



(86) **Date de dépôt PCT/PCT Filing Date:** 2012/12/21
(87) **Date publication PCT/PCT Publication Date:** 2013/06/27
(85) **Entrée phase nationale/National Entry:** 2014/06/20
(86) **N° demande PCT/PCT Application No.:** KR 2012/011328
(87) **N° publication PCT/PCT Publication No.:** 2013/095071
(30) **Priorité/Priority:** 2011/12/21 (KR10-2011-0139527)

(51) **Cl.Int./Int.Cl. C12N 15/52** (2006.01),
C12N 15/63 (2006.01), **C12N 15/74** (2006.01),
C12P 13/08 (2006.01)

(71) **Demandeur/Applicant:**
CJ CHEILJEDANG CORPORATION, KR

(72) **Inventeurs/Inventors:**
LEE, KWANG HO, KR;
LIM, SANG JO, KR;
MOON, JUN OK, KR;
JANG, JAE WOO, KR;
PARK, SU JIN, KR;
PARK, SANG HEE, KR

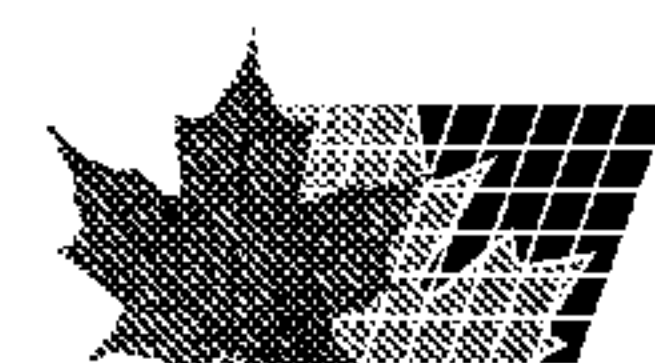
(74) **Agent:** BERESKIN & PARR LLP/S.E.N.C.R.L., S.R.L.

(54) **Titre : METHODE DE PRODUCTION DE LA L-LYSINE AU MOYEN DE MICRO-ORGANISMES CAPABLES DE PRODUIRE
L'ACIDE AMINE**

(54) **Title: METHOD FOR PRODUCING L-LYSINE USING MICROORGANISMS HAVING ABILITY TO PRODUCE L-LYSINE**

(57) **Abrégé/Abstract:**

The present invention relates to variant polynucleotides in which an initiation codon of each polynucleotide coding aspartate kinase; EC:2.7.2.4 (hereinafter, LysC), transketolase; EC:2.2.1.1 (hereinafter, Tkt) or pyruvate carboxylase; EC:6.4.1.1 (hereinafter, Pyc) is substituted by ATG. The present invention also relates to a vector comprising the variant polynucleotides, microorganisms transformed by the vector and to a method for producing L-lysine using the microorganisms.



ABSTRACT OF THE DISCLOSURE

The present invention relates to a modified polynucleotide encoding aspartate kinase (EC:2.7.2.4; hereinafter, referred to as LysC), transketolase (EC:2.2.1.1; hereinafter, referred to as Tkt) or pyruvate carboxylase (EC:6.4.1.1; hereinafter, referred to as Pyc), in which the initiation codon is substituted with ATG, a vector including the same, a microorganism transformed with the vector, and a method for producing L-lysine using the same.

**Method for producing L-lysine using microorganisms having
ability to produce L-lysine**

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a modified polynucleotide, in which an initiation codon is substituted with ATG, a vector comprising the same, a microorganism comprising the polynucleotide, and a method for producing L-lysine using the same.

2. Description of the Related Art

Strains of the genus *Corynebacterium*, specifically *Corynebacterium glutamicum*, are Gram-positive microorganisms which are extensively used to produce L-lysine. L-lysine is used for animals feed, medicines and cosmetics for humans, and is produced through fermentation of the *Corynebacterium* strain.

Conventionally, a strain of the genus *Corynebacterium* having enhanced lysine biosynthesis genes and a method of producing L-lysine using the same are known. For example, US Patent No. 6,746,855 discloses a method for producing L-lysine by culturing *Corynebacterium* which has an enhanced

expression of *lysE* gene (lysine export carrier gene), and additionally introduced genes selected from the group consisting of a *dapA* gene, a *lysC* gene, a *pyc* gene and a *dapB* gene.

Another method is to amplify genes on the lysine biosynthesis pathway or to modify a promoter. For example, Korean Patent application laid-open Nos. 2009-0082702 and 2009-0084099 disclose a method for producing L-lysine by introducing improved promoters of *ddh* and *lysC*-*asd* operon into *Corynebacterium*. Korean Patent application laid-open No. 2008-0025355 discloses a method for improving lysine productivity by amplifying the copy number of the genes on the lysine biosynthesis pathway, which are *aspB*, *lysC*, *asd*, *dapA*, *dapB*, *lysA*, and *pyc*, in the chromosome.

