

[DESCRIPTION]

[Invention Title]

MICROORGANISM PRODUCING O-ACETYL-HOMOSERINE AND METHOD FOR PRODUCING O-ACETYLMOMOSERINE USING THE SAME

[Technical Field]

The present disclosure relates to a microorganism producing *O*-acetylhomoserine in high yield and a method for producing *O*-acetylhomoserine using the microorganism.

[Background Art]

O-acetylhomoserine acts as a precursor of methionine, which is a kind of essential amino acid in the body. Methionine is used not only as an additive for feeds and foods but also as a raw material for infusion solutions and pharmaceutical drugs.

Methionine is produced by chemical and biological syntheses. Recently, a two-step process for producing *L*-methionine from an *L*-methionine precursor, which was produced via fermentation, by enzymatic conversion was published (International Publication No. WO 2008/013432).

The two-step process employs *O*-succinylhomoserine and *O*-acetylhomoserine as precursors, and it is very important to produce *O*-acetylhomoserine in high yield for the purpose of economical mass production.

[Disclosure]

[Technical Problem]

Under the circumstances, the present inventors have made efforts to improve the production of *O*-acetylhomoserine, and as a result, they have discovered a protein capable of exporting *O*-acetylhomoserine, thereby completing the present disclosure.

[Technical Solution]

An object of the present disclosure is to provide a microorganism having enhanced *O*-acetylhomoserine productivity.

Another object of the present disclosure is to provide a method for efficient production

of *O*-acetylhomoserine using the microorganism having enhanced *O*-acetylhomoserine productivity.

[Advantageous Effects of the Invention]

The microorganism of the present disclosure with an enhanced activity of YjeH, *i.e.*, an inner membrane protein, has an improved ability of exporting *O*-acetylhomoserine and thus the efficiency of *O*-acetylhomoserine production can be increased. Therefore, the microorganism of the present disclosure can be widely used for the production of *O*-acetylhomoserine.

[Description of Drawings]

Fig. 1 shows a cleavage map of the *yjeH* vector (*pBAC-yjeH* vector) according to the present disclosure.

[Best Mode]

An aspect of the present disclosure provides a microorganism having *O*-acetylhomoserine productivity, in which the activity of YjeH, *i.e.*, an inner membrane protein, is enhanced compared to a non-modified microorganism.

As used herein, the term “*O*-acetylhomoserine” refers to an acetyl derivative of *L*-homoserine, which is a specific intermediate material in the biosynthetic pathway of methionine in microorganisms. *O*-Acetylhomoserine is known to be produced by a reaction between homoserine and acetyl-CoA catalyzed by homoserine acetyltransferase. It has a chemical formula of $C_6H_{11}NO_4$.

As used herein, the term “a microorganism having *O*-acetylhomoserine productivity” refers to a microorganism which, when cultured in a culture medium, can produce *O*-acetylhomoserine within a bioorganism and secrete it into the culture medium. The *O*-acetylhomoserine productivity may be provided or enhanced by improvement of species. Specifically, the microorganism having *O*-acetylhomoserine productivity may be a microorganism of the genus *Escherichia* having *O*-acetylhomoserine productivity, and more specifically, *E. coli*. For example, the microorganism may be *E. coli* having lysine, threonine, isoleucine, or methionine productivity, but is not limited thereto. As used herein, the term

“YjeH” is known as a protein which, being a member of the Amino Acid-Polyamine-Organocation (APC) family for amino acid transporters, is present in an inner membrane. YjeH is expected to act as an amino acid transporter but its exact function is not known yet. As such, the present inventors have confirmed first that YjeH specifically exports *O*-acetylhomoserine.

Specifically, YjeH may be derived from a microorganism of the genus *Escherichia*, and more specifically, from *E. coli*. In particular, YjeH may be a protein having an amino acid sequence of SEQ ID NO: 1, or any amino acid sequence having a homology of 70% or higher, specifically 80% or higher, or more specifically 90% or higher, to the amino acid sequence of SEQ ID NO: 1. Additionally, it is obvious that any amino acid sequence which has the same amino acid sequence as that of SEQ ID NO: 1, or any amino acid sequence having the activity of exporting *O*-acetylhomoserine, can belong to the scope of the present disclosure, although the sequence may have a partial deletion, modification, substitution, or addition therein. Additionally, based on genetic codon degeneracy, nucleotide sequences encoding the same amino acid and variants thereof belong to the scope of the present disclosure, for example, a nucleotide sequence represented by SEQ ID NO: 2, but are not limited thereto.

As used herein, the term “homology” refers to a degree of identity between two different sequences of nucleotides or amino acid residues in a particular comparison region of the nucleotide or amino acid sequences of a gene encoding a protein, after aligning both sequences to a maximal match. When the homology is sufficiently high, the expression products of the corresponding gene may have the same or similar activity. The homology may be determined using a sequence comparison program known in the art, *e.g.*, BLAST (NCBI), CLC Main Workbench (CLC bio), MegAlignTM (DNASTAR Inc.), *etc.*

As used herein, the term “a non-modified microorganism” refers to a microorganism which is not introduced with a modification in the activity of the corresponding protein, and the microorganism refers to a base strain to be introduced with the modification in the activity of the corresponding protein, which may be a native or modified microorganism.

As used herein, the term “enhancement” of a protein activity refers to improving the activity state of proteins possessed by a microorganism. The enhancement of a protein activity

is not limited as long as the activity of each protein can be enhanced compared to that of a non-modified microorganism, as is the case with the enhancement of the activity of a target protein. For example, the enhancement may be performed by methods selected from the group consisting of i) increasing the copy number of a polynucleotide encoding each protein, ii) modifying the expression control sequence for increasing the expression of the polynucleotide, iii) modifying the polynucleotide sequence on the chromosome for enhancing the activity of each protein; and iv) a combination thereof. Specifically, the enhancement of a protein activity may be performed by a method selected from the group consisting of a method of introducing a nucleotide sequence encoding each protein into the chromosome, a method of introducing the nucleotide sequence into a microorganism after introducing it into a vector system, a method of introducing a promoter exhibiting an improved activity into the upstream of the nucleotide sequence encoding each protein or introducing each protein with a modification on the promoter, a method of modifying the nucleotide sequence of the 5'-UTR region, and a method of introducing a variant of the nucleotide sequence encoding each protein, but the method is not limited thereto.

In a specific embodiment of the present disclosure, the YjeH activity may be enhanced compared to that of a non-modified microorganism, by increasing the copy number or enhancing the activity of a given promoter. Specifically, the YjeH activity may be enhanced by introducing a promoter exhibiting an improved activity to YjeH, which is an inner membrane. In a specific embodiment of the present disclosure, the promoter with an improved activity may include without limitation any promoter having an improved activity compared to that of the self-promoter of *yjeH*. Examples of such promoter may include without limitation any promoter of a gene having a higher activity of gene expression compared to that of the self-promoter of *yjeH*, or a modified promoter with an improved activity due to modification of the gene in the self-promoter of *yjeH*, etc. Specifically, the promoter of the present disclosure exhibiting an improved activity may be selected from the group consisting of *icd* promoter, *pro* promoter, and *cysk* promoter. Specifically, the *icd* promoter may consist of the nucleotide sequence of SEQ ID NO: 51; the *pro* promoter may consist of the nucleotide sequence of SEQ ID NO: 52; and the *cysk* promoter may consist of the nucleotide sequence of SEQ ID NO: 53, but each of the promoters may consist of a nucleotide sequence having a homology of 70% or higher, specifically 80% or higher, and more specifically 90% or higher, to each of the above

nucleotide sequences.

In a specific aspect of the present disclosure, the microorganism of the genus *Escherichia* having *O*-acetylhomoserine productivity may be one in which the activity of cystathionine synthase is further weakened or inactivated. Specifically, the microorganism may be one in which the activity of cystathionine synthase is weakened or inactivated compared to that of a non-modified microorganism, and specifically the gene, *metB* which encodes cystathionine synthase, is deleted, but is not limited thereto. The amino acid sequence of *metB* may be obtained from a known database, and any amino acid sequence having the activity of cystathionine synthase may be included without limitation, for example, it may refer to a protein having the amino acid sequence of SEQ ID NO: 3. The protein having the amino acid sequence of SEQ ID NO: 3 may be a protein encoded by the nucleotide sequence of SEQ ID NO: 4, but is not limited thereto. Additionally, in another aspect of the present disclosure, the microorganism of the genus *Escherichia* may be one in which the activity of homoserine kinase is further weakened or inactivated. Specifically, the microorganism may be one in which the activity of homoserine kinase is weakened or inactivated compared to the endogenous activity of a non-modified microorganism, and specifically one in which the gene, *thrB* which encodes homoserine kinase, is deleted, but is not limited thereto. The amino acid sequence of *thrB* may be obtained from a known database, and any amino acid sequence having the activity of homoserine kinase may be included without limitation, for example, it may refer to 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.

In the present disclosure, “weakening” of the activity of the protein may be performed by a method selected from the group consisting of i) deleting a part or entirety of the gene encoding each protein, ii) modifying the expression control sequence for decreasing the expression of the gene, iii) modifying the gene sequence on the chromosome for weakening the activity of the protein; and iv) a combination thereof, but is not limited thereto.

Specifically, the term “weakening of a protein activity” refers to a decrease in the activity of an enzyme compared to the endogenous activity of the enzyme possessed by a microorganism in its natural or base strain state. The weakening is a concept including a case when there is a decrease in the activity of an enzyme in a microorganism compared to that

originally possessed by the enzyme itself due to a modification of the enzyme-encoding gene, *etc.*, a case when the level of overall enzyme activity in a cell is lower than that of the wild-type strain due to inhibition of expression or inhibition of translation of the enzyme-encoding gene, or a combined case thereof, but is not limited thereto.

