11*, *Charon4A*, *Charon21A*, etc., may be used; and as a plasmid vector, *pBR*-based, *pUC*-based, *pBluescriptII*-based, *pGEM*-based, *pTZ*-based, *pCL*-based, *pET*-based, etc., may be used. Specifically, *pDZ*, *pACYC177*, *pACYC184*, *pCL*, *pECCG117*, *pUC19*, *pBR322*, *pMW118*, *pCC1BAC* vectors, etc., may be used.

Additionally, a polynucleotide encoding an endogenous target protein may be substituted with a modified polynucleotide using a vector for inserting into the chromosome of a microorganism. The insertion of the polynucleotide into the chromosome may be performed using a known method in the art, for example, by homologous recombination. Since the vector of the present invention can be inserted into the chromosome via homologous recombination, a selection marker for confirmation of the insertion into the chromosome may be additionally included. The selection marker is used for selection of a transformed cell, i.e., in order to confirm whether the target polynucleotide has been inserted, and markers providing selectable phenotypes such as drug resistance, nutrient requirement, resistance to cytotoxic agents, and expression of surface proteins may be used, but are not limited thereto. Under the circumstances where selective agents are treated, only the cells expressing the selection markers can survive or express other phenotypic traits, and thus the transformed cells can be easily selected.

As used herein, the term “transformation” refers to a process of introducing a vector including a polynucleotide encoding a target protein into a host cell thereby enabling the expression of the polynucleotide encoded by the protein in the host cell. For the transformed polynucleotide, it does not matter whether it is inserted into the chromosome of a host cell and located therein and located outside the chromosome, as long as it can be expressed in the host cell. Additionally, the polynucleotide includes DNA and RNA which encode the target protein. The polynucleotide may be inserted in any form insofar as it can be introduced into a host cell and expressed therein. For example, the polynucleotide may be introduced into a host cell in the form of an expression cassette, which is a genetic construct including all essential elements required for self-expression. The expression cassette may conventionally include a promoter operably connected to the polynucleotide, a transcription termination signal, a ribosome-binding domain, and a translation termination signal, and may be in the form of an expression vector capable of self-replication. Additionally, the polynucleotide may be introduced into a host cell as it is, and operably connected to a sequence essential for its expression in the host cell.

Additionally, as used herein, the term “operably connected” refers to a functional connection between a promoter sequence, which initiates and mediates the transcription of the polynucleotide encoding the target protein, and the gene sequence.

Then, the modification of the expression regulation sequence for increasing the expression of the polynucleotide, although not particularly limited thereto, may be performed by inducing a variation in the polynucleotide sequence via deletion, insertion, conservative substitution, non-conservative substitution, or a combination thereof so as to further enhance the activity of the expression regulation sequence; or by replacing the polynucleotide sequence with a polynucleotide sequence with a stronger activity. The expression regulation sequence, although not particularly limited thereto, may include a promoter, an operator sequence, a sequence encoding a ribosome-binding domain, and a sequence for regulating termination of transcription and translation, etc. Additionally, a strong exogenous promoter, instead of the original promoter, may be connected to the upper end of the expression unit of the polynucleotide.

Furthermore, the modification of the polynucleotide sequence on the chromosome, although not particularly limited thereto, may be performed by inducing a variation on the expression regulation sequence of the polynucleotide sequence via deletion, insertion, conservative substitution, non-conservative substitution, or a combination thereof so as to further enhance the activity of the polynucleotide sequence; or by replacing the polynucleotide sequence with an enhanced polynucleotide sequence with a stronger activity.

Generally, the introduction and enhancement of the protein activity may increase the activity or concentration of the corresponding protein relative to the activity or concentration of a wild-type protein or in a microorganism from at least 1%, 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400%, or 500%, to a maximum of 1000% or 2000%.

Additionally, the microorganism may be one in which the activity of the endogenous homoserine *O*-succinyltransferase was attenuated or inactivated compared to that of the endogenous activity, in order to enhance the biosynthesis pathway of *O*-acetyl homoserine by blocking the pathway of biosynthesizing *O*-succinyl homoserine from homoserine.

The attenuation and inactivation of the homoserine *O*-succinyltransferase activity may be performed according to the method explained above.

In an exemplary embodiment of the present invention, the *O*-acetyl homoserine-producing microorganism may be one in which the activity of an enzyme involved

in the biosynthesis pathway from phosphoenolpyruvate to homoserine is additionally introduced or enhanced, in order to further increase the amount of homoserine, a substrate for the biosynthesis of *O*-acetyl homoserine.

Specifically, the above microorganism may be one, in which the activity of at least one protein selected from the group consisting of phosphoenolpyruvate carboxylase (*ppc*, EC 4.1.1.31), aspartate aminotransferase (*aspC*, EC 2.6.1.1), and aspartate semialdehyde dehydrogenase (*asd*, EC 1.2.1.11) is further introduced or enhanced.

For example, *ppc* gene encoding phosphoenolpyruvate carboxylate including an amino acid sequence represented by SEQ ID NO: 20, *aspC* gene encoding aspartate aminotransferase including an amino acid sequence represented by SEQ ID NO: 21, and *asd* gene encoding aspartate semialdehyde dehydrogenase including an amino acid represented by SEQ ID NO: 22 may be introduced into a microorganism. For example, the activities of the three different enzymes may be introduced and enhanced by making all the genes encoding the three different enzymes present in the chromosome of a host cell with a copy number of at least 2, but is not limited thereto. The introduction and enhancement of the activities may be performed according to the method described above.