Meanwhile, the initiation codon which is recognized by ribosomes to initiate translation in the chromosome is usually ATG. Translation can be controlled according to the initiation codon of the gene, and the sequence of the initiation codon is important in the regulation of protein activity. However, while ATG is the common initiation codon among the lysine biosynthesis genes derived from *Corynebacterium glutamicum*, the initiation codon of *lysC* and *pyc* genes is GTG, and TTG is for *tkt* gene on pentose

phosphate pathway contains the initiation codon of TTG (reference: J. Biotechnol., 104: 5-

The present inventors have made many efforts to find a method for improving lysine productivity, and as a result, they found that the initiation codon of the wild-type *lysC*, *tkt* and *pyc* genes can be substituted with ATG to enhance the activities of aspartate kinase, transketolase and pyruvate carboxylase over their endogenous activities, thereby completing the present invention.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a modified polynucleotide encoding aspartate kinase (EC:2.7.2.4; hereinafter, referred to as LysC), transketolase (EC:2.2.1.1; hereinafter, referred to as Tkt) or pyruvate carboxylase (EC:6.4.1.1; hereinafter, referred to as Pyc), wherein the initiation codon of the polynucleotide is substituted with ATG.

Another object of the present invention is to provide a vector comprising one or more modified polynucleotides encoding aspartate kinase, transketolase, or pyruvate carboxylase, in which the initiation codon of the polynucleotide is substituted with ATG

Still another object of the present invention is to provide a microorganism, in which one or more the enzymes have enhanced activity compared to their endogenous activity.

Still another object of the present invention is to provide a method for producing L-lysine, comprising the steps of culturing the microorganism, and recovering L-lysine from the cultured microorganism or the culture broth.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In one aspect, the present invention provides a modified polynucleotide encoding aspartate kinase (EC:2.7.2.4; hereinafter, referred to as LysC), transketolase (EC:2.2.1.1; hereinafter, referred to as Tkt) or pyruvate carboxylase (EC:6.4.1.1; hereinafter, referred to as Pyc), in which the initiation codon of the polynucleotide is substituted with ATG.

Each polynucleotide encoding aspartate kinase, transketolase or pyruvate carboxylase may also include a partial substitution, deletion, insertion or addition in the polynucleotide, as long as each of them has the enzymatic activity, and may have 70% or more homology, specifically 80% or more homology, more specifically 90% or more homology, and much more specifically 95% or more homology, and most specifically 100% homology based on the known polynucleotide.

As used herein, the term "homology (homologous)" means the degree of similarity between nucleotide sequences of *lysC* gene, *tkt* gene or *pyc* gene of the wild-type and nucleotide sequences of the corresponding modified genes thereof, that is, modified *lysC* gene, *tkt* gene or *pyc* gene, in which a part of the polynucleotides is substituted, deleted, inserted or added.

As used herein, the term "initiation codon" means 3 nucleotides corresponding to a translation start point when a coding sequence of mRNA (messenger RNA) is translated into a protein. In general, initiation codons found in the chromosome of microorganisms are ATG (AUG on mRNA), GTG (GUG on mRNA) and TTG (UUG on mRNA), and they exist in a ratio of 62.5%~66.5%, 23.1%~24.3% and 10.3%~13.2% according to the analysis result of the entire genome sequence of *Corynebacterium glutamicum* (reference: Handbook of *Corynebacterium glutamicum*, 40p, Lothar Eggeling & Michael Bott, 2005).

Among the lysine biosynthesis genes derived from *Corynebacterium* reported until now, *lysC* and *pyc* have the initiation codon of GTG, and *tkt* has the initiation codon of TTG. The initiation codon of these genes is not ATG, which is regarded as a unique characteristic of *Corynebacterium*.

The modified polynucleotide according to the present invention is characterized in that the initiation codon of the *lysC*, *tkt* or *pyc* gene is substituted with ATG, and these modified polynucleotides having such substituted initiation codon have been first modified by the present inventors. In more detail, GTG initiation codon of the polynucleotide encoding aspartate kinase (LysC) or pyruvate carboxylase (Pyc) is substituted with ATG, and TTG initiation codon of the polynucleotide encoding transketolase (Tkt) is substituted with ATG in the present invention. More specifically, the sequences of *lysC*, *tkt*, and *pyc* genes are SEQ ID NOs. 13, 14, and 15, respectively, the sequences of the genes having the substituted initiation codon as ATG are represented by SEQ ID NOs. 16, 17, and 18, respectively.

The base substitutions of the initiation codons may be performed by any method known in the art, for example, site-specific mutagenesis, homologous recombination, but is not limited thereto.

In another aspect, the present invention provides a vector comprising one or more modified polynucleotides of the modified polynucleotides encoding aspartate kinase, transketolase or pyruvate carboxylase, in which the initiation codon of the polynucleotide is substituted with ATG.