The “inactivation” refers to a case when the enzyme-encoding gene in a microorganism is not expressed at all and a case when the gene is expressed but exhibits no activity, compared to that of the wild-type strain.

The inactivation of an enzyme may be achieved by applying various methods known in the art. Examples of the methods may include a method of substituting the enzyme-encoding gene on the chromosome with a gene mutated to reduce the activity of the enzyme, including the case when the enzyme activity is eliminated; a method of introducing a modification in the expression control sequence of the enzyme-encoding gene on the chromosome; a method of substituting the expression control sequence of the enzyme-encoding gene with a sequence having weak or no activity; a method of deleting the entirety or a part of the enzyme-encoding gene 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 complementary sequence to the SD sequence into the upstream of the SD sequence of the enzyme-encoding gene, 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) of the corresponding sequence to induce a reverse transcription (reverse transcription engineering (RTE)), *etc.*, and also a combination thereof, but are not limited thereto.

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

As used herein, the term “a 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 a locus of a gene chain having a mutual homology.

Specifically, the expression regulatory sequence may be modified by inducing a modification of the expression control sequence by deletion, insertion, non-conservative or conservative substitution, or a combination thereof to further weaken the activity of the expression control sequence; or by substituting with a promoter having much 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 a weaker activity or a gene sequence which was improved to have no activity, but is not limited thereto.

In a specific embodiment of the present disclosure, the activity of each protein was weakened by deleting the *metB* gene encoding cystathionine synthase and/or the *thrB* gene by homologous recombination.

Additionally, in a specific embodiment of the present disclosure, the microorganism of the genus *Escherichia* may be one in which, additionally, the activity of homoserine acetyltransferase is enhanced compared to that of a non-modified microorganism. Specifically, the microorganism may be one in which the activity of homoserine acetyltransferase is increased compared to that of a non-modified microorganism, and in particular, the microorganism may be one in which a modified *metA* gene encoding homoserine acetyltransferase having an enhanced activity is introduced. The modified *metA* gene may be a gene encoding homoserine acetyltransferase, in which the 111th amino acid of homoserine acetyltransferase is substituted with glutamic acid and the 112th amino acid is substituted with histidine, and in particular, a gene which consists of the nucleotide sequence of SEQ ID NO: 8, but is not limited thereto. The modified *metA* gene may include without limitation any amino acid in which the activity of homoserine acetyltransferase is enhanced compared to that of wild-type, e.g., a protein having the amino acid sequence of SEQ ID NO: 7. An exemplary embodiment with respect to the preparation, utilization of the modified *metA* gene, a strain with an enhanced activity of

homoserine acetyltransferase, *etc.*, is disclosed in Korean Patent No. 10-1335841, and the entirety of the Korean Patent may be included as a reference to the present disclosure.

Additionally, in a specific aspect of the present disclosure, the microorganism of the genus *Escherichia* may be one in which the activity of aspartate kinase (EC 2.7.2.4) is enhanced compared to that of a non-modified microorganism. Specifically, the microorganism may be one in which the activity of aspartate kinase is increased compared to that of the endogenous activity of the microorganism, but is not limited thereto.

In a specific embodiment of the present disclosure, the biosynthetic pathway was further enhanced for the purpose of maximizing the *O*-acetylhomoserine productivity. Specifically, aspartate kinase and homoserine *O*-acetyltransferase were introduced using a plasmid, and the change in *O*-acetylhomoserine productivity was measured after further enhancing YjeH in the strain with an enhanced biosynthetic pathway for homoserine. As a result of the simultaneous enhancement of both biosynthetic pathway and *yjeH*, it was confirmed that the *O*-acetylhomoserine productivity was further improved. In particular, it was confirmed that when the YjeH activity was enhanced using the *cysK* promoter, the YjeH productivity was increased by about 93% (2.8 g/L -> 5.4 g/L) (Table 5).

Additionally, in a specific embodiment of the present disclosure, it was examined whether the enhancement of YjeH activity in an existing strain having high-yield *O*-acetylhomoserine productivity can further increase its *O*-acetylhomoserine productivity. More specifically, the amount of *O*-acetylhomoserine production was examined using a strain *KCCM11146P* (International Publication No. WO2012/087039) producing *O*-acetylhomoserine prepared from the strain having threonine productivity by NTG mutation, which a wild-type W3110-derived strain after substituting the promoter of the endogenous *yjeH* gene with a promoter with high expression-inducing activity. As a result, it was confirmed that the strain, which already has high-yield *O*-acetylhomoserine productivity, can further increase the activity of producing *O*-acetylhomoserine by the enhancement of YjeH activity by about 14% (14.2 g/L -> 18.2 g/L), and in particular, by the enhancement of YjeH activity using the *cysK* promoter (Table 7).

Information on the genes used in the present disclosure, the sequences of the proteins encoded by the genes, and promoter sequences can be obtained from a known database, *e.g.*,

NCBI GenBank, but is not limited thereto.

The genes encoding each of the proteins and promoters of the present disclosure include not only the nucleotide sequences represented by each of the SEQ ID NOS. but also include without limitation any gene sequence having a homology of 80% or higher, specifically 90% or higher, more specifically 95% or higher, even more specifically 98% or higher, and most specifically 99% or higher, to these sequences, as long as the gene sequence encodes an enzyme which exhibits an effect substantially the same as or corresponding to that of each of the enzymes described above. Additionally, it is obvious that any nucleotide sequence having the above sequence homology must also belong to the scope of the present disclosure, although the amino acid may have deletion, modification, substitution, or addition in part of the sequence.

Additionally, it is obvious that any amino acid constituting each of the above proteins of the present disclosure must also belong to the scope of the present disclosure although the amino acid may have deletion, modification, substitution, or addition in part of the sequence, as long as the amino acid has the same sequence represented by each of the SEQ ID NOS. or has a homology thereto while having an effect substantially the same as or corresponding to that of each of the proteins.

The effect of uniformly increasing *O*-acetylhomoserine productivity by the enhancement of YjeH activity was confirmed based on microorganisms having various genetic backgrounds and *O*-acetylhomoserine productivities. This may occur by promoting the production of a specific intermediate material by YjeH in the biosynthetic pathway. However, considering the characteristics of YjeH, which is an inner membrane and a kind of amino acid transporter family, the increase of *O*-acetylhomoserine productivity is thought to be achieved by increasing the ability of exporting *O*-acetylhomoserine, which is a final product and a kind of amino acid, thereby promoting intracellular reactions.

In still another aspect of the present disclosure, the present disclosure provides a method for producing *O*-succinylhomoserine including culturing a microorganism of the genus *Escherichia* producing *O*-acetylhomoserine of the present disclosure and recovering the cultured medium.

The medium used for culturing the microorganism of the present disclosure and other

culture conditions are not particularly limited but any medium used for the conventional culturing of the microorganism of the genus *Escherichia* may be used. Specifically, the microorganism of the present disclosure may be cultured in a conventional medium containing appropriate carbon sources, nitrogen sources, phosphorous sources, inorganic compounds, amino acids and/or vitamins, *etc.*, in an aerobic condition while adjusting temperature, pH, *etc.*

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

Examples of the nitrogen sources may include inorganic nitrogen sources (*e.g.*, ammonia, ammonium sulfate, ammonium chloride, ammonium carbonate, ammonium phosphate, 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 combination of at least two kinds, but are not limited thereto.

Examples of the phosphorus sources may include monopotassium phosphate, dipotassium phosphate, and corresponding sodium-containing salts. 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. These media or precursors may be added in a batch culture process or a continuous culture process to a culture, but are not limited thereto.

In the present disclosure, the pH of a culture may be adjusted during the cultivation by adding a compound such as ammonium hydroxide, potassium hydroxide, ammonia, phosphoric acid, and sulfuric acid to the culture in an appropriate manner. During the cultivation, an antifoaming agent, such as fatty acid polyglycol ester, may also be added to prevent foam

generation. Additionally, oxygen or an oxygen-containing gas may be injected into the culture in order to maintain an aerobic state of the culture; or nitrogen, hydrogen, or carbon dioxide gas may be injected without the injection of a gas in order to maintain an anaerobic or microaerobic state of the culture.

The culture temperature may generally be in a range from 27°C to 37°C, and more specifically, from 30°C to 35°C, but is not limited thereto. The cultivation may be continued until the desired amount of useful materials is obtained, and specifically for from 10 hours to 100 hours, but is not limited thereto.

The method of producing *O*-acetylhomoserine of the present disclosure may further include a method of recovering *O*-acetylhomoserine from the cultured microorganism or the cultured medium.

Specifically, the recovering of *O*-acetylhomoserine may be performed by a method of culturing microorganisms of the present disclosure, for example, an appropriate method known in the art such as a batch process, a continuous batch process, or a fed-batch process.

The recovering may include a purification process.

The thus-recovered *O*-acetylhomoserine may be used to produce methionine by a two-step process developed by the present inventors (Korean Patent No. 10-0905381), *i.e.*, a two-step process.

The two-step process includes a process for producing *L*-methionine and organic acids by an enzymatic reaction using an enzyme having the activity of *O*-acetylhomoserine sulfhydrylase or a strain containing the enzyme by utilizing *O*-acetylhomoserine produced by a strain producing the *L*-methionine precursor and methyl mercaptan as substrates.

More specifically, the present disclosure provides a method for producing *L*-methionine by an enzyme reaction using an enzyme such as *O*-acetylhomoserine sulfhydrylase by utilizing *O*-acetylhomoserine accumulated by the above method as a substrate.

In the two-step process, when *O*-acetylhomoserine is used as a precursor of *L*-methionine, specifically, *O*-acetylhomoserine sulfhydrylase derived from a microorganism strain belonging to the genus *Leptospira*, the genus *Chromobacterium*, and the genus *Hyphomonas*, and more specifically, from a microorganism strain belonging to *Leptospira meyeri*, *Pseudomonas aurogenosa*, *Hyphomonas neptunium*, and *Chromobacterium violaceum* may be used.