In an exemplary embodiment of the present invention, the activity of citrate synthase protein was attenuated or inactivated by various methods, which includes deleting the citrate synthase gene in a microorganism of *E. coli* producing *O*-acetyl homoserine; introducing the gene encoding the modified citrate synthase protein, whose activity was attenuated compared to that of a wild-type, into the position of the citrate synthase gene; and introducing an expression vector for citrate synthase gene antisense RNA. As a result, the thus-constructed *O*-acetyl homoserine-producing microorganism, in which the activity of citrate synthase protein was attenuated or inactivated, showed an improved *O*-acetyl homoserine production capability, compared to that of the parent microorganism (Examples 1 to 4).

In another aspect, the present invention provides a method for producing *O*-acetyl homoserine using an *O*-acetyl homoserine-producing microorganism with an improved production capability of *O*-acetyl homoserine. Specifically, the present invention provides a method for producing *O*-acetyl homoserine including (a) culturing the microorganism; and (b) recovering *O*-acetyl homoserine produced during the cultivation of the microorganism.

The method of cultivation of the *E. coli* having *O*-acetyl homoserine production

capability according to the present invention may be performed according to the suitable media and culture conditions known in the art. The cultivation process may be easily adjusted by a skilled person in the art depending on the microorganism to be selected. In particular, since the microorganism of the present invention is a microorganism where the activity of the citrate synthase, which is an enzyme mediating the first step of the TCA cycle, is attenuated or inactivated, the cultivation medium may include glutamate, but is not specifically limited thereto.

Examples of the culture methods may include a batch culture, a continuous culture, and a fed-batch culture, but are not limited thereto. These various methods are, for example, disclosed in "Biochemical Engineering" by James M. Lee, Prentice-Hall International Editions, pp 138-176.

The medium used in the cultivation may appropriately meet the requirement of a specific microorganism. Specifically, examples of the microorganism culture media are disclosed in "Manual of Methods for General Bacteriology" by the American Society for Bacteriology, Washington, DC, 1981. The culture media may be those which include an appropriate carbon source, phosphorous source, inorganic compound, amino acid, and/or vitamins, etc, and cultivation may be performed in aerobic conditions while adjusting temperature, pH, etc.

Examples of the carbon source may include carbohydrates such as glucose, lactose, sucrose, lactic acid, fructose, maltose, starch, and cellulose; fats such as soybean oil, sunflower oil, castor oil, berber oil, and coconut oil; fatty acids such as palmitic acid, stearic acid, and linoleic acid; alcohols such as glycerol and ethanol; and organic acids such as acetic acid. These carbon sources may be used alone or in combination, but are not limited thereto.

Examples of the nitrogen source may include organic nitrogen sources such as peptone, yeast extract, gravy, malt extract, corn steep liquor (CSL), and bean flour; and inorganic nitrogen sources such as urea, 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. The culture media may further include, as a phosphorous source, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and corresponding sodium-containing salts. The culture media may include metals such as magnesium sulfate and iron sulfate. Additionally, amino acids, vitamins and appropriate precursors may be included. These culture media or precursors may be added to the culture in the form of a batch culture or continuous culture, but are not limited thereto.

Additionally, the pH of the culture may be adjusted by adding a compound such as ammonium hydroxide, potassium hydroxide, ammonia, phosphoric acid, and sulfuric acid during

cultivation in an appropriate manner. Additionally, bubble formation may be prevented during the cultivation using an antifoaming agent such as fatty acid polyglycol ester. Additionally, in order to maintain aerobic conditions in a culture liquid, an oxygen gas or a gas (e.g., air) containing an oxygen gas may be added to a culture. The culture temperature may be from 20°C to 45°C, and specifically from 25°C to 40°C, but is not limited thereto. The cultivation may be continued until the production of *O*-acetyl homoserine reaches the intended level, and specifically for 10 hours to 160 hours, but is not limited thereto.

The method of producing *O*-acetyl homoserine of the present invention may further include recovering *O*-acetyl homoserine from the cultured microorganism or a cultured product thereof. The recovery of the intended *O*-acetyl homoserine may be performed by a microorganism cultivation method according to the present invention, for example, an appropriate method known in the art such as a batch culture, a continuous culture, and a fed-batch culture.

The recovery may include a purification step.

The thus-recovered *O*-acetyl homoserine can produce methionine 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 *O*-acetyl homoserine sulfhydrylase activity or a microorganism possessing the enzyme, while using the *O*-acetyl homoserine, which was produced by the L-methionine precursor-producing microorganism, and methyl mercaptan as substrates.

More specifically, the present invention provides a method for producing L-methionine by an enzyme reaction of *O*-acetyl homoserine sulfhydrylase, etc., using *O*-acetyl homoserine, which was accumulated by the above method, as a substrate.

In the second step, when *O*-acetyl homoserine is used as an L-methionine precursor, *O*-acetyl homoserine sulfhydrylase derived from a microorganism, specifically belonging to *Leptospira* sp., *Chromobacterium* sp., and *Hyphomonas* sp., and more specifically belonging to *Leptospira meyeri*, *Pseudomonas aurogenosa*, *Hyphomonas Neptunium*, and *Chromobacterium Violaceum* may be used.

The reaction is the same as shown below.



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

MODE FOR INVENTION

Hereinafter, the present invention will be described in more detail with reference to the following Examples. However, these Examples are for illustrative purposes only, and the invention is not intended to be limited by these Examples.

Reference Example : Construction of O-acetyl homoserine-producing microorganism

<1-1> Deletion of *metB* gene derived from wild-type *E. coli* (International Publication No. WO 2008/013432)

An O-acetyl homoserine-producing microorganism was constructed using *E. coli*, a representative microorganism among *Escherichia* sp. To this end, wild-type *E. coli* K12 W3110 (ATCC27325) obtained from American Type Culture Collection (ATCC) was used. First, in order to block the synthesis pathway from O-succinyl-L-homoserine to cystathionine, cystathionine synthase-encoding *metB* gene (SEQ ID NO: 10) was deleted. Specifically, the cystathionine synthase-encoding *metB* gene was deleted via an FRT-one-step PCR deletion method (PNAS (2000) vol 97: P6640-6645).