The modified polynucleotides having the substituted initiation codon as ATG and encoding aspartate kinase, transketolase or pyruvate carboxylase, which is comprised in the vector of the present invention, may include those in which a part of the polynucleotides is substituted, deleted, inserted or added, as long as they have enzymatic activity, and may have 70% or more homology, specifically 80% or more homology, more specifically 90% or more homology, much more specifically 95% or more homology, and most specifically 100% homology.

Further, the modified polynucleotides having the substituted initiation codon as ATG and encoding aspartate kinase, transketolase or pyruvate carboxylase, which is comprised in the vector of the present invention, may also include only a part of the gene encoding aspartate kinase, transketolase or pyruvate carboxylase, as long as they have the substituted initiation codon as ATG.

As used herein, the term "vector" refers to a DNA construct that contains a base sequence which is operably linked to a suitable control sequence, to expresses a target gene in a suitable host. The control sequences may include a promoter to initiate transcription, a certain operator sequence to control such transcription, a sequence encoding

a suitable ribosome-binding site on the mRNA, and a sequence to control termination of transcription and translation. The vector used in the present invention is not particularly limited, and may be any vector known in the art, as long as it is replicable in the host. For example, the vector may be a plasmid, a phage particle, or a potential genome insert, and is specifically pDZ (Korean Patent No. 10-0924065), but is not limited thereto. Once transformed into a suitable host, the vector may be replicated or function independently of the host genome, or may be integrated into the genome itself.

Specifically, a nucleotide sequence (SEQ ID NO. 13, 14 or 15) comprising the initiation codon of the *lysC*, *tkt* or *pyc* gene was acquired, and based on this sequence, primers having the substituted initiation codon as ATG were synthesized. PCR was carried out using the primers and a chromosomal DNA of an L-lysine-producing strain as a template, so as to obtain DNA of which one end is substituted with ATG. This DNA fragment thus obtained was cloned into a vector to obtain a final recombinant vector. More specifically, in the present invention, pDZ-*lysC*(ATG), pDZ-*tkt*(ATG) and pDZ-*pyc*(ATG) vectors were constructed, respectively.

In still another aspect, the present invention provides a microorganism which includes one or more modified polynucleotides having the substituted initiation codon as ATG and encoding an enzymes selected from the group consisting of aspartate kinase, transketolase and pyruvate carboxylase, and then improved in translation levels of mRNAs transcribed from the polynucleotides into proteins, resulting in enhanced activities of one or more of the enzymes compared to endogenous activities thereof. The microorganism of the present invention may have enhanced activities of aspartate kinase, transketolase or pyruvate carboxylase by using the modified polynucleotides in combinations of one, two, or three thereof, in which the modified polynucleotides encode the corresponding enzymes and are substituted with ATG on initiation codon.

In order to substitute ATG for the initiation codons of the target genes on the chromosome of the microorganism, various methods known in the art can be used. For example, the initiation codon sequences of endogenous *lysC*, *tkt* and *pyc* genes in the microorganism can be substituted on the chromosome. Alternatively, the corresponding genes having substituted initiation codon sequences can be introduced into the microorganism in the form of a plasmid.

The microorganism may be any strain, as long as it has L-lysine productivity. Specifically it may be a microorganisms of *Corynebacterium* sp. or *Brevibacterium* sp. Examples of the microorganisms of *Corynebacterium* sp. or *Brevibacterium* sp. include *Corynebacterium glutamicum* ATCC13032, *Corynebacterium thermoaminogenes* FERM BP-1539, *Brevibacterium flavum* ATCC 14067, *Brevibacterium lactofermentum* ATCC 13869. Further, L-lysine-producing variants or stains derived therefrom, for example, *Corynebacterium glutamicum* KCCM11016P (this microorganism was disclosed as KFCC10881, and re-deposited to an International Depositary Authority under the Budapest Treaty with Accession No. KCCM11016P, Korean Patent No. 10-0159812, Korean Patent No. 10-0397322) and *Corynebacterium glutamicum* KFCC 11001, may be included. Futher more may be *Corynebacterium glutamicum* with Accession No. KCCM11016P.

Specifically, *Corynebacterium glutamicum* with Accession No. KCCM11016P was transformed with a vector comprising the polynucleotide encoding aspartate kinase, transketolase or pyruvate carboxylasewherein the initiation codon is substituted with ATG, to obtain recombinant *Corynebacterium glutamicum*. More Specifically, pDZ-lysC(ATG), pDZ-tkt(ATG) or pDZ-pyc(ATG) vector was introduced into *Corynebacterium*

glutamicum with Accession No. KCCM11016P, respectively so as to obtain each recombinant *Corynebacterium glutamicum*.