The reaction is shown below.



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

[Mode for Invention]

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

Example 1: Preparation of strains having *O*-acetylhomoserine productivity

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

For the preparation of a strain producing *O*-acetylhomoserine, *E. coli*, a representative microorganism of the genus *Escherichia*, was used. To this end, a wild-type *E. coli* (*K12*) *W3110* (ATCC27325) was obtained from the American Type Culture Collection (ATCC) and used. First, for blocking the pathway of producing cystathionine from *O*-succinyl-*L*-homoserine, the *metB* gene (SEQ ID NO: 4) encoding cystathionine synthase was deleted.

Specifically, an FRT one-step PCR deletion method was used for the deletion of *metB* gene (Wanner BL., *Proc. Natl. Acad. Sci. USA* 97: 6640 - 6645, 2000). A deletion cassette was prepared by performing PCR based on the *pKD3* vector (Wanner BL., *Proc. Natl. Acad. Sci. USA* 97: 6640 - 6645, 2000) as a template using the primers TKd of SEQ ID NOS: 13 and 14. In particular, PCR was performed for a total of 30 cycles under the following conditions: denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min.

The thus-obtained PCR product was electrophoresed on a 1.0% agarose gel, and the DNA obtained from a 1.2 kb band was purified. The recovered DNA fragment was introduced into the *E. coli* (*K12*) *W3110* strain, which was already transformed with the *pKD46* vector (Wanner BL., *Proc. Natl. Acad. Sci. USA* 97: 6640 - 6645, 2000), by electroporation. The *W3110* strain transformed with *pKD46* was cultured using LB medium containing ampicillin

(100 µg/L) and *L*-arabinose (5 mM) until the culture reached an OD₆₀₀ = 0.6 at 30°C, and used after washing twice with sterile distilled water and once with 10% glycerol. The electroporation was performed at 2500 V. The recovered strain was spread on LB plate medium containing chloramphenicol (25 µg/L), cultured overnight at 37°C, and the resistant strains were selected.

PCR was performed using the thus-selected strain as a template along with the same primers (SEQ ID NOS: 13 and 14), and the deletion of the *metB* gene was confirmed by observing the presence of a 1.2 kb gene band on a 1.0% agarose gel. The strain, in which the deletion of *metB* gene was confirmed, was transformed with *pCP20* vector (Wanner BL., *Proc. Natl. Acad. Sci. USA* 97: 6640 - 6645, 2000) and cultured in the same LB medium. Then, PCR was performed under the same conditions followed by electrophoresis on a 1.0% agarose gel to observe the presence of a 1.2 kb gene band, thus confirming the deletion of *metB* gene. The strain, upon confirmation of the deletion, was transformed with *pCP20* vector (Wanner BL., *Proc. Natl. Acad. Sci. USA* 97: 6640 - 6645, 2000) and cultured in the same LB medium. Then, PCR was performed under the same conditions followed by electrophoresis on a 1.0% agarose gel to select the final strain with the deletion of *metB* gene represented by the gene band, which was reduced to a size of 150 bp, thereby confirming the removal of the chloramphenicol marker from the strain.

The thus prepared and selected strain, with the deletion of *metB* gene, encoding cystathionine synthase was named as “*W3-B*”.

1-2. Deletion of *thrB* gene

For increasing the amount of *O*-succinylhomoserine synthesis from the *W3-B* strain prepared in Example 1-1, *thrB* gene (SEQ ID NO: 6) encoding homoserine kinase was deleted from the strain. For the deletion of *thrB* gene, the same FRT one-step PCR deletion method, which was used in the deletion of *metB* gene in Example 1-1, was used.

First, for the preparation of a *thrB* deletion cassette, PCR was performed using the *pKD4* vector (Wanner BL., *Proc. Natl. Acad. Sci. USA* 97: 6640 - 6645, 2000) as a template and thereby a deletion cassette was prepared. Specifically, PCR was performed using the primers of SEQ ID NOS: 15 and 16 for a total of 30 cycles under the following conditions: denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min.

The thus-obtained PCR product was electrophoresed on a 1.0% agarose gel, and the

DNA obtained from a 1.6 kb band was purified. The recovered DNA fragment was introduced into the *W3-B* strain, which was already transformed with the *pKD46* vector by electroporation. The recovered strain was spread on LB plate medium containing kanamycin (50 µg/L), cultured overnight at 37°C, and the resistant strains were selected.

PCR was performed under the same conditions using the strain selected through the above process as a template along with primers (SEQ ID NOS: 15 and 16), and the deletion of the *thrB* gene was confirmed by observing the presence of a 1.6 kb gene on a 1.0% agarose gel. The strain confirmed was transformed again with the *pCP20* vector and cultured on LB medium, and then, PCR was performed under the same conditions followed by electrophoresis on a 1.0% agarose gel to select the final strain with the deletion of *thrB* gene represented by the gene band, which was reduced to a size of 150 bp, thereby confirming the removal of the kanamycin marker from the strain. The thus-prepared and selected strain, with the deletion of *thrB* gene, encoding homoserine kinase was named as “*W3-BT*”.

Exemplary embodiments with respect to the strains with the deletion of *metB* gene and *thrB* gene, etc., are disclosed in Korean Patent No. 10-0905381 or International Publication No. WO 2008/013432, and the entire specification of the Korean patent or the international patent publication may be included herein as a reference to the present disclosure.

1-3. Preparation of a strain with modified *metA* gene having an activity of homoserine acetyltransferase

For enhancing the activity of homoserine acetyltransferase in the strain prepared in Example 1-2, an attempt was made to introduce a modified *metA* gene (SEQ ID NO: 8), which encodes homoserine acetyltransferase with an enhanced activity, to the strain.

In this regard, for the preparation of a modified *metA* gene with an enhanced activity, the *metA* gene was first amplified and obtained by PCR using the chromosome of the *W3110* strain as a template along with primers (SEQ ID NOS: 17 and 18). The primers (SEQ ID NOS: 17 and 18) used in the PCR were prepared to include an *EcoRV* restriction site and a *HindIII* restriction site, respectively, based on the nucleotide sequence of *E. coli* chromosome of NC_000913 registered in the NIH GenBank.

The thus-obtained PCR product and the *pCL1920* plasmid including *pcj1* were treated with *EcoRV* and *HindIII* and cloned. *E. coli DH5α* was transformed with the cloned plasmid and cultured in LB plate medium containing spectinomycin (50 µg/L), and the transformed *E.*

coli DH5α was selected and thereby the plasmid was obtained. The thus-obtained plasmid was named as “*pCL_Pcj1_metA*”.

A modified *metA* gene was prepared using the site-directed mutagenesis kit (Stratagene, USA) based on the *pCL_Pcj1_metA* obtained above. Specifically, the 111th amino acid (Gly) of homoserine acetyltransferase was substituted with glutamic acid (Glu) (G111E). The thus-prepared plasmid was named as “*pCL_Pcj1_metA(EL)*”.

Additionally, the 111th amino acid (Gly) of homoserine acetyltransferase was substituted with glutamic acid (Glu), and additionally, the 112th amino acid (Leu) was substituted with histidine (His) using the primers (SEQ ID NOS: 21 and 22). Accordingly, the plasmid including the *metA* gene, in which the 111th amino acid was changed from glycine to glutamic acid and the 112th amino acid was changed from leucine to histidine, was named as “*pCL_Pcj1_metA(EH)*”.

Then, for preparing a replacement cassette to substitute with the modified *metA* gene after introducing it into a strain, PCR was performed using the *pKD3* vector as a template along with primers (SEQ ID NOS: 27 and 28) for a total of 30 cycles under the following conditions: denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 2 min. The *metA(EH)* part of the replacement cassette was obtained as a PCR product using *pCL-Pcj1-metA(EH)* as a template along with primers (SEQ ID NOS: 23 and 24) and the part of the *metA* wild-type was obtained using primers (SEQ ID NOS: 25 and 26), respectively. The three PCR products were used for the preparation of the *metA(EH)* replacement cassette, which includes a chloramphenicol marker, using primers (SEQ ID NOS: 23 and 26), and the modified *metA* gene was introduced into the *W3-BT* strain, which was already transformed with *pKD46* vector, prepared in Example 1-2 by electroporation. The strain, in which the introduction was confirmed, was again transformed with *pCP20* vector, cultured in LB medium, and the strain in which the chloramphenicol marker was removed and the *metA* gene was substituted with *metA(EH)* was named as “*W3-BTA*”.

Exemplary embodiments with respect to the strains with enhanced activity of homoserine acetyltransferase, *etc.*, are disclosed in Korean Patent No. 10-1335841 or International Publication No. WO 2012/087039, and the entire specification of the Korean patent or the international patent publication may be included herein as a reference to the present disclosure.

1-4. Preparation of strains including 2 copies of *ppc*, *aspC*, and *asd* genes

For increasing the *O*-acetylhomoserine productivity of *W3-BTA* prepared in Example 1-3, the existing strategy for enhancing biosynthetic pathway was introduced. Efforts were made to prepare a strain, in which 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 aspartate-semialdehyde dehydrogenase (*i.e.*, *ppc* gene, *aspC* gene, and *asd* gene) were amplified into 2 copies.

Accordingly, *ppc* gene was amplified into 2 copies using the primers of SEQ ID NOS: 29, 30, 31, and 32; *aspC* gene was amplified into 2 copies using the primers of SEQ ID NOS: 33 and 34; and *asd* gene was amplified into 2 copies using the primers of SEQ ID NOS: 35, 36, 37, and 38, respectively.

The strain, in which the biosynthetic pathway for *O*-acetylhomoserine was enhanced based on *W3-BTA* strain by the above process, was named as “*W3-BTA2PCD* (= *WCJM*)”.