Specifically, the *metB* deletion cassette was constructed via a PCR reaction based on the *pKD3* vector (PNAS (2000) vol 97: P6640-6645) as a template using primers of SEQ ID NOS: 30 and 31 as follows: 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. The resulting PCR product was electrophoresed on a 1.0% agarose gel, and a 1.2 kb DNA band obtained therefrom was purified. The recovered DNA fragment was electroporated into *E. coli* (K12) W3110, which was already transformed with the *pKD46* vector (PNAS (2000) vol 97: P6640-6645). For electroporation, the W3110 strain transformed with the *pKD46* was cultured in an LB medium containing 100 µg/L ampicillin and 5 mM arabinose (L-arabinose) at 30°C until OD₆₀₀=0.6, and used after washing twice with sterile distilled water and once with 10% glycerol. Electroporation was performed at 2500 V. The recovered strain was streaked on an LB plate containing 25 µg/L chloramphenicol, cultured at 37°C overnight, and the strain showing resistance was selected. The selected strain was subjected to a PCR reaction under the same conditions based on the strain as a template

using the same primers, and the deletion of *metB* gene was confirmed by observing the gene size of 1.2 kb on a 1.0% agarose gel. The thus-confirmed strain was cultured in an LB medium after transforming again with the *pCP20* vector (PNAS (2000) vol 97: P6640-6645), and the final *metB* gene-deleted strain, where the gene size was reduced to 150 bp on a 1.0% agarose gel through PCR performed under the same conditions, was constructed and the removal of the chloramphenicol marker was confirmed. The thus-constructed strain was designated as “W3-B”.

<1-2> Deletion of *thrB* gene (International Publication No. WO 2008/013432)

In an effort to increase the amount of *O*-succinylhomoserine synthesis from homoserine, *thrB* gene, which is a homoserine kinase-encoding gene, was deleted. For deletion of the *thrB* gene from the W3-B strain constructed in Example 1, the FRT one step PCR deletion method used in the deletion of *metB* gene was used.

A *thrB* deletion cassette was constructed via PCR based on the *pKD4* vector (PNAS (2000) vol 97: P6640-6645) as a template using primers of SEQ ID NOS: 32 and 33 as follows: 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute.

The resulting PCR product was electrophoresed on a 1.0% agarose gel, and a 1.6 kb DNA band obtained therefrom was purified. The recovered DNA fragment was electroporated into W3-B strain, which was already transformed with the *pKD46* vector. The recovered strain was streaked on an LB plate containing 50 µg/L kanamycin, cultured at 37°C overnight, and the strain showing resistance was selected.

The selected strain was subjected to a PCR reaction under the same conditions directly based on the strain as a template using the same primers of SEQ ID NOS: 32 and 33, and confirmed the deletion of *thrB* gene by selecting the strain having the gene size of 1.6 kb on a 1.0% agarose gel. The thus-confirmed strain was cultured in an LB medium after transforming again with the *pCP20* vector, and the final *thrB* gene-deleted strain, where the gene size was reduced to 150 bp on a 1.0% agarose gel through PCR performed under the same conditions, was constructed and the removal of the kanamycin marker was confirmed. The thus-constructed strain was designated as “W3-BT”.

<1-3> A variant *metA* with homoserine acetyltransferase activity (International Publication No. WO 2012/087039)

In order to strengthen the homoserine acetyltransferase activity in the strain obtained in

Reference Example <1-2>, it was intended to introduce mutant type *metA* gene (SEQ ID NOS: 24 and 26) encoding homoserine acetyltransferase.

First, in order to construct the variant of *metA* gene with a strengthened activity, a PCR reaction was performed based on the chromosome of a wild-type strain *W3110* as a template using the primers of SEQ ID NOS: 34 and 35, and the *metA* gene encoding homoserine *O*-succinyltransferase was amplified.

The primers used in the PCR reaction were prepared based on the polynucleotide sequence of the *E. coli* chromosome, NC_000913, registered in the NIH Gene Bank, and the primers of SEQ ID NOS: 34 and 35 have the *EcoRV* and *HindIII* restriction sites, respectively. The thus-obtained PCR product and the *pCL1920* plasmid including *Pcj1* were respectively treated with *EcoRV* and *HindIII*, and the PCR product was cloned into the *pCL1920* plasmid. *E. coli* DH5 α was transformed using the cloned plasmid, and the transformed *E. coli* DH5 α was selected on LB plates containing 50 μ g/mL spectinomycin, and the plasmid was obtained therefrom. The thus-obtained plasmid was designated as “*pCL_Pcj1_metA*”.

Then, the 111th amino acid, glycine (Gly), of *O*-succinyltransferase was substituted with glutamic acid (Glu) (G111E) based on the above-constructed *pCL_Pcj1_metA* plasmid as a template using a site directed mutagenesis kit (Stratagene, USA). The thus-constructed plasmid including the variant of G111E *metA* gene was designated as “*pCL_Pcj1_metA* (EL)”.

Additionally, in order to substitute the 111th amino acid of *O*-succinyltransferase from glycine to glutamic acid, and the 112nd amino acid from leucine to histidine, primers of SEQ ID NOS: 38 and 39 were used. The plasmid including *metA* gene, in which the 111th amino acid was substituted from glycine to glutamic acid, and the 112nd amino acid was substituted from leucine to histidine was designated as “*pCL_Pcj1_metA* (EH)”.