Further, the microorganism can be transformed with a vector comprising two or more polynucleotides of the polynucleotides encoding aspartate kinase, transketolase or pyruvate carboxylase and having the substituted initiation codon as ATG. Specifically, the pDZ-tkt(ATG) vector comprising the gene of which the initiation codon has been substituted with ATG and encodes transketolase is transformed into *Corynebacterium glutamicum* KCCM11016P-lysC, in which GTG initiation codon of *lysC* is substituted with ATG, and through second crossover, the initiation codons of *lysC* and *tkt* on the chromosome are substituted with ATG so as to obtain *Corynebacterium glutamicum* KCCM11016P-lysC-tkt. Further, the pDZ-pyc(ATG) vector comprising the polynucleotide of which the initiation codon has been substituted with ATG and encodes pyruvate carboxylase is transformed into KCCM11016P-lysC, in which GTG initiation codon of *lysC* is substituted with ATG, and through second crossover, the initiation codons of *lysC* and *pyc* on the chromosome are substituted with ATG so as to obtain KCCM11016P-lysC-pyc. Furthermore, the pDZ-tkt(ATG) vector is transformed into KCCM11016P-lysC-pyc as prepared above, in which GTG initiation codon of *lysC* and *pyc* is substituted

with ATG, and through second crossover, the initiation codons of *lysC*, *pyc* and *tkt* on the chromosome are substituted with ATG so as to obtain KCCM11016P-*lysC*-*pyc*-*tkt*. It was confirmed that the initiation codons of *lysC*, *pyc* and *tkt* can be also substituted with ATG in another lysine-producing strains belonging to *Corynebacterium glutamicum*, KFCC10750 (Korean Patent No. 10-0073610), KCCM10770P (Korean Patent No. 10-0924065), CJ3P (Genome Biology 2012, 13:R40) in the same manner. These results suggest that the modified polynucleotides of the present invention can be stably introduced into various strains belonging to *Corynebacterium* sp., thereby increasing L-lysine productivity.

Specifically, the transformant KCCM11016P-*lysC*-*pyc*-*tkt*, which was obtained by introducing pDZ-*lysC*(ATG), pDZ-*tkt*(ATG) and pDZ-*pyc*(ATG) vectors including the polynucleotides encoding aspartate kinase, transketolase and pyruvate carboxylase into *Corynebacterium glutamicum* KCCM11016P, was designated as *Corynebacterium glutamicum* CA01-2059, and deposited on May 2, 2011 at the Korean Culture Center of Microorganisms (hereinafter, abbreviated to "KCCM"), which is an International Depositary Authority under the Budapest Treaty, under Accession No. KCCM11188P. It is deposited by an International Depositary Authority under the Budapest Treaty.

The microorganism according to the present invention is characterized in that the initiation codons of the wild-type genes encoding the above enzymes are substituted with ATG, and therefore, the microorganism has enhanced activities of aspartate kinase, transketolase and pyruvate carboxylase compared to endogenous activities thereof, resulting from remarkable improvement in translation levels of mRNAs transcribed from the *lysC*, *tkt* and *pyc* genes into the proteins.

As used herein, the term "endogenous activity" means the activity of an enzyme in a native microorganism, and for example, the activity of aspartate kinase, transketolase or pyruvate carboxylase in a native microorganism belonging to *Corynebacterium* sp. The term "enhanced endogenous activity" means that the activity is further improved compared to that of the native enzyme.

Specifically, when the aspartate kinase activity of the strain in which the initiation codon of *lysC* gene was substituted with ATG was compared to that of the parent strain KCCM11016P, 2.73-fold increase in the aspartate kinase activity was observed (Table 4). Further, when the transketolase activity of the strain in which the initiation

codon of *tkt* gene was substituted with ATG was compared to that of the parent strain KCCM11016P, 3.5-fold increase in the transketolase activity was observed (Table 5). Furthermore, when the pyruvate carboxylase activity of the strain in which the initiation codon of *pyc* gene is substituted with ATG was compared to that of the parent strain KCCM11016P, 1.89-fold increase in the pyruvate carboxylase activity was observed (Table 6).

It was found that L-lysine productivity of the microorganism can be increased by improving the activity of aspartate kinase, transketolase, pyruvate carboxylase or a combination thereof.