Exemplary embodiments with respect to the strains with enhanced activity of homoserine acetyltransferase, *etc.*, are disclosed in Korean Patent No. 10-0905381 or International Publication No. WO 2008/013432, and the entire specification of the Korean patent or the international patent publication may be included herein as a reference to the present disclosure.

1-5. Experiment of flask culture

For the experiment of the amount of *O*-acetylhomoserine production by the strains prepared in Examples 1-3 and 1-4, an Erlenmeyer flask culture was performed. The *W3110*, *W3-BTA*, and *WCJM* strains were inoculated into LB medium, cultured at 33°C overnight. Then, a single colony was inoculated into LB medium (3 mL), cultured at 33°C for 5 hours, again diluted 200-fold in a 250 mL Erlenmeyer flask containing 25 mL of medium for producing *O*-acetylhomoserine, cultured at 33°C at a rate of 200 rpm for 30 hours, and the amount of *O*-acetylhomoserine production was confirmed by HPLC analysis. The medium composition used is summarized in Table 1 below.

[Table 1] Composition for flask medium for 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 strains were cultured in the above medium for 30 hours and the amounts of *O*-acetylhomoserine production were confirmed by HPLC analysis. The results are shown in Table 2 below.

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

	OD (562 nm)	Glucose Consumption (g/L)	<i>O</i> -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 confirmed from Table 2, wild-type *W3110* strain did not produce *O*-acetylhomoserine at all, however, the *W3-BTA* strain produced *O*-acetylhomoserine in a concentration of 0.9 g/L, and the *WCJM* strain, in which the biosynthetic pathway is enhanced, produced *O*-acetylhomoserine in a concentration of 1.2 g/L.

Example 2: Identification of membrane proteins increasing the *O*-acetylhomoserine productivity

The present inventors have applied yjeH (SEQ ID NO: 1), of which the association with *O*-acetylhomoserine exportation and *O*-acetylhomoserine productivity has never been disclosed.

The *yjeH* gene of a strain was enhanced by cloning the *yjeH* gene into the *bac* vector using the *Hind*III restriction site present in the *bac* vector. For the *bac* vector, the CopyControl BAC Cloning kit (Cat. No. CCBAC1H-*Hind*III, Epicentre) was used.

First, for obtaining the *yjeH* gene, PCR was performed using primers (SEQ ID NOS: 9 and 10) for a total of 30 cycles under the following conditions: denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 68°C for 1 min. The resulting PCR product was electrophoresed on a 1.0% agarose gel, and the DNA obtained from a 1.2 kb band was purified. The purified DNA was treated with *Hind*III at 37°C overnight, purified one more time, and *yjeH* and the *BAC* vector were cloned using T4 ligase. *E. coil DH5α* was transformed with the cloned plasmid, and the transformed *E. coil DH5α* was selected from LB plate medium containing chloramphenicol 50 µg/mL. The thus-prepared plasmid was introduced into *W3-BTA* and *WCJM* strains, which produce *O*-acetylhomoserine, and the flask evaluation on their *O*-acetylhomoserine productivity was performed.

The resulting PCR product was electrophoresed on a 1.0% agarose gel, and the DNA obtained from a 1.2 kb band was purified. The purified DNA was treated with *Hind*III at 37°C overnight, purified one more time, and *yjeH* and the *BAC* vector were cloned using T4 ligase. *E. coil DH5α* was transformed with the cloned plasmid, and the transformed *E. coil DH5α* was selected from LB plate medium containing chloramphenicol 50 µg/mL and a plasmid was obtained therefrom. The thus-prepared plasmid was introduced into *W3-BTA* and *WCJM* strains, which produce *O*-acetylhomoserine, and the flask evaluation on their *O*-acetylhomoserine productivity was performed.

Specifically, each strain was spread on solid LB medium and cultured in a 33°C incubator overnight. Single colonies for each strain cultured on solid LB medium overnight were inoculated to LB medium (3 mL), cultured at 33°C for 5 hours. Again, the resultant was diluted 200-fold in a 250 mL Erlenmeyer flask containing 25 mL of medium for producing *O*-acetylhomoserine, cultured at 33°C at a rate of 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)	<i>O</i> -AH (g/L)

<i>W3-BTA/pBAC</i>	9.5	35	0.8
<i>WCJM/pBAC</i>	9.6	35	1.2
<i>W3-BTA/pBAC-yjeH</i>	9.8	36	1.5
<i>WCJM/pBAC-yjeH</i>	10.1	37	2.3

As can be confirmed from Table 3, the introduction of the *yjeH* plasmid into the *WCJM* strain resulted in a higher OD value compared to that of the control strain, and also a higher rate of glucose consumption. The *WCJM/pBAC-yjeH* strain produced *O*-acetylhomoserine in a concentration of 2.3 g/L, thus confirming that the strain can have an increased *O*-acetylhomoserine productivity by the *yjeH* introduction.

Example 3: Preparation of a plasmid with enhanced *yjeH* promoter and evaluation of *O*-acetylhomoserine productivity

3-1. Preparation of a plasmid with enhanced *yjeH* promoter

An experiment was performed to replace the endogenous *yjeH* promoter with 3 different promoters having strong expression-inducing activities compared to that of the endogenous *yjeH* promoter, based on the plasmid prepared in Example 2.

Specifically, *PCL* vectors with promoters having an enhanced activity were prepared using *pro*, *cysk*, or *icd* promoter. The *PCL* vectors were prepared using the *Sma*I restriction site of the *PCL* vector, and the *icd* promoter (SEQ ID NO: 51) was prepared by PCR amplification using the primers of SEQ ID NOS: 39 and 40; the *pro* promoter (SEQ ID NO: 52) using the primers of SEQ ID NOS: 41 and 42; and the *cysk* promoter (SEQ ID NO: 53) using the primers of SEQ ID NOS: 43 and 44. The thus-prepared plasmids were introduced into the *WCJM* strain, respectively, and their *O*-acetylhomoserine productivities were evaluated in flasks.

Specifically, each strain was spread on LB plate medium and cultured in a 33°C incubator overnight. Each strain cultured overnight on LB plate medium was inoculated into titer medium 25 mL and cultured in a 33°C incubator at 200 rpm overnight. The results are shown in Table 4 below.

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

	OD (562 nm)	Glucose Consumption (g/L)	<i>O</i> -AH (g/L)
--	----------------	------------------------------	-----------------------

<i>WCJM/pCL1920</i>	9.6	35	1.2
<i>WCJM/pCL-yjeH</i>	10.1	37	2.3
<i>WCJM/pCL-Picd-yjeH</i>	10.5	38	3.1
<i>WCJM/pCL-Ppro-yjeH</i>	10.7	38	3.5
<i>WCJM/pCL-Pcysk-yjeH</i>	9.4	39	4.4

As can be confirmed from Table 4, the strain introduced with the *pCL-Pcysk-yjeH* plasmid showed a decrease in OD value compared to that of the self-promoter, but the strain showed a higher rate of glucose consumption and the highest *O*-acetylhomoserine production (4.4 g/L).

3-2. Preparation of a plasmid for enhancing a gene and promoter in a biosynthetic pathway

For maximizing *O*-acetylhomoserine productivity, a plasmid for enhancing the biosynthetic pathway up to homoserine was prepared. For cloning aspartate kinase, homoserine *O*-acetyltransferase, and *yjeH* into the PCL vector, the *pCL-thrA-metX* plasmid, which was already prepared, was used.

Specifically, for obtaining the *yjeH* gene, PCR was performed using the primers (SEQ ID NOS: 11 and 12) for a total of 30 cycles under the following conditions: denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 68°C for 1 min.

The resulting PCR product was electrophoresed on a 1.0% agarose gel, and the DNA obtained from a 1.2 kb band was purified. The purified DNA was treated with *KpnI* at 37°C overnight, purified one more time, and *yjeH* and the *BAC* vector were cloned using T4 ligase. *E. coil DH5α* was transformed with the cloned plasmid, and the transformed *E. coil DH5α* was selected from LB plate medium containing spectinomycin 50 µg/mL and plasmid was obtained therefrom. The thus-prepared plasmid was introduced into the *WCJM* strain, which produces *O*-acetylhomoserine, and the flask evaluation on their *O*-acetylhomoserine productivity was performed. The thus-prepared plasmids were all 3 kinds and they were prepared using the 3 different promoters prepared in Example 3-1. The 3 different kinds of plasmids were introduced into the *WCJM* strain and the flask evaluation was performed in the same manner as in Example 3-1. 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)	<i>O</i> -AH (g/L)
<i>WCJM/pC2</i>	9.6	35	1.5
<i>WCJM/pC2-yjeH</i>	10.1	37	2.8
<i>WCJM/pC2-Picd-yjeH</i>	10.5	38	4.2
<i>WCJM/pC2-Ppro-yjeH</i>	10.7	38	4.5
<i>WCJM/pC2-Pcysk-yjeH</i>	9.4	39	5.4

As can be confirmed from Table 5, when the biosynthetic pathway and *yjeH* were enhanced simultaneously, the *O*-acetylhomoserine production was improved further. The order of the increase of the *O*-acetylhomoserine production was as follows: in the same manner as described above, the strain introduced with the *pC2-Pcysk-yjeH* plasmid showed a decrease in OD value compared to that of the strain, in which the self-promoter was used, but the strain introduced with the *pC2-Pcysk-yjeH* plasmid showed the highest glucose consumption rate, and also the highest *O*-acetylhomoserine production (5.4 g/L).