Then, a replacement cassette, for the replacement with *metA* (EH) into a strain, was constructed via PCR using *pKD3* vector as a template along with primers of SEQ ID NOS: 40 and 41 as follows: 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 2 minutes. The respective PCR product was obtained using *pCL_Pcj1-metA* (EH) as a template for the *metA* (EH) part of the replacement cassette along with primers of SEQ ID NOS: 42 and 43, and primers of SEQ ID NOS: 42 and 45 for the wild-type *metA* part. *metA* (EH) replacement cassettes including the chloramphenicol marker part were constructed using the three different PCR products along with the primers of SEQ ID NOS: 42 and 45, and electroporated into *W3-BT* strain, which was already transformed with the *pKD46* vector, constructed in Reference Example <1-2>. The thus-confirmed strain was cultured in an LB medium after transforming again with the *pCP20* vector, and the strain, where the

chloramphenicol marker was removed, and *metA* gene was substituted with *metA* (EH) was designated as “W3-BTA”.

<1-4> Construction of a strain with 2 copies of *ppc*, *aspC*, and *asd* genes (European Patent Application Publication No. EP 2290051)

In order to increase the *O*-acetyl homoserine-producing capability of W3-BTA strain constructed in Reference Example <1-3>, the biosynthetic pathway was enhanced by citing the prior filed patent EP 2290051. In the same manner as in the above EP patent, a strain having 2 amplified copies each of the genes, i.e., the *ppc* gene encoding phosphoenolpyruvate carboxylase using primers of SEQ ID NOS: 46, 47, 48, and 49; the *aspC* gene encoding aspartate aminotransferase using primers of SEQ ID NOS: 50 and 51; and the *asd* gene encoding aspartate semialdehyde dehydrogenase using primers of SEQ ID NOS: 52, 53, 54, and 55, was constructed. In particular, the above strain with an enhanced biosynthetic pathway while producing *O*-acetyl homoserine was designated as “W3-BTA2PCD” (also called “WCJM”).

<1-5> Flask-culture Experiments

The amount of *O*-acetyl homoserine production by the strain constructed in Reference Examples <1-3> and <1-4> was tested by an Erlenmeyer flask-culture.

Specifically, W3110, W3-BTA, and WCJM strains were inoculated into LB media, and cultured at 33°C overnight. Then, a single colony thereof was inoculated into 3 mL of LB medium, cultured at 33°C for 5 hours, diluted 200-fold in a 250 mL Erlenmeyer flask containing 25 mL of an *O*-acetyl homoserine-producing medium, cultured at 33°C at a rate of 200 rpm for 30 hours, and the amount of *O*-acetyl homoserine production was examined via HPLC analysis. The media compositions used are shown in Table 1 below, and the amount of *O*-acetyl homoserine production examined is shown in Table 2 below.

[Table 1] Composition of *O*-acetyl homoserine-producing flask-culture

Composition	Conc. (per liter)
glucose	40 g
(NH ₄) ₂ SO ₄	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
CaCO ₃	30 g
yeast extract	2 g
methionine	0.15 g
threonine	0.15 g

[Table 2]

	OD (562nm)	Glucose consumption (g/L)	O-Acetyl homoserine (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

The result revealed that the wild-type *W3110* did not produce *O*-acetyl homoserine at all, whereas the *W3-BTA* strain produced 0.9 g/L of *O*-acetyl homoserine and the *WCJM* strain, which was strengthened with the biosynthesis pathway, produced 1.2 g/L of *O*-acetyl homoserine.

Example 1: Deletion of citrate synthase activity

<1-1> Construction of a citrate synthase gene-deleted microorganism in an *O*-acetyl homoserine-producing microorganism

Citrate synthase (GltA) is the enzyme in the first step of the TCA cycle, and starts with the reaction between oxaloacetate and acetyl-CoA. The growth inhibition by decrease in the TCA cycle is well known (Meded Rijksuniv Gent Fak Landbouwkde Toegep Biol Wet. 2001; 66(3a): 333-6). However, in order to increase the amount of acetyl-CoA used as a substrate for *O*-acetyl homoserine, *W3-BTA* and *WCJM* strains where citrate synthase activity is deleted were to be produced.

Specifically, the citrate synthase gene in *W3-BTA* and *WCJM* strains was deleted via PCR based on *pKD4* vector as a template using primers of SEQ ID NOS: 56 and 57 as follows: 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 2 minutes. The resulting PCR product was electrophoresed on a 1.0% agarose gel,

and the size of the gene was confirmed to be 1.6 kb, and its DNA was purified. The recovered DNA fragments were electroporated into *W3-BTA* and *WCJM* strains, which were already transformed with the *pKD46* vector. For electroporation, *W3-BTA* and *WCJM* strains, transformed with the *pKD46* vector, were cultured in an LB medium containing 100 µg/L ampicillin and 5 mM arabinose at 30°C until OD₆₀₀=0.6, and washed twice with distilled water and once with 10% glycerol to be used. Electroporation was performed at 2500 V. The thus-recovered strains were streaked on LB plates containing 50 µg/L kanamycin, cultured at 37°C, and the strains showing resistance were selected.

The selected strains were subjected to PCR using the primers of SEQ ID NOS: 58 and 59 under the same conditions, electrophoresed on a 1.0% agarose gel, and the size of the gene was observed to be 2.5 kb, thereby confirming that a deletion cassette was inserted into the citrate synthase gene portion on the chromosome. The thus-confirmed strains were transformed again with the *pCP20* vector, cultured in LB media, and strains having a deletion of the citrate synthase gene, whose size was reduced to 1.1 kb on a 1.0% agarose gel, were constructed by PCR, and it was confirmed that kanamycin markers were removed. The thus-constructed strains were designated as “*W3-BTA-AD*” and “*WCJM-AD*”, respectively.

<1-2> Evaluation of a citrate synthase gene-deleted microorganism in an *O*-acetyl homoserine-producing microorganism

W3-BTA-AD and *WCJM-AD* strains can grow in an LB medium, but due to the deletion of the citrate synthase gene, they could not grow in an *O*-acetyl homoserine-containing medium. In order to test the amount of *O*-acetyl homoserine production, an Erlenmeyer flask-culture was performed under the condition (Table 3 – a composition adding glutamate in the medium) of adding 3 g/L of glutamate into the existing composition of the culture medium.