In the present invention, the amounts of L-lysine produced in the L-lysine-producing strains, *Corynebacterium glutamicum* KCCM11016P-*lysC*, KCCM11016P-*tkt*, KCCM11016P-*pyc*, KCCM11016P-*lysC*-*tkt*, KCCM11016P-*lysC*-*pyc*, and KCCM11016P-*lysC*-*pyc*-*tkt* were measured, and as a result, they showed a remarkable improvement in the L-lysine production compared to the parent strain KCCM11016P (Table 7). Also, another lysine-producing strains belonging to *Corynebacterium glutamicum*, KFCC10750 (Korean Patent No. 10-0073610), KCCM10770P (Korean Patent No. 10-0924065), and CJ3P (Genome Biology 2012, 13:R40), in which initiation codons of *lysC*,

pyc and *tkl* were substituted with ATG, showed a remarkable improvement in the L-lysine production compared to the parent strain (Tables 8~10). These results suggest that microorganisms having one type of modified polynucleotides encoding *LysC*, *Tkt*, or *Pyc*, or having two types of the modified polynucleotides encoding the enzymes, or having three types of modified polynucleotides encoding the enzymes and having the substituted initiation codon as ATG on the chromosome, also showed remarkably improved L-lysine productivity compared to the wild-type microorganism having GTG or TTG initiation codon.

In still another aspect, the present invention provides a method for producing L-lysine, comprising the steps of culturing the microorganism as described above; and recovering L-lysine from the cultured microorganism or the culture broth.

For the cultivation, various methods of producing L-lysine using a microorganism which are widely known in the art can be used. The cultivation can be carried out according to the widely known method, and conditions for the cultivation, including temperature, time, pH of medium, etc. may be controlled properly. A detailed description of the cultivation is given in the following document [Chmiel;

Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991), and Storhas; Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig / Wiesbaden, 1994)]. Further, the cultivation may include batch culture, continuous culture, and fed-batch culture. Specifically, a batch, fed batch or repeated fed batch process may be operated in a continuous manner, but the present invention is not limited thereto.

For use in the cultivation, a medium must satisfy the requirement of a particular strain employed. Culture media for microorganisms belonging to *Corynebacterium* sp. are well known (e.g., Manual of Methods for General Bacteriology. American Society for Bacteriology. Washington D.C., USA, 1981). Carbon sources to be used may include saccharides and carbohydrates such as glucose, sucrose, lactose, fructose, maltose, starch and cellulose; oils and lipids such as soybean oil, sunflower seed oil, castor oil and coconut oil; fatty acids such as palmitic acid, stearic acid, linoleic acid; alcohols such as glycerol and ethanol; and organic acids such as acetic acid. These materials may be used separately or in combination. Nitrogen sources to be used may include peptone, yeast extract, meat broth, malt extract, corn steep liquor, soybean meal and urea, or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and

ammonium nitrate. These nitrogen sources may be used separately or in combination. Phosphorus sources to be used may include dipotassium hydrogen phosphate, potassium dihydrogen phosphate, and corresponding sodium salts. In addition, culture media may contain metal salts such as magnesium sulfate or iron sulfate essential for the growth. Lastly, essential nutrients such as amino acids and vitamins may be used in addition to the above substances. In addition, proper precursors may be added into the culture media. These materials may be properly added into the culture during cultivations in a batch or continuous mode.

The pH of the culture media may be adjusted with a basic compound such as sodium hydroxide, potassium hydroxide or ammonia, or an acidic compound such as phosphoric acid or sulfuric acid. The generation of foam may be restrained using an anti-foaming agent such as fatty acid polyglycol ester. The culture media may be kept under aerobic conditions by introducing oxygen or an oxygen-containing gas (e.g., air) thereto. The culture temperature is typically between 20 and 45°C and specifically between 25 and 40°C. The culturing is continued until a maximal amount of L-lysine is produced. In this regard, it may be accomplished within 10 to 160 hrs. After being produced, L-lysine may be exported into the culture media or may remain within the cells.

Further, the method for producing L-lysine of the present invention comprises the step of recovering L-lysine from the cultured microorganism or the culture broth. The method of recovering L-lysine from the microorganism or the culture broth is widely known in the art. Examples of the L-lysine recovering method may include filtration, anion exchange chromatography, crystallization and HPLC, but are not limited thereto.

Examples

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

In the Examples, recombinant vectors for substituting ATG for GTG or TTG initiation codon of *lysC* gene encoding aspartate kinase, *tkt* gene encoding transketolase, and *pyc* gene encoding pyruvate carboxylase derived from the lysine-producing strain, *Corynebacterium glutamicum* KCCM11016P, were constructed. The vector was transformed into *Corynebacterium glutamicum* KCCM11016P strain to obtain a strain having the substituted initiation codon on the

chromosome, thereby preparing a strain having improved lysine productivity.