Example 4: Preparation of a strain with enhanced endogenous *yjeH* promoter and evaluation of *O*-acetylhomoserine productivity

4-1. Preparation of a strain with enhanced endogenous *yjeH* and evaluation thereof

For preparing a strain with an enhanced activity of the endogenous *yjeH* gene of the *WCJM* strain, which produces *O*-acetylhomoserine, an experiment of replacing a promoter was performed. The *WCJM* strain of the present disclosure has one copy of the *yjeH* gene, and the strain was prepared to increase the *yjeH* gene by enhancing the promoter instead of increasing the copy number of the *yjeH* gene.

Specifically, as a promoter for the replacement, the *icd*, *cysK*, and *pro* promoters (*Picd*, *Pcysk*, and *Ppro*), whose activities were confirmed in Example 3, and the FRT one-step PCR deletion method specified above was used (Wanner BL., *Proc. Natl. Acad. Sci. USA* 97: 6640 - 6645, 2000). Insertion cassettes were prepared using the *pKD4* vector as a template along with the primers of SEQ ID NOS: 45 and 46 for the *icd* promoter, the primers of SEQ ID NOS: 47 and 48 for the *cysk* promoter, and the primers of SEQ ID NOS: 49 and 50 for the *pro* promoter. PCR was performed for a total of 30 cycles under the following conditions: denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min.

The thus-obtained PCR product was electrophoresed on a 1.0% agarose gel, and the DNA obtained from a 2.5 kb band was purified. The recovered DNA fragment was introduced into the *WCJM* strain, which was already transformed with the *pKD46* vector (Wanner BL., *Proc. Natl. Acad. Sci. USA* 97: 6640 - 6645, 2000), by electroporation. The *WCJM* strain transformed with *pKD46* was cultured using LB medium containing ampicillin 100 µg/L and *L*-arabinose 5 mM until the culture reached an OD₆₀₀ = 0.6 at 30°C, and used after washing twice with sterile distilled water and once with 10% glycerol. The electroporation was performed at 2500 V. The recovered strain was spread on LB plate medium containing chloramphenicol 25 µg/L, cultured overnight at 37°C, and the resistant strains were selected.

PCR was performed using the thus-selected strain as a template along with the primers of SEQ ID NOS: 45 and 46 for the *icd* promoter, the primers of SEQ ID NOS: 47 and 48 for the *cysk* promoter, and the primers of SEQ ID NOS: 49 and 50 for the *pro* promoter under the same conditions described above, and the replacement of the endogenous promoter of the *yjeH* gene with each of the foreign promoter was confirmed by observing the presence of a 2.5 kb gene band on a 1.0% agarose gel. The strains, in which the promoter replacement was confirmed, were transformed with the *pCP20* vector (Wanner BL., *Proc. Natl. Acad. Sci. USA* 97: 6640 - 6645, 2000) and cultured in LB medium. Then, PCR was performed under the same conditions followed by electrophoresis on a 1.0% agarose gel to prepare the final strains with the promoter replacement represented by the gene band, which was reduced to a size of 1 kb, thereby confirming the removal of the kanamycin marker from the strains. The thus-prepared strains were named according to their respective promoter: *i.e.*, “*WCJM-PIY*” for the strain with the *icd* replaced promoter of *icd*, “*WCJM-PCY*” for the strain with the replaced promoter of *cysk*, and “*WCJM-PPY*” for the strain with the replaced promoter of *pro*. The *O*-acetylhomoserine productivities of the strains, in which the promoter of the *yjeH* gene was replaced were measured by performing flask culture evaluation and the results are shown in Table 6 below.

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

	OD (562 nm)	Glucose Consumption (g/L)	<i>O</i> -AH (g/L)
<i>WCJM</i>	9.6	35	1.2
<i>WCJM-PIY</i>	9.2	38	1.8
<i>WCJM-PCY</i>	10.5	38	3.1
<i>WCJM-PPY</i>	10.1	38	1.9

As can be confirmed from Table 6, when the *yjeH* gene expression of the WCJM strain was enhanced by replacing the endogenous promoter of the *yjeH* gene within the chromosome with a promoter having a strong expression activity, the *O*-acetylhomoserine productivity was not rapidly changed compared to the result of Example 3, in which the strain was transformed by introducing with a plasmid (5 copies), but the *O*-acetylhomoserine productivity of each of the strains was shown to increase compared to that of the WCJM strain, which is the strain producing *O*-acetylhomoserine.

4-2. Preparation of a strain with an enhanced promoter of the *yjeH* gene in a strain with a high yield of *O*-acetylhomoserine and evaluation of *O*-acetylhomoserine productivity of the strain

The method of preparing a strain producing *O*-acetylhomoserine using a wild-type W3110-derived strain, which has threonine productivity by NTG mutation, is known (International Publication No. WO 2012/087039). In particular, the thus-prepared strain producing *O*-acetylhomoserine in high yield was deposited at Korean Culture Center of Microorganisms (KCCM) with Accession No. KCCM11468P.

The strain with Accession No. KCCM11146P has high-yield *O*-acetylhomoserine productivity, which consumes 40 g/L of glucose and produces about 15 g/L to 16 g/L during flask culture. In this regard, the present inventors examined whether the strains, which already have high *O*-acetylhomoserine productivity, can further improve their *O*-acetylhomoserine productivity by enhancing the *yjeH* gene in the strains.

Specifically, the promoter of the *yjeH* gene was replaced with a promoter with a high expression-inducing activity, and this was performed in the same manner as in Example 4-1. The thus-prepared strains, in which the promoter of the *yjeH* gene of the KCCM11146P strain, were named as “KCCM11146P-PIY” for the strain with the replaced promoter of *icd*, “KCCM11146P-PCY” for the strain with the replaced promoter of *cysK*, and “KCCM11146P-PPY” for the strain with the replaced promoter of *pro*, respectively.

The *O*-acetylhomoserine productivities of the strains, in which the promoter of the *yjeH* gene was replaced, were measured by flask culture evaluation. Specifically, KCCM11146P, KCCM11146P-PIY, KCCM11146P-PCY, and KCCM11146P-PPY strains were inoculated into LB medium and cultured at 33°C overnight. The resulting single colonies were 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 medium for producing *O*-acetylhomoserine, cultured at 33°C for at a rate of 200 rpm for 30 hours, and the amount of *O*-acetylhomoserine production was analyzed by HPLC. The results are shown in Table 7 below.

[Table 7] Measurement of *O*-acetylhomoserine production by flask culture

	OD (562 nm)	Glucose Consumption (g/L)	<i>O</i> -AH (g/L)
<i>KCCM11146P</i>	18.3	40	14.2
<i>KCCM11146P-PIY</i>	16.2	40	16.3
<i>KCCM11146P-PCY</i>	19.2	40	18.2
<i>KCCM11146P-PPY</i>	18.8	40	16.2

As can be confirmed from Table 7, the *KCCM11146P* strain produced 14.2 g/L of *O*-acetylhomoserine, whereas, in the cases of the strains with a replaced promoter, the PCY strain showed the highest production (18.2 g/L) and the *PIY* and *PPY* strains showed an increased production of *O*-acetylhomoserine compared to that of the original strain.

The present inventors have confirmed that the strain based on the *KCCM11146P* strain, in which the *yjeH* gene activity is enhanced, can increase *O*-acetylhomoserine production. As a result, the inventors have named the strain as “CA05-4008”, and deposited the strain with the Korean Culture Center of Microorganisms (KCCM), under the Budapest Treaty, on November 22, 2013, with Accession No. *KCCM11484P*.

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.

[CLAIMS]

[Claim 1]

A microorganism of the genus *Escherichia* producing *O*-acetylhomoserine, wherein an activity of the protein comprising the amino acid sequence of SEQ ID NO: 1 is enhanced compared to a non-modified microorganism.

[Claim 2]

The microorganism of the genus *Escherichia* according to claim 1, wherein the microorganism is *Escherichia coli*.

[Claim 3]

The microorganism of the genus *Escherichia* according to claim 1, wherein an activity of cystathionine synthase is further weakened or inactivated.

[Claim 4]

The microorganism of the genus *Escherichia* according to claim 1, wherein an activity of homoserine kinase is further weakened or inactivated.

[Claim 5]

The microorganism of the genus *Escherichia* according to claim 1, wherein an activity of homoserine acetyltransferase is further enhanced compared to a non-modified microorganism.

[Claim 6]

The microorganism of the genus *Escherichia* according to claim 1, wherein the activity of at least one enzyme selected from the group consisting of phosphoenolpyruvate carboxylase, aspartate aminotransferase, and aspartate semialdehyde dehydrogenase is further enhanced.

[Claim 7]

The microorganism of the genus *Escherichia* according to claim 1, wherein an activity of aspartate kinase is enhanced compared to a non-modified microorganism.

[Claim 8]

A method for producing *O*-succinylhomoserine comprising
culturing the microorganism of the genus *Escherichia* producing *O*-acetylhomoserine of
any of claims 1 to 7 to obtain a cultured medium.

[Claim 9]

The method according to claim 8, further comprising recovering *O*-acetylhomoserine
from the cultured microorganism or the cultured medium.