Specifically, *W3-BTA-AD* and *WCJM-AD* strains were inoculated into LB media and cultured at 33°C overnight. Then, a single colony thereof was inoculated into 3 mL of LB medium, cultured at 33°C for 5 hours, diluted 200-fold in a 250 mL Erlenmeyer flask containing 25 mL of an *O*-acetyl homoserine-producing medium (with glutamate added), cultured at 33°C at a rate of 200 rpm for 30 hours, and the amount of *O*-acetyl homoserine production was examined via HPLC analysis. The amount of *O*-acetyl homoserine production examined is shown in Table 4 below.

[Table 3] Composition of a medium with glutamate added to a basal medium

Composition	Conc. (per liter)
glucose	40 g
(NH ₄) ₂ SO ₄	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
CaCO ₃	30 g
yeast extract	2 g
methionine	0.15 g
threonine	0.15 g
glutamate	3 g

[Table 4] Production of *O*-acetyl homoserine via flask-culture

	OD (562 nm)	Glucose consumption (g/L)	<i>O</i>-acetyl homoserine (g/L)	Glutamate (g/L)
<i>W3-BTA</i>	9.9	38	0.9	3.2
<i>W3-BTA-AD</i>	6.1	34	1.4	2.3
<i>WCJM</i>	9.2	37	1.3	3.5
<i>WCJM-AD</i>	5.6	33	2.1	1.7

The result of the *O*-acetyl homoserine production via flask-culture revealed that the *W3-BTA* strain produced 0.9 g/L of *O*-acetyl homoserine, and *W3-BTA-AD* produced 1.4 g/L of *O*-acetyl homoserine, which is a 55.6% increase, although it showed a decrease in its glucose consumption. The *WCJM* strain produced 1.3 g/L of *O*-acetyl homoserine while the *WCJM-AD* strain produced 2.1 g/L of *O*-acetyl homoserine, thus confirming that the *O*-acetyl homoserine production capability was improved by 61.5% due to the deletion of the citrate synthase gene.

Example 2: Attenuation of the activity of citrate synthase protein

<2-1> Kinds of citrate synthase gene modifications

The *WCJM*-AD strain constructed in Example <1-1> showed a low culture rate, and three different types of variants, which showed an attenuated activity and a reduced binding ability to acetyl-CoA according to various modificationss of citrate synthase known in numerous references (The Journal of Biological Chemistry, 2003, 278, 35435-35443), were selected. The information on the three different types of variants is shown in Table 5, which shows modified genes in which the 145th amino acid, tyrosine (Y), was substituted with alanine (A), and the 167th amino acid, lysine(K), was substituted with alanine (A), and the 204th amino acid, threonine (T), was substituted with alanine (A).

[Table 5]

Evaluation on citrate synthase (gltA) variants

	KM VALUE [mM]	
	Acetyl-CoA	OAA
WT	0.12	0.026
Y145A	0.23	0.051
K167A	0.22	0.037
T204A	0.21	0.004

<2-2> Construction of a microorganism with attenuated citrate synthase protein activity in an O-acetyl homoserine-producing microorganism

The present inventors intended to increase the production capability by introducing the variants, in which the activity of citrate synthase protein was attenuated as explained in Example <2-1>, into the *O*-acetyl homoserine-producing microorganism.

In order to introduce the three different types of citrate synthase gene variants into the *WCJM*-AD strain, a modification replacement cassette was designed as shown in FIG. 1. Each variant was synthesized by substituting a primer with a nucleotide, and each cassette was constructed through 3 PCR products. For the citrate synthase gene portion, the *W3110* strain was used as a template, and the modification on the 145th amino acid produced PCR reactions were performed using the primers of SEQ ID NOS: 60 and 63 and SEQ ID NOS: 62 and 61, respectively, and obtained PCR products with a size of 514 bp and 1,112 bp.

Likewise, the modification on the 167th amino acid produced PCR products with a size of 580 bp and 1,046 bp using the primers of SEQ ID NOS: 60 and 65, and the primers of SEQ ID

NOS: 64 and 61, and the modification on the 204th amino acid produced PCR products with a size of 688 bp and 936 bp using the primers of SEQ ID NOS: 60 and 67 and SEQ ID NOS: 66 and 61. For the common kanamycin portion, PCR reactions were performed based on the *pKD4* vector as a template using the primers of SEQ ID NOS: 68 and 69. In particular, for the insertion into the position of the citrate synthase gene, the cassette was constructed so as to include the polynucleotide sequence downstream of the citrate synthase gene in the SEQ ID NO: 69, and a PCR product with a size of 1,571 bp was obtained via electrophoresis. A sewing PCR reaction was performed based on the kanamycin DNA fragment, which is the common part with each of the two DNA fragments collected according to the modifications, respectively, using the primers of SEQ ID NOS: 60 and 69, as follows: 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 4 minutes. Each of the final PCR products was confirmed on a 1.0% agarose gel, and the DNA fragments with a size of 3,115 bp for the three different kinds of cassettes of citrate synthase gene modifications. The collected DNA fragments were electroporated into the *WCJM-AD* strain, which was already transformed with the *pKD46* vector. For electroporation, the *W3110* strain transformed with the *pKD46* was cultured in an LB medium containing 100 µg/L ampicillin and 5 mM arabinose at 30°C until OD₆₀₀=0.6, and used after washing twice with sterile distilled water and once with 10% glycerol. Electroporation was performed at 2500 V. The recovered strain was streaked on an LB plate containing 25 µg/L kanamycin, cultured at 37°C overnight, and the strain showing resistance was selected. The selected strain was subjected to a PCR reaction under the same conditions based on the strain as a template using the same primers of SEQ ID NOS: 58 and 59, and confirmed the deletion of *metB* gene by observing the gene size of 3.7 kb on a 1.0% agarose gel, thereby confirming that a modification cassette, in which the amino acid of the citrate synthase gene was substituted, was inserted. The thus-confirmed strain was cultured in an LB medium after transforming again with the *pCP20* vector, and the three variant strains regarding the citrate synthase activity, where the gene size was reduced to 2.5 kb on a 1.0% agarose gel through PCR performed under the same conditions, were constructed and the removal of the kanamycin marker was confirmed. The thus-constructed strains were designated as “*WCJM-A145*”, “*WCJM-A167*”, and “*WCJM-A204*”, and the sequence information of the citrate synthase gene introduced with modifications are shown in SEQ ID NOS: 1 to 3 (amino acid sequences) and SEQ ID NOS: 5 to 7 (nucleotide sequences), respectively.