Corynebacterium glutamicum KCCM11016P strain used in the present invention is a strain which is resistant to S-(2-aminoethyl) cysteine (hereinafter, referred to as AEC) and is homoserine-leaky, prepared by artificial mutation using wild-type *Corynebacterium glutamicum* (ATCC 13032) as a parent strain (disclosed as KFCC10881. See Korean Patent No. 10-0159812 and Korean Patent No. 10-0397322). Further, KFCC10750 strain is a *Corynebacterium glutamicum* L-lysine-producing strain which is homoserine auxotroph and resistant to an L-leucine analog, 4-azaleucine and an antibiotic rifampicin, prepared by artificial mutation (Korean Patent No. 10-0073610), KCCM10770P strain is an L-lysine-producing strain derived from KCCM11016P, which retains two copies of 6 types of the genes constituting the lysine biosynthesis pathway on the chromosome (Korean Patent No. 10-0924065), and CJ3P strain is a *Corynebacterium glutamicum* strain which has L-lysine productivity by introducing each of P458S, V59A, and T311I mutations into three types of *pyc*, *hom*, and *lysC* genes of the wild type, based on the description by Binder et al. (Genome Biology 2012, 13:R40).

Example 1: Construction of recombinant vector (pDZ-lysC(ATG)) having substituted ATG initiation codon in lysC

derived from *Corynebacterium glutamicum* and Preparation of strain having substituted initiation codon

pDZ (see Korean Patent No. 10-0924065) was used as a basic vector for the recombinant vector, which was constructed as follows.

(1) Construction of pDZ-lysC(ATG) recombinant vector

In order to acquire *lysC* gene derived from *Corynebacterium glutamicum*, chromosomal DNA of a lysine-producing strain (*Corynebacterium glutamicum* KCCM11016P) prepared by artificial mutation was used as a template. Based on the genbank at the U.S. National Institutes of Health (NIH GenBank), a nucleotide sequence (SEQ ID NO. 13) containing the initiation codon region of *lysC* gene (NCBI Accession No. NC_003450, Ncgl0247) was acquired, and based on this sequence, two pairs of primers (Table 1, SEQ ID NOs. 1 ~ 4) for substituting the initiation codon GTG with ATG were synthesized.

PCR was carried out using chromosomal DNA of KCCM11016P as a template and primers of the following Table 1. PfuUltraTM High-Fidelity DNA polymerase (Stratagene) was used as a polymerase, and PCR was performed with 30 cycles of denaturation at 96°C for 30 seconds; annealing at 55°C for 30 seconds; and polymerization at 72°C for 30 seconds. Two DNA fragments thus obtained were cloned into pDZ vector

treated with restriction enzyme XbaI using an In-Fusion PCR cloning kit (Clontech), and finally, a pDZ-lysC(ATG) recombinant vector was constructed.

(2) Preparation of strain

The pDZ-lysC(ATG) vector thus constructed was transformed into KCCM11016P by an electric pulse method (by use of the transformation method according to Appl. Microbiol. Biotechnol. (1999) 52:541-545), and then strains, in which the gene was inserted into the chromosome by homologous recombination, were selected on a selection medium containing 25 mg/L of kanamycin. The successful chromosomal insertion of the vector was confirmed by the blue color of the colonies on a solid medium containing X-gal (5-bromo-4-chloro-3-indolyl-(β -D-galactoside). The strain with the first chromosomal insertion was shaking-cultured (30°C, 8 hours) in a nutrient medium. Then, the cultured strain was serially diluted from 10^{-4} to 10^{-10} , and the diluted culture was plated on a solid medium containing X-gal. Most colonies exhibited blue color, whereas white colonies also existed at a low level. By selecting the white colonies, strains in which the nucleotide sequence at the initiation codon region of *lysC* was substituted via a second crossover were selected. The nucleotide substitution of the initiation codon in the selected strain was finally

confirmed by PCR using the primers of SEQ ID NOs. 1 and 4 and then by analyzing the nucleotide sequence of the target site.

[Table 1]

Primer	Nucleotide sequence	SEQ ID NO.
lysC/ATG/FX	CCGGGGATCCTCTAGActtagggagccatcttttgg	1
lysC/ATG/R	CCAGGGCCATCTTTGTGC	2
lysC/ATG/F	GCACAAAGATGGCCCTGG	3
lysC/ATG/RX	GCAGGTCGACTCTAGAAGTGACATCAACAATGCGTG	4

Example 2: Construction of recombinant vector (pDZ-tkt(ATG)) having substituted ATG initiation codon in tkt derived from *Corynebacterium glutamicum* and Preparation of strain having substituted initiation codon

There are two codons which are expected as an initiation codon on the sequence of tkt gene. Based on the distance from RBS (Ribosomal binding site) and proteomics, the downstream codon was determined as the initiation codon in the present invention.

(1) Construction of pDZ-tkt(ATG) recombinant vector

In order to acquire tkt gene derived from *Corynebacterium glutamicum*, chromosomal DNA of KCCM11016P was used as a template. Based on the genbank at the U.S.

National Institutes of Health (NIH GenBank), a nucleotide sequence (SEQ ID NO. 14) containing the initiation codon region of *tkl* gene (NCBI Accession No. NC_003450, Ncgl1512) was acquired, and based on this sequence, two pairs of primers (Table 2, SEQ ID NOs. 5 ~ 8) for substituting the initiation codon TTG with ATG were synthesized.