OPA15135_Sequence Listing

<110> CJ CheilJedang Corporation

<120> Microorganism producing O-acetyl-homoserine and method for producing O-Acetyl-homoserine using the same

<130> OPA15135

<150> KR10-2014-0068613

<151> 2014-06-05

<160> 53

<170> KopatentIn 2.0

<210> 1

<211> 418

<212> PRT

<213> E.coli yjeH

<400> 1

Met Ser Gly Leu Lys Gln Glu Leu Gly Leu Ala Gln Gly Ile Gly Leu
1 5 10 15

Leu Ser Thr Ser Leu Leu Gly Thr Gly Val Phe Ala Val Pro Ala Leu
20 25 30

Ala Ala Leu Val Ala Gly Asn Asn Ser Leu Trp Ala Trp Pro Val Leu
35 40 45

Ile Ile Leu Val Phe Pro Ile Ala Ile Val Phe Ala Ile Leu Gly Arg
50 55 60

His Tyr Pro Ser Ala Gly Gly Val Ala His Phe Val Gly Met Ala Phe
65 70 75 80

Gly Ser Arg Leu Glu Arg Val Thr Gly Trp Leu Phe Leu Ser Val Ile
85 90 95

Pro Val Gly Leu Pro Ala Ala Leu Gln Ile Ala Ala Gly Phe Gly Gln
100 105 110

Ala Met Phe Gly Trp His Ser Trp Gln Leu Leu Leu Ala Glu Leu Gly
115 120 125

Thr Leu Ala Leu Val Trp Tyr Ile Gly Thr Arg Gly Ala Ser Ser Ser
130 135 140

Ala Asn Leu Gln Thr Val Ile Ala Gly Leu Ile Val Ala Leu Ile Val
145 150 155 160

Ala Ile Trp Trp Ala Gly Asp Ile Lys Pro Ala Asn Ile Pro Phe Pro
165 170 175

Ala Pro Gly Asn Ile Glu Leu Thr Gly Leu Phe Ala Ala Leu Ser Val
180 185 190

Met Phe Trp Cys Phe Val Gly Leu Glu Ala Phe Ala His Leu Ala Ser
195 200 205

Glu Phe Lys Asn Pro Glu Arg Asp Phe Pro Arg Ala Leu Met Ile Gly
210 215 220

Leu Leu Leu Ala Gly Leu Val Tyr Trp Gly Cys Thr Val Val Val Leu
225 230 235 240

His Phe Asp Ala Tyr Gly Glu Lys Met Ala Ala Ala Ala Ser Leu Pro
245 250 255

OPA15135_Sequence Listing

Lys Ile Val Val Gln Leu Phe Gly Val Gly Ala Leu Trp Ile Ala Cys
260 265 270
Val Ile Gly Tyr Leu Ala Cys Phe Ala Ser Leu Asn Ile Tyr Ile Gln
275 280 285
Ser Phe Ala Arg Leu Val Trp Ser Gln Ala Gln His Asn Pro Asp His
290 295 300
Tyr Leu Ala Arg Leu Ser Ser Arg His Ile Pro Asn Asn Ala Leu Asn
305 310 315 320
Ala Val Leu Gly Cys Cys Val Val Ser Thr Leu Val Ile His Ala Leu
325 330 335
Glu Ile Asn Leu Asp Ala Leu Ile Ile Tyr Ala Asn Gly Ile Phe Ile
340 345 350
Met Ile Tyr Leu Leu Cys Met Leu Ala Gly Cys Lys Leu Leu Gln Gly
355 360 365
Arg Tyr Arg Leu Leu Ala Val Val Gly Gly Leu Leu Cys Val Leu Leu
370 375 380
Leu Ala Met Val Gly Trp Lys Ser Leu Tyr Ala Leu Ile Met Leu Ala
385 390 395 400
Gly Leu Trp Leu Leu Leu Pro Lys Arg Lys Thr Pro Glu Asn Gly Ile
405 410 415
Thr Thr

<210> 2
<211> 1257
<212> DNA
<213> E.coli yjeH

<400> 2
atgagtggac tcaaacaaga actggggctg gccagggca ttggcctgct atcgacgtca 60
ttattaggca ctggcgtgtt tgccgttcct gcgttagctg cgctggtagc gggcaataac 120
agcctgtggg cgtggcccg tttgattatc ttagtggtcc cgattgcat tgtgtttgctg 180
attctgggtc gccactatcc cagcgcaggc ggcgtcgcgc acttcgtcgg tatggcgttt 240
ggttcgcggc ttgagcgagt caccggctgg ctgtttttat cggtcattcc cgtggggttg 300
cctgccgcac taaaattgc cgccgggttc ggccaggcga tgtttggtg gcatactgg 360
caactgttgt tggcagaact cggtagctg gcgctggtgt ggtatatcgg tactcgcggt 420
gccagttcca gtgctaactt acaaaccgtt attgccggac ttatcgtcgc gctgattgtc 480
gctatctggt gggcgggcga tatcaaacct gcgaatatcc cttttccggc acctggaat 540
atcgaactta ccgggttatt tgctgcgtta tcagtgatgt tctggtgttt tgcggtctg 600
gaggcatttg ccatctcgc ctcggaattt aaaaatccag agcgtgattt tcctcgtgct 660
ttgatgattg gtctgctgct ggcaggatta gtctactggg gctgtacggt agtcgtctta 720
cacttcgacg cctatggtga aaaaatggcg gcggcagcat cgcttcaaaa aattgtagtg 780
cagttgttcg gtgtaggagc gttatggatt gcctgcgtga ttggctatct ggcctgcttt 840

OPA15135_Sequence Listing

gccagtctca acatttatat acagagcttc gcccgcttg tctggctgca ggcgcaacat 900
aatcctgacc actacctggc acgcctctct tctcgccata tcccgaataa tgccctcaat 960
gcggtgctcg gctgctgtgt ggtgagcact ttggtgattc atgctttaga gatcaatctg 1020
gacgctctta ttatttatgc caatggcatc tttattatga tttatctgtt atgcatgctg 1080
gcaggctgta aattattgca aggacgttat cgactactgg cggtggttgg cgggctgtta 1140
tgcgttctgt tactggcaat ggtcggctgg aaaagtctct atgcgctgat catgctggcg 1200
gggttatggc tgttgctgcc aaaacgaaaa acgccggaac atggcataac cacataa 1257

<210> 3
<211> 386
<212> PRT
<213> E.coli metB

<400> 3
Met Thr Arg Lys Gln Ala Thr Ile Ala Val Arg Ser Gly Leu Asn Asp
1 5 10 15
Asp Glu Gln Tyr Gly Cys Val Val Pro Pro Ile His Leu Ser Ser Thr
20 25 30
Tyr Asn Phe Thr Gly Phe Asn Glu Pro Arg Ala His Asp Tyr Ser Arg
35 40 45
Arg Gly Asn Pro Thr Arg Asp Val Val Gln Arg Ala Leu Ala Glu Leu
50 55 60
Glu Gly Gly Ala Gly Ala Val Leu Thr Asn Thr Gly Met Ser Ala Ile
65 70 75 80
His Leu Val Thr Thr Val Phe Leu Lys Pro Gly Asp Leu Leu Val Ala
85 90 95
Pro His Asp Cys Tyr Gly Gly Ser Tyr Arg Leu Phe Asp Ser Leu Ala
100 105 110
Lys Arg Gly Cys Tyr Arg Val Leu Phe Val Asp Gln Gly Asp Glu Gln
115 120 125
Ala Leu Arg Ala Ala Leu Ala Glu Lys Pro Lys Leu Val Leu Val Glu
130 135 140
Ser Pro Ser Asn Pro Leu Leu Arg Val Val Asp Ile Ala Lys Ile Cys
145 150 155 160
His Leu Ala Arg Glu Val Gly Ala Val Ser Val Val Asp Asn Thr Phe
165 170 175
Leu Ser Pro Ala Leu Gln Asn Pro Leu Ala Leu Gly Ala Asp Leu Val
180 185 190
Leu His Ser Cys Thr Lys Tyr Leu Asn Gly His Ser Asp Val Val Ala
195 200 205
Gly Val Val Ile Ala Lys Asp Pro Asp Val Val Thr Glu Leu Ala Trp
210 215 220
Trp Ala Asn Asn Ile Gly Val Thr Gly Gly Ala Phe Asp Ser Tyr Leu
225 230 235 240
Leu Leu Arg Gly Leu Arg Thr Leu Val Pro Arg Met Glu Leu Ala Gln
245 250 255

OPA15135_Sequence Listing

Arg Asn Ala Gln Ala Ile Val Lys Tyr Leu Gln Thr Gln Pro Leu Val
260 265 270
Lys Lys Leu Tyr His Pro Ser Leu Pro Glu Asn Gln Gly His Glu Ile
275 280 285
Ala Ala Arg Gln Gln Lys Gly Phe Gly Ala Met Leu Ser Phe Glu Leu
290 295 300
Asp Gly Asp Glu Gln Thr Leu Arg Arg Phe Leu Gly Gly Leu Ser Leu
305 310 315 320
Phe Thr Leu Ala Glu Ser Leu Gly Gly Val Glu Ser Leu Ile Ser His
325 330 335
Ala Ala Thr Met Thr His Ala Gly Met Ala Pro Glu Ala Arg Ala Ala
340 345 350
Ala Gly Ile Ser Glu Thr Leu Leu Arg Ile Ser Thr Gly Ile Glu Asp
355 360 365
Gly Glu Asp Leu Ile Ala Asp Leu Glu Asn Gly Phe Arg Ala Ala Asn
370 375 380
Lys Gly
385

<210> 4
<211> 1161
<212> DNA
<213> E.coli metB

<400> 4
atgacgcgta aacaggccac catcgcagtg cgtagcgggt taaatgacga cgaacagtat 60
ggttgcgttg tcccaccgat ccatctttcc agcacctata actttaccgg atttaatgaa 120
ccgcgcgcgc atgattactc gcgtcgcggc aaccacacgc gcgatgtggt tcagcgtgcg 180
ctggcagaac tggaaggttg tgctggtgca gtacttacta ataccggcat gtccgcgatt 240
cacctggtaa cgaccgtctt ttgaaacct ggcgatctgc tggttgcgcc gcacgactgc 300
tacggcggtg gctatcgctt gttcgacagt ctggcgaaac gcggttgcta tcgctgtttg 360
tttgttgatc aaggcgatga acaggcatta cgggcagcgc tggcagaaaa acccaactg 420
gtactggtag aaagcccaag taatccattg ttacgcgtcg tggatattgc gaaaatctgc 480
catctggcaa gggaagtcgg ggcggtgagc gtggtggata acaccttctt aagcccggca 540
ttacaaaatc cgctggcatt aggtgccgat ctggtggtgc attcatgcac gaaatatctg 600
aacggtcact cagacgtagt ggccggcgtg gtgattgcta aagaccgga cgttgtcact 660
gaactggcct ggtgggcaaa caatattggc gtgacgggcg gcgcgtttga cagctatctg 720
ctgctacgtg ggttgcgaaac gctggtgccg cgtatggagc tggcgcagcg caacgcgcag 780
gcgattgtga aatacctgca aaccagccg ttggtgaaaa aactgtatca cccgtcgttg 840
ccggaaaatc aggggcatga aattgccgcg cgccagcaaa aaggctttgg cgcaatgttg 900
agttttgaac tggatggcga tgagcagacg ctgcgtcgtt tcctgggagg gctgtcgttg 960
tttacgctgg cggaatcatt agggggagtg gaaagtttaa tctctcacgc cgcaaccatg 1020