<2-3> Evaluation of microorganisms with attenuated citrate synthase activity in O-acetyl homoserine-producing microorganisms

An Erlenmeyer flask-culture was performed in order to examine the amount of *O*-acetyl homoserine production by three different strains of *WCJM-A145*, *WCJM-A167*, and *WCJM-A204*, in which the activity of the citrate synthase gene was attenuated. Four kinds of strains, i.e., *WCJM-A145*, *WCJM-A167*, and *WCJM-A204* strains including the *WCJM* strain, were inoculated into LB media, and cultured at 33°C overnight. Then, a single colony thereof was inoculated into 3 mL of LB medium, cultured at 33°C for 5 hours, diluted 200-fold in a 250 mL Erlenmeyer flask containing 25 mL of an *O*-acetyl homoserine-producing medium, cultured at 33°C at a rate of 200 rpm for 30 hours, and the amount of *O*-acetyl homoserine production was examined via HPLC analysis. The results are shown in Table 6 below.

[Table 6] Production of *O*-acetyl homoserine via flask-culture

	OD (562 nm)	Glucose consumption (g/L)	<i>O</i>-acetyl homoserine (g/L)	Glutamate (g/L)
<i>WCJM</i>	8.9	35	1.3	1.3
<i>WCJM-A145</i>	7.4	35	2.0	0
<i>WCJM-A167</i>	6.3	29	1.9	0
<i>WCJM-A204</i>	9.1	40	1.1	1.8

The result of *O*-acetyl homoserine production via flask-culture revealed that the *WCJM* strain produced 1.3 g/L of *O*-acetyl homoserine, and the two strains, *WCJM-A145* and *WCJM-A167*, produced 2.0 g/L and 1.9 g/L of *O*-acetyl homoserine, respectively, while the amount of their glucose consumption decreased along with the decrease in their absorbance (OD). Considering the specific decrease of glutamate from 1.3 g/L to 0 g/L, it was confirmed that the result is due to the decrease in TCA cycle flow caused by the attenuation in the citrate synthase activity. However, the *WCJM-A204* strain showed an increase in glutamate while showing a decrease in the amount of *O*-acetyl homoserine production to 0.2 g/L, thus confirming the modification is one with a strengthened activity.

Example 3: Attenuation in expression of citrate synthase protein

<3-1> Construction of expression vector for citrate synthase gene antisense RNA (asRNA)

The present inventors made an effort to apply an antisense RNA (asRNA) technology in

order to attenuate the expression of citrate synthase protein. The antisense RNA technology is a method for reducing protein expression by neutralizing the binding between citrate synthase mRNA and ribosome, via overexpression of the complementary binding portion to the citrate synthase mRNA of the target gene. This method has the advantage in that it can regulate the level of inhibition by controlling the binding force with the mRNA of the citrate synthase gene, and this method is also useful for the construction of a recombinant microorganism because this method can effectively construct and reduce gene expression via antisense RNA controlling gene expression, not necessitating the conventional process of gene deletion.

The vector construction was performed referring to a reference (Methods Mol Biol. 2012; 815:307-19. doi: 10.1007/978-1-61779-424-7_23.), and for overexpression, the antisense RNA region of the synthase gene was to be introduced into the *pBAD24* plasmid capable of induction. The *pBAD24*-citrate synthase asRNA vector map is shown in FIG. 2. The region where the antisense RNA of the citrate synthase gene was expressed has a size of 100 bp including the 52 bp region of the promoter region and the 48 bp region from the initiation codon of the citrate synthase, and a 38 bp paired termini (PT) structure, which reduces the instability of the antisense RNA (asRNA), is connected to both flanking regions. The antisense RNA region of the citrate synthase gene was obtained using the primers of SEQ ID NOS: 70 and 71, and the *Nco*I and *Hind*III restriction sites were included to be cloned into a vector.

The thus-obtained PCR product had a size of 194 bp, and the PCR product was cloned into the *pBAD24* plasmid after treating them with *Eco*RV and *Hind*III, respectively. The thus-cloned plasmid was used to transform *E. coli* DH5 α , and the transformed *E. coli* DH5 α was selected from the LB plates containing 100 μ g/mL of ampicillin, and obtained the plasmid therefrom. The thus-obtained plasmid was designated as “*pBAD24-gltA* asRNA”.

<3-2> Introduction of an expression vector of antisense RNA of citrate synthase gene into an *O*-acetyl homoserine-producing microorganism and evaluation thereof

The *pBAD24-gltA*-asRNA, an expression vector of antisense RNA of citrate synthase gene, was transformed into the *WCJM* strain, which is an *O*-acetyl homoserine-producing microorganism. Here, the transformed strain was designated as “*WCJM/A*-asRNA”. In particular, it was attempted to control the expression amount of the citrate synthase protein by controlling the expression amount of the antisense RNA of the citrate synthase, and here, the expression amount of the antisense RNA can be controlled according to the concentration of arabinose.

As a result, it was confirmed that the amount of the *O*-acetyl homoserine production

increased when the activity of the citrate synthase was attenuated as in Example 2.