PCR was carried out using chromosomal DNA of KCCM11016P as a template and primers of the following Table 2 under the conditions of Example 1-1. Two DNA fragments thus obtained were cloned into pDZ vector treated with restriction enzyme *Xba*I using an In-Fusion PCR cloning kit (Clontech), and finally, a pDZ-*tkl*(ATG) recombinant vector was constructed.

(2) Preparation of strain

The pDZ-*tkl*(ATG) vector thus constructed was transformed into the lysine-producing strain KCCM11016P in the same manner as in Example 1-2, and KCCM11016P-*tkl*, in which the initiation codon of *tkl* on the chromosome was substituted with ATG via a second crossover, was obtained. The nucleotide substitution of the initiation codon of the gene was finally confirmed by PCR using the primers of SEQ ID NOs. 5 and 8 and then by analyzing the nucleotide sequence of the target site.

[Table 2]

Primer	Nucleotide sequence	SEQ ID NO.
tkt/ATG/FX	CCG GGG ATC <u>CTC TAG A</u> GAA ATA GAT GGG TGT AGA CG	5
tkt/ATG/R4	GTGACAGCGTCATGGTGGTCAAT	6
tkt/ATG/F4	ATTGACCACCATGACGCTGTCAC	7
tkt/ATG/RX	GCA GGT CGA <u>CTC TAG A</u> CGC AGA GCC TTC AGG TCA TC	8

Example 3: Construction of recombinant vector (pDZ-*pyc*(ATG)) having substituted ATG initiation codon in *pyc* derived from *Corynebacterium glutamicum* and Preparation of strain having substituted initiation codon

(1) Construction of pDZ-*pyc*(ATG) recombinant vector

In order to acquire *pyc* gene derived from *Corynebacterium glutamicum*, chromosomal DNA of KCCM11016P was used as a template. Based on the genbank at the U.S. National Institutes of Health (NIH GenBank), a nucleotide sequence (SEQ ID NO. 15) containing the initiation codon region of *pyc* gene (NCBI Accession No. NC_003450, Ncgl0659) was acquired, and based on this sequence, two pairs of primers (Table 3, SEQ ID NOs. 9 ~ 12) for substituting the initiation codon GTG with ATG were synthesized.

PCR was carried out using chromosomal DNA of KCCM11016P as a template and primers of the following Table 3 under the conditions of Example 1-1. Two DNA fragments thus obtained were cloned into pDZ vector treated with restriction enzyme

XbaI using an In-Fusion PCR cloning kit (Clontech), and finally, a pDZ-*pyc*(ATG) recombinant vector was constructed.

(2) Preparation of strain

The pDZ-*pyc*(ATG) vector thus constructed was transformed into the lysine-producing strain KCCM11016P in the same manner as in Example 1-2, and KCCM11016P-*pyc*, in which the initiation codon of *pyc* on the chromosome was substituted with ATG via a second crossover, was obtained. The nucleotide substitution of the initiation codon of the gene was finally confirmed by PCR using the primers of SEQ ID NOs. 9 and 12 and then by analyzing the nucleotide sequence of the target site.

[Table 3]

Primer	Nucleotide sequence	SEQ ID NO.
<i>pyc</i> /ATG/FX	CCGGGGATCCTCTAGATTTTGGGGAAAA GTGCAAAG	9
<i>pyc</i> /ATG/R	GAGTCGACAtTAGAGTAAT	10
<i>pyc</i> /ATG/F	ATTACTCTAaTGTCGACTC	11
<i>pyc</i> /ATG/RX	GCAGGTCGACTCTAGAGGGCATTTCAGACAGGAAG	12

Example 4: Measurement of enzymatic activity of aspartate kinase in strain having substituted ATG initiation codon in *lysC* gene

The cells in the exponential phase were collected by centrifugation (5,000 rpm, 15 minutes) and washed three times with 0.1% Tris.HCl (pH 8.0) buffer, and then suspended in the same buffer for a turbidity at 610 nm of 160. The cells were disrupted for 6 minutes using a bead beater after glass beads added at 1.25 g/1.5 ml of the suspension. The supernatant was collected by centrifugation (15,000 rpm, 20 minutes), and quantitatively measured for protein content by a Bradford method (Bradford, M.M 1976. Anal. Biochem. 72:248-254) and used as a crude protein solution for measuring the enzymatic activity of aspartate kinase (LysC). In order to quantify the enzymatic activity of LysC, about 0.05 mL of the crude protein solution was added to a reaction solution containing 0.1 M Tris.HCl (pH 8.0), 0.01 M magnesium chloride (MgCl_2), 0.6 M hydroxylamine.HCl (pH 7.0), 4 mM ATP, and 0.2 M aspartate to initiate the reaction. The mixture was allowed to react at 30°C for 30 minutes, and a stop solution (10% FeCl_2 , 3.3% TCA, 0.7 N HCl) was added to terminate the reaction. The supernatant was collected by centrifugation, and absorbance at 540 nm was measured. The unit (U) of the LysC enzymatic activity was defined as nmole of the aspartate hydroxamate produced by 1 mg of protein for 1 minute.