OPA15135_Sequence Listing

acacatgcag gcatggcacc agaagcgcgt gctgccgccg ggatctccga gacgctgctg 1080
 cgtatctcca ccggtattga agatggcgaa gatttaattg cgcacctgga aaatggcttc 1140
 cgggctgcaa acaaggggta a 1161

<210> 5
 <211> 310
 <212> PRT
 <213> E.coli thrB

<400> 5
 Met Val Lys Val Tyr Ala Pro Ala Ser Ser Ala Asn Met Ser Val Gly
 1 5 10 15
 Phe Asp Val Leu Gly Ala Ala Val Thr Pro Val Asp Gly Ala Leu Leu
 20 25 30
 Gly Asp Val Val Thr Val Glu Ala Ala Glu Thr Phe Ser Leu Asn Asn
 35 40 45
 Leu Gly Arg Phe Ala Asp Lys Leu Pro Ser Glu Pro Arg Glu Asn Ile
 50 55 60
 Val Tyr Gln Cys Trp Glu Arg Phe Cys Gln Glu Leu Gly Lys Gln Ile
 65 70 75 80
 Pro Val Ala Met Thr Leu Glu Lys Asn Met Pro Ile Gly Ser Gly Leu
 85 90 95
 Gly Ser Ser Ala Cys Ser Val Val Ala Ala Leu Met Ala Met Asn Glu
 100 105 110
 His Cys Gly Lys Pro Leu Asn Asp Thr Arg Leu Leu Ala Leu Met Gly
 115 120 125
 Glu Leu Glu Gly Arg Ile Ser Gly Ser Ile His Tyr Asp Asn Val Ala
 130 135 140
 Pro Cys Phe Leu Gly Gly Met Gln Leu Met Ile Glu Glu Asn Asp Ile
 145 150 155 160
 Ile Ser Gln Gln Val Pro Gly Phe Asp Glu Trp Leu Trp Val Leu Ala
 165 170 175
 Tyr Pro Gly Ile Lys Val Ser Thr Ala Glu Ala Arg Ala Ile Leu Pro
 180 185 190
 Ala Gln Tyr Arg Arg Gln Asp Cys Ile Ala His Gly Arg His Leu Ala
 195 200 205
 Gly Phe Ile His Ala Cys Tyr Ser Arg Gln Pro Glu Leu Ala Ala Lys
 210 215 220
 Leu Met Lys Asp Val Ile Ala Glu Pro Tyr Arg Glu Arg Leu Leu Pro
 225 230 235 240
 Gly Phe Arg Gln Ala Arg Gln Ala Val Ala Glu Ile Gly Ala Val Ala
 245 250 255
 Ser Gly Ile Ser Gly Ser Gly Pro Thr Leu Phe Ala Leu Cys Asp Lys
 260 265 270
 Pro Glu Thr Ala Gln Arg Val Ala Asp Trp Leu Gly Lys Asn Tyr Leu
 275 280 285

OPA15135_Sequence Listing

Gln Asn Gln Glu Gly Phe Val His Ile Cys Arg Leu Asp Thr Ala Gly
 290 295 300

Ala Arg Val Leu Glu Asn
 305 310

<210> 6
 <211> 933
 <212> DNA
 <213> E,coli thrB

<400> 6
 atggttaaag tttatgcccc ggcttccagt gccaatatga gcgctcgggtt tgatgtgctc 60
 ggggcggcgg tgacacctgt tgatggtgca ttgctcggag atgtagtcac ggttgaggcg 120
 gcagagacat tcagtctcaa caacctcgga cgctttgccg ataagctgcc gtcagaacca 180
 cgggaaaata tcgtttatca gtgctgggag cgtttttgcc aggaactggg taagcaaatt 240
 ccagtggcga tgacctgga aaagaatatg ccgatcgggtt cgggcttagg ctccagtgcc 300
 tgttcgggtg tcgcggcgct gatggcgatg aatgaacact gcggcaagcc gcttaatgac 360
 actcgtttgc tggctttgat gggcgagctg gaaggccgta tctccggcag cattcattac 420
 gacaacgtgg caccgtgttt tctcggtggt atgcagttga tgatcgaaga aaacgacatc 480
 atcagccagc aagtgccagg gtttgatgag tggctgtggg tgctggcgta tccggggatt 540
 aaagtctcga cggcagaagc cagggtatt ttaccggcgc agtatcgccg ccaggattgc 600
 attgcgcacg ggcgacatct ggcaggcttc attcacgcct gctattcccg tcagcctgag 660
 cttgccgcga agctgatgaa agatgttatc gctgaaccct accgtgaacg gttactgcca 720
 ggcttccggc aggcgcggca ggcggtcgcg gaaatcggcg cggtagcgag cggtatctcc 780
 ggctccggcc cgacctgtt cgctctgtgt gacaagccgg aaaccgcca gcgcgttgcc 840
 gactggttgg gtaagaacta cctgcaaaat caggaagggt ttgttcatat ttgccggctg 900
 gatacggcgg gcgcacgagt actggaaaac taa 933

<210> 7
 <211> 309
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> meta

<400> 7
 Met Pro Ile Arg Val Pro Asp Glu Leu Pro Ala Val Asn Phe Leu Arg
 1 5 10 15
 Glu Glu Asn Val Phe Val Met Thr Thr Ser Arg Ala Ser Gly Gln Glu
 20 25 30
 Ile Arg Pro Leu Lys Val Leu Ile Leu Asn Leu Met Pro Lys Lys Ile
 35 40 45
 Glu Thr Glu Asn Gln Phe Leu Arg Leu Leu Ser Asn Ser Pro Leu Gln
 50 55 60

OPA15135_Sequence Listing

Val Asp Ile Gln Leu Leu Arg Ile Asp Ser Arg Glu Ser Arg Asn Thr
65 70 75 80
Pro Ala Glu His Leu Asn Asn Phe Tyr Cys Asn Phe Glu Asp Ile Gln
85 90 95
Asp Gln Asn Phe Asp Gly Leu Ile Val Thr Gly Ala Pro Leu Gly Leu
100 105 110
Val Glu Phe Asn Asp Val Ala Tyr Trp Pro Gln Ile Lys Gln Val Leu
115 120 125
Glu Trp Ser Lys Asp His Val Thr Ser Thr Leu Phe Val Cys Trp Ala
130 135 140
Val Gln Ala Ala Leu Asn Ile Leu Tyr Gly Ile Pro Lys Gln Thr Arg
145 150 155 160
Thr Glu Lys Leu Ser Gly Val Tyr Glu His His Ile Leu His Pro His
165 170 175
Ala Leu Leu Thr Arg Gly Phe Asp Asp Ser Phe Leu Ala Pro His Ser
180 185 190
Arg Tyr Ala Asp Phe Pro Ala Ala Leu Ile Arg Asp Tyr Thr Asp Leu
195 200 205
Glu Ile Leu Ala Glu Thr Glu Glu Gly Asp Ala Tyr Leu Phe Ala Ser
210 215 220
Lys Asp Lys Arg Ile Ala Phe Val Thr Gly His Pro Glu Tyr Asp Ala
225 230 235 240
Gln Thr Leu Ala Gln Glu Phe Phe Arg Asp Val Glu Ala Gly Leu Asp
245 250 255
Pro Asp Val Pro Tyr Asn Tyr Phe Pro His Asn Asp Pro Gln Asn Thr
260 265 270
Pro Arg Ala Ser Trp Arg Ser His Gly Asn Leu Leu Phe Thr Asn Trp
275 280 285
Leu Asn Tyr Tyr Val Tyr Gln Ile Thr Pro Tyr Asp Leu Arg His Met
290 295 300
Asn Pro Thr Leu Asp
305

<210> 8
<211> 933
<212> DNA
<213> Artificial Sequence
<220>
<223> meta

<400> 8
atgggttaaag tttatgcccc ggcttccagt gccaatatga gcgtcggggtt tgatgtgctc 60
ggggcgggcgg tgacacctgt tgatggtgca ttgctcggag atgtagtcac ggttgaggcg 120
gcagagacat tcagtctcaa caacctcgga cgctttgccg ataagctgcc gtcagaacca 180
cgggaaaata tcgtttatca gtgctgggag cgtttttgcc aggaactggg taagcaaatt 240
ccagtggcga tgacctgga aaagaatatg ccgatcggtt cgggcttagg ctccagtgcc 300