Additionally, an Erlenmeyer flask-culture was performed to examine whether the amount of *O*-acetyl homoserine production increases as the expression amount of the citrate synthase decreases.

Specifically, *WCJM* and *WCJM/A-asRNA* strains were inoculated into LB media, and cultured at 33°C overnight. Then, a single colony thereof was inoculated into 3 mL of LB medium, cultured at 33°C for 5 hours, diluted 200-fold in a 250 mL Erlenmeyer flask containing 25 mL of an *O*-acetyl homoserine-producing medium. In particular, in order to control the expression amount of antisense RNA of the citrate synthase, arabinose was added at concentrations of 0 mM, 2 mM, and 5 mM, and cultured at 33°C at a rate of 200 rpm for 15 hours and 30 hours. The amount of *O*-acetyl homoserine production was examined via HPLC analysis, and the results are shown in Tables 7 and 8 below.

[Table 7]

15 Hours	Arabinose	OD (562nm)	Glucose consumption (g/L)	<i>O</i>-acetyl homoserine (g/L)
<i>WCJM</i>	0 mM	4.2	9.7	0.5
<i>WCJM</i>	2 mM	4.5	8.9	0.6
<i>WCJM</i>	5 mM	4.7	8.9	0.5
<i>WCJM/A-asRNA</i>	0 mM	4.5	10.1	0.6
<i>WCJM/A-asRNA</i>	2 mM	4.2	8.8	0.6
<i>WCJM/A-asRNA</i>	5 mM	3.4	6.9	0.5

[Table 8]

30 Hours	Arabinose	OD (562nm)	Glucose consumption (g/L)	<i>O</i>-acetyl homoserine (g/L)
<i>WCJM</i>	0 mM	8.9	32	1.4
<i>WCJM</i>	2 mM	9.1	34	1.3

<i>WCJM</i>	5 mM	8.9	33	1.3
<i>WCJM/A-asRNA</i>	0 mM	9.2	33	1.3
<i>WCJM/A-asRNA</i>	2 mM	8.8	32	1.6
<i>WCJM/A-asRNA</i>	5 mM	7.1	29	1.7

As a result, it was confirmed that, when cultured for 15 hours, the *WCJM/A-asRNA* strain showed a decrease in OD by about 1 according to the concentration of arabinose, whereas the concentration of *O*-acetyl homoserine was similar. However, when cultured for 30 hours, the *WCJM* strain, which is a control strain, showed the same OD and *O*-acetyl homoserine concentration even when the concentration of arabinose increased, whereas the *WCJM/A-asRNA* strain, which is a strain introduced with the expression vector for citrate synthase RNA antisense, showed a marked difference as the concentration of arabinose increased. The OD was 9.2 when the arabinose concentration was 0 mM, whereas the OD was 7.1 at 5 mM of the arabinose concentration, a decrease of 5.1, and the amount of *O*-acetyl homoserine increased by 30.8% although the glucose consumption was small. From these results, it was confirmed that not only the attenuation in citrate synthase activity but also in the attenuation in protein expression exhibit the same results.

Example 4: Attenuation and inactivation of citrate synthase activity in a microorganism with high production yield of *O*-acetyl homoserine

<4-1> Construction of a microorganism with high *O*-acetyl homoserine production yield with inactivated citrate synthase activity and evaluation thereof

International Publication No. WO 2012/087039 discloses in detail a method for constructing an *O*-acetyl homoserine-producing microorganism from a threonine-producing microorganism derived from a wild-type *W3110* strain, due to NTG mutation. In particular, the constructed strain producing *O*-acetyl homoserine with high yield was deposited at Korean Culture Center of Microorganisms (KCCM) under the Accession No of *KCCM 11146P*.

The *KCCM11146P* strain can consume 40 g/L of glucose during a flask-culture and produces about 15 g/L to 16 g/L of *O*-acetyl homoserine and is thus regarded as having high

O-acetyl homoserine production capability. Accordingly, in order to examine whether the strain produces a higher yield of *O*-acetyl homoserine when the citrate synthase activity is deleted, the same was applied to the *KCCM11146P* strain. The construction method was the same as in Example <1-1>, and by this method, the *KCCM11146P* strain, where the citrate synthase activity was deleted, was constructed and designated as “*KCCM11146P-AD*”.

The amount of *O*-acetyl homoserine production by the *KCCM11146P* strain, where the citrate synthase activity was deleted, was tested by an Erlenmeyer flask-culture. The *KCCM11146P* or *KCCM11146P-AD* strain was inoculated into an LB medium and cultured at 33°C overnight. Then, a single colony thereof was inoculated into 3 mL of LB medium, cultured at 33°C for 5 hours, diluted 200-fold in a 250 mL Erlenmeyer flask containing 25 mL of an *O*-acetyl homoserine-producing medium (with glutamate added), and cultured at 33°C at a rate of 200 rpm for 30 hours. The amount of *O*-acetyl homoserine production was examined via HPLC analysis, and the results are shown in Table 9 below.

[Table 9] Production of *O*-acetyl homoserine via flask-culture

	OD (562nm)	Glucose consumption (g/L)	<i>O</i> -acetyl homoserine (g/L)	Glutamate (g/L)
<i>KCCM11146P</i>	18.3	60	14.2	4.6
<i>KCCM11146P-AD</i>	14.6	60	16.7	1.8

The result of *O*-acetyl homoserine production via flask-culture revealed that the *KCCM11146P* strain produced 14.2 g/L of *O*-acetyl homoserine, and the *KCCM11146P-AD* strain produced 16.7 g/L of *O*-acetyl homoserine, a 17.6% increase, although it showed a decrease in absorbance (OD).

<4-2> Construction of a microorganism with high *O*-acetyl homoserine production yield with attenuated citrate synthase activity and evaluation thereof

In order to examine whether the *KCCM11146P* strain, which is a strain with high production yield of *O*-acetyl homoserine, produces a higher yield of *O*-acetyl homoserine even when the citrate synthase activity is attenuated, the modification on the 145th amino acid (from tyrosine (Y) to alanine (A)) and the modification on the 167th amino acid (from lysine (K) to alanine (A)), which showed the highest *O*-acetyl homoserine-producing capabilities among the three variant types attenuating the protein activities explained in Example <2-1>, were applied to

the *KCCM11146P* strain.

The construction method was the same as in Example <2-2>, and by the method, two *KCCM11146P* strains, where the citrate synthase activity was attenuated, were constructed and designated as “*KCCM11146P*-A145” and “*KCCM11146P*-A167”, respectively.

The amount of *O*-acetyl homoserine production by the two strains of *KCCM11146P*-A145 and *KCCM11146P*-A167, where the citrate synthase activity was attenuated, was tested by an Erlenmeyer flask-culture. The three strains, i.e., *KCCM11146P*-A145 and *KCCM11146P*-A167 strains and the *KCCM11146P* strain, were inoculated into LB media, and cultured at 33°C overnight. Then, a single colony thereof was inoculated into 3 mL of LB medium, cultured at 33°C for 5 hours, diluted 200-fold in a 250 mL Erlenmeyer flask containing 25 mL of an *O*-acetyl homoserine-producing medium, and cultured at 33°C at a rate of 200 rpm for 30 hours. The amount of *O*-acetyl homoserine production was examined via HPLC analysis, and the results are shown in Table 10 below.

[Table 10] Production of *O*-acetyl homoserine via flask-culture

	OD (562nm)	Glucose consumption (g/L)	<i>O</i>-acetyl homoserine (g/L)	Glutamate (g/L)
<i>KCCM11146P</i>	16.3	60	15.0	1.6
<i>KCCM11146P</i> -A145	14.6	60	17.5	0
<i>KCCM11146P</i> -A167	14.2	60	17.3	0

The result of *O*-acetyl homoserine production via flask-culture revealed that the *KCCM11146P* strain produced 15.0 g/L of *O*-acetyl homoserine, and the two strains of *KCCM11146P*-A145 and *KCCM11146P*-A167 showed similar results as in Example <2-3>. The two strains respectively produced 17.5 g/L and 17.3 g/L of *O*-acetyl homoserine, an increase of about 16.7%, although they both showed a decrease in absorbance (OD).

The strain with high production yield of *O*-acetyl homoserine also showed a decrease in glutamate from 1.6 g/L to 0 g/L, according to the decrease in TCA cycle flow caused by the attenuation in the citrate synthase activity.

These results demonstrate that citrate synthase activity enables production of *O*-acetyl

homoserine by applying the attenuated modification. Additionally, they also indicate that when a conversion reaction is conducted based on the *O*-acetyl homoserine, which was produced according to the International Publication No. WO2008/013432, as a template, and using a conversion enzyme, which additionally has the activities of cystathionine gamma synthase, *O*-succinylhomoserine sulfhydrylase, and *O*-acetyl homoserine sulfhydrylase, it is possible to simultaneously synthesize L-methionine and acetate.

The present inventors confirmed that the *KCCM11146P* strain, the variant on the 167th amino acid of the citrate synthase, has an improved production of *O*-acetyl homoserine, designated the *KCCM11146P-A167* strain as “CA05-4007”, and deposited it at the Korean Culture Center of Microorganisms (KCCM), an international depositary authority under the Budapest Treaty, on Nov. 22, 2013 (Accession No: *KCCM 11483P*).

From the foregoing, a skilled person in the art to which the present invention pertains will be able to understand that the present invention may be embodied in other specific forms without modifying the technical concepts or essential characteristics of the present invention. 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 invention. On the contrary, the present invention 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 invention 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> CA05-4007	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM11483P
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 November. 22. 2013. (date of the original deposit) ¹	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : Yurim B/D 45, Hongjena-e-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: November. 22. 2013.

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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한국미생물보존센터

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CLAIMS

1. An isolated microorganism of *Escherichia* sp. producing O-acetyl homoserine, wherein the endogenous activity of citrate synthase is attenuated or inactivated.
2. The isolated microorganism of *Escherichia* sp. according to claim 1, wherein the microorganism with the attenuated endogenous activity of citrate synthase has an amino acid sequence respresented by SEQ ID NO: 1 or SEQ ID NO: 2.
3. The isolated microorganism of *Escherichia* sp. according to claim 1, wherein the activity of cystathionine gamma synthase, homoserine kinase, or both is further attenuated or inactivated compared to their endogenous activities.
4. The isolated microorganism of *Escherichia* sp. according to claim 1, wherein the activity of homoserine O-acetyltransferase is further introduced or enhanced, or the endogenous homoserine O-succinyltransferase is further modified to have the activity of homoserine O-acetyltransferase.
5. The isolated microorganism of *Escherichia* sp. according to claim 1, wherein the activity of at least one protein selected from the group consisting of phosphoenolpyruvate carboxylase, aspartate aminotransferase, and aspartate semialdehyde dehydrogenase is further introduced or enhanced.
6. The isolated microorganism of *Escherichia* sp. according to claim 1, wherein the microorganism is *Escherichia coli*.
7. A method for producing O-acetyl homoserine, comprising:
 - (a) culturing the isolated microorganism of any of claims 1 to 6; and
 - (b) recovering O-acetyl homoserine produced during the cultivation of the microorganism.
8. A method for producing methionine, comprising:
 - (a) culturing the isolated microorganism of any of clams 1 to 6;
 - (b) recovering O-acetyl homoserine produced during the cultivation of the microorganism; and
 - (c) reacting the recovered O-acetyl homerine and methyl mercaptan by an enzyme reaction using an enzyme having an O-acetyl homoserine sulfhydrylase activity or a microorganism possessing the enzyme.