OPA15135_Sequence Listing

tgttcgggtgg tcgcggcgct gatggcgatg aatgaacact gcggcaagcc gcttaatgac	360
actcgtttgc tggctttgat gggcgagctg gaaggccgta tctccggcag cattcattac	420
gacaacgtgg caccgtgttt tctcgggtgg atgcagttga tgatcgaaga aaacgacatc	480
atcagccagc aagtgccagg gtttgatgag tggctgtggg tgctggcgta tccggggatt	540
aaagtctcga cggcagaagc cagggctatt ttaccggcgc agtatcgccg ccaggattgc	600
attgcgcacg ggcgacatct ggcaggcttc attcacgcct gctattcccg tcagcctgag	660
cttgccgcga agctgatgaa agatgttatc gctgaaccct accgtgaacg gttactgcc	720
ggcttccggc aggcgcggca ggcggtcgcg gaaatcggcg cggtagcgag cggtatctcc	780
ggctccggcc cgacctgtt cgctctgtgt gacaagccgg aaaccgcca gcgcgttgcc	840
gactggttgg gtaagaacta cctgcaaat caggaagggt ttgttcatat ttgccggctg	900
gatacggcgg gcgcacgagt actggaaaac taa	933

<210> 9
 <211> 22
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> yjeH primer

<400> 9	
aagctttgat aactctcctt tg	22

<210> 10
 <211> 22
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> yjeH primer

<400> 10	
aagctttatg tggttatgcc at	22

<210> 11
 <211> 28
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> yjeH gene primer

<400> 11	
ggtaccaggt cgactctaga ggatcccc	28

<210> 12
 <211> 23
 <212> DNA
 <213> Artificial Sequence

OPA15135_Sequence Listing

<220>		
<223>	yjeH gene primer	
<400>	12	
	ggtaccttat gtggttatgc cat	23
<210>	13	
<211>	70	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	MetB primer	
<400>	13	
	ttacccttg tttgcagccc ggaagccatt ttccaggctg gcaattaaat catatgaata	60
	tcctccttag	70
<210>	14	
<211>	70	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	metB primer	
<400>	14	
	ttactctggg gcctgacatt tcaccgacaa agcccaggga acttcatcac gtgtaggctg	60
	gagctgcttc	70
<210>	15	
<211>	70	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	thrB primer	
<400>	15	
	aaagaatatg ccgatcggtt cgggcttagg ctccagtgcc tgttcggtgg gtgtaggctg	60
	gagctgcttc	70
<210>	16	
<211>	70	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	thrB primer	
<400>	16	
	agacaaccga catcgctttc aacattggcg accggagccg ggaaggcaaa catatgaata	60
	tcctccttag	70

OPA15135_Sequence Listing

<210>	17	
<211>	29	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	metaA primer	
<400>	17	
aattgatatc atgccgattc gtgtgccgg		29
<210>	18	
<211>	34	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	metaA primer	
<400>	18	
aattaagctt ttaatccagc gttggattca tgtg		34
<210>	19	
<211>	44	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	meta G111E mutagenesis primer	
<400>	19	
ttgtaactgg tgcgccgctg gaactggtgg ggtttaatga gtgc		44
<210>	20	
<211>	44	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	meta G111E mutagenesis primer	
<400>	20	
gacatcatta aacccaccca gttccagcgg cgcaccagtt acaa		44
<210>	21	
<211>	44	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	meta EH mutagenesis primer	
<400>	21	
tgtaactggt gcgccgctgg aacatgtggg gtttaatgat gtcg		44
<210>	22	
<211>	44	
<212>	DNA	

OPA15135_Sequence Listing

<213> Artificial Sequence

<220>

<223> metaA EH mutagenesis primer

<400> 22
cgacatcatt aaacccaca tgttccagcg gcgcaccagt taca 44

<210> 23
<211> 29
<212> DNA
<213> Artificial Sequence

<220>

<223> metaA EH primer

<400> 23
aattgatatc atgccgattc gtgtgccgg 29

<210> 24
<211> 27
<212> DNA
<213> Artificial Sequence

<220>

<223> metaA EH primer

<400> 24
aattaagcct gctgaggtac gtttcgg 27

<210> 25
<211> 23
<212> DNA
<213> Artificial Sequence

<220>

<223> metaA WT primer

<400> 25
cagcaggtga ataaatttta ttc 23

<210> 26
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

<223> metaA WT primer

<400> 26
cgcgaatgga agctgtttcc 20

<210> 27
<211> 42
<212> DNA
<213> Artificial Sequence

<220>

OPA15135_Sequence Listing

<223> pKD3 primer

<400> 27
 tttccgaaac gtacctcagc aggtgtaggc tggagctgct tc 42

<210> 28
 <211> 43
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> pKD3 primer

<400> 28
 gaataaaatt tattcacctg ctgcatatga atatcctcct tag 43

<210> 29
 <211> 31
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> ppc 2 copy primer

<400> 29
 gccggaattc tgtcggatgc gatacttgcg c 31

<210> 30
 <211> 30
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> ppc 2 copy primer

<400> 30
 gaaggagctc agaaaaccct cgcgcaaag 30

<210> 31
 <211> 31
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> ppc 2 copy primer

<400> 31
 gccggagctc tgtcggatgc gatacttgcg c 31

<210> 32
 <211> 30
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> ppc 2 copy primer

OPA15135_Sequence Listing

<400>	32		
gaagggtacc agaaaaccct cgcgcaaaag			30
<210>	33		
<211>	30		
<212>	DNA		
<213>	Artificial Sequence		
<220>			
<223>	aspC 2 copy primer		
<400>	33		
tccgagctca taagcgtagc gcatcaggca			30
<210>	34		
<211>	30		
<212>	DNA		
<213>	Artificial Sequence		
<220>			
<223>	aspC 2 copy primer		
<400>	34		
tccgagctcg tccacctatg ttgactacat			30
<210>	35		
<211>	27		
<212>	DNA		
<213>	Artificial Sequence		
<220>			
<223>	asd 2 copy primer		
<400>	35		
ccggaattcc caggagagca ataagca			27
<210>	36		
<211>	28		
<212>	DNA		
<213>	Artificial Sequence		
<220>			
<223>	asd 2 copy primer		
<400>	36		
ctagtctaga tgctctatTT aactcccG			28
<210>	37		
<211>	28		
<212>	DNA		
<213>	Artificial Sequence		
<220>			
<223>	asd 2 copy primer		
<400>	37		
ctagtctaga ccaggagagc aataagca			28

OPA15135_Sequence Listing

<210>	38	
<211>	27	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	asd 2 copy primer	
<400>	38	
	ccggaattct gctctattta actcccg	27
<210>	39	
<211>	23	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	icd promoter primer	
<400>	39	
	cccgggggtat tttcagagat tat	23
<210>	40	
<211>	30	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	icd promoter primer	
<400>	40	
	cccgggatcc tccttcgagc gctactggtt	30
<210>	41	
<211>	32	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	pro promoter primer	
<400>	41	
	cccgggggatc ctctgtgtgg aattctcgga ca	32
<210>	42	
<211>	30	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	pro promoter primer	
<400>	42	
	cccgggaatt cattaaagag gagaaaggat	30
<210>	43	
<211>	29	

OPA15135_Sequence Listing

<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	cysk promoter primer	
<400>	43	
	cccgaggagct tccagcctgt ttacgatga	29
<210>	44	
<211>	29	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	cysk promoter primer	
<400>	44	
	cccggtccc aatttcatac agttaagga	29
<210>	45	
<211>	44	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	icd promoter primer	
<400>	45	
	gaaaaagaaa aaaaattctg gcgattgtgt aggctggagc tgct	44
<210>	46	
<211>	48	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	icd promoter primer	
<400>	46	
	cggcaattca taatctctga aaatacgcca tggccatat gaatatcc	48
<210>	47	
<211>	44	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	cysk promoter primer	
<400>	47	
	gaaaaagaaa aaaaattctg gcgattgtgt aggctggagc tgct	44
<210>	48	
<211>	44	
<212>	DNA	
<213>	Artificial Sequence	

OPA15135_Sequence Listing

```

<220>
<223>    cysk  promoter primer

<400>    48
gcgggatcat cgtaaacagg ctgccatggt ccatatgaat atcc                44

<210>    49
<211>    44
<212>    DNA
<213>    Artificial Sequence

<220>
<223>    pro promoter primer

<400>    49
gaaaaagaaa aaaaattctg gcgattgtgt aggctggagc tgct                44

<210>    50
<211>    48
<212>    DNA
<213>    Artificial Sequence

<220>
<223>    pro  promoter primer

<400>    50
tgtccgagaa ttccacacag aggatcgcca tggccatat gaatatcc            48

<210>    51
<211>    197
<212>    DNA
<213>    Artificial Sequence

<220>
<223>    icd promoter

<400>    51
gtatttttcag agattatgaa ttgccgcatt atagcctaataacgcgcatac tttcatgacg        60
gcaaacaata gggtagtatt gacaagccaa ttacaaatca ttaacaaaaa attgctctaa        120
agcatccgta tcgcaggacg caaacgcata tgcaacgtgg tggcagacga gcaaaccagt        180
agcgctcgaa ggaggat                                                197

<210>    52
<211>    197
<212>    DNA
<213>    Artificial Sequence

<220>
<223>    pro promoter

<400>    52
gtatttttcag agattatgaa ttgccgcatt atagcctaataacgcgcatac tttcatgacg        60
gcaaacaata gggtagtatt gacaagccaa ttacaaatca ttaacaaaaa attgctctaa        120
agcatccgta tcgcaggacg caaacgcata tgcaacgtgg tggcagacga gcaaaccagt        180

```

OPA15135_Sequence Listing

agcgctcgaa ggaggat 197

<210> 53
 <211> 299
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> cysk promoter

<400> 53	
agcttcagc ctgtttacga tgatcccgct gcttaatctg ttcacatgc ccgttgccgt	60
ttgtggcgcg acggcgatgt gggtcgattg ctatcgcat aaacacgcga tgtggcggt	120
acaatctacc ggttatcttg taaaccgttt gtgtgaaaca ggggtggctt atgccgcccc	180
ttattccatc ttgcatgtca ttatttcct tctgtatata gatatgctaa atccttactt	240
ccgcatattc tctgagcggg tatgctacct gttgtatccc aatttcatac agttaagga	299