KCCM11016P-lysC strain was observed to have 2.73-fold higher LysC activity than that of the parent strain KCCM11016P (Table 4).

[Table 4]

LysC enzymatic activity

Strain	Enzymatic activity (Fold)
	LysC
KCCM11016P	1.00
KCCM11016P-lysC	2.73

Example 5: Measurement of enzymatic activity of transketolase in strain having substituted ATG initiation codon in *tkt* gene

The cells in the exponential phase were collected by centrifugation (5,000 rpm, 15 minutes) and washed three times with 0.1% Tris.HCl (pH 7.5) buffer, and then suspended in the same buffer for a turbidity at 610 nm of 160. The cells were disrupted for 6 minutes using a bead beater after glass beads added at 1.25 g/1.5 ml of the suspension. The supernatant was collected by centrifugation (15,000 rpm, 20 minutes), and quantitatively measured for protein content by a Bradford method and used as a crude protein solution for measuring the enzymatic activity of transketolase (Tkt). In order to quantify the enzymatic activity of Tkt, the crude

protein solution was added to a reaction solution containing 0.1 M Tris.HCl (pH7.5), 10 mM D-R5P, 2 mM D-Xu5P, 10 μ M ThDP, 1.2 mM MgCl₂, 100 μ M NADH, 1 unit triosephosphate isomerase, 1 unit glycerol-3-phosphate dehydrogenase per 1 ml to initiate the reaction. The mixture was allowed to react at 30°C for 20~30 minutes, and absorbance at 340 nm was measured. The unit (U) of the Tkt enzymatic activity was defined as the amount (mg) of the enzyme catalyzing production of 1 μ mol of glyceraldehyde 3-phosphate for 1 minute, and specific activity was defined as units/mg (Biochem.J. (2004)382, 759-767).

KCCM11016P-tkt strain was observed to have 3.5-fold higher Tkt activity than that of the parent strain KCCM11016P (Table 5).

[Table 5]

Tkt enzymatic activity

Strain	Enzymatic activity
	(Fold)
	Tkt
KCCM11016P	1.00
KCCM11016P-tkt	3.5

Example 6: Measurement of enzymatic activity of pyruvate carboxylase in strain having substituted ATG initiation codon in pyc gene

The cells in the exponential phase were collected by centrifugation (5,000 rpm, 15 minutes) and washed twice with 50 mM Tris.HCl (pH 6.3) buffer containing 50 mM sodium chloride (NaCl), and then suspended in 100 mM HEPES (pH 7.5) buffer containing 20% glycerol. CTAB was added to the suspension to a concentration of 0.3%, and left on ice for 1 minute. The cells were collected by centrifugation (5,000 rpm, 10 minutes) and then suspended in 100 mM Tris.HCl (pH 7.3) buffer. The protein content was quantitatively measured by a Bradford method and used as a crude protein solution for measuring the enzymatic activity of pyruvate carboxylase (Pyc). In order to quantify the enzymatic activity of Pyc, the crude protein solution was added to a reaction solution containing 25 mM NaHCO₃, 5 mM MgCl₂, 3 mM pyruvate, and 4 mM ATP to initiate the reaction. The mixture was allowed to react at 30°C for 1.5 minutes, and 80 µl of a stop solution (30% o-phosphoric acid) was added to terminate the reaction. The supernatant was collected by centrifugation (12,000 rpm, 15 min, 4°C). 50 µl of the supernatant, 150 mM Tris.HCl (pH 7.8), 150 µM NADH, and 2.5 U lactate dehydrogenase were added per 1 ml and absorbance at 340 nm was measured at 37°C. The unit (U) of the Pyc

enzymatic activity was defined as nmole of the lactate produced by 1 mg of protein for 1 minute.

KCCM11016P-*pyc* strain was observed to have 1.89-fold higher *Pyc* activity than that of the parent strain KCCM11016P (Table 6).

[Table 6]

Pyc enzymatic activity

Strain	Enzymatic activity (Fold)
	<i>Pyc</i>
KCCM11016P	1.00
KCCM11016P- <i>pyc</i>	1.89

Example 7: Development of KCCM11016P-derived strains having substituted ATG initiation codons in two or more genes of *lysC*, *tkt*, and *pyc* genes

As the recombinant vectors, pDZ-*lysC*(ATG) pDZ-*tkt*(ATG) pDZ-*pyc*(ATG) prepared in Examples 1, 2, and 3 were used, and the preparation process is as follows.

The pDZ-*tkt*(ATG) vector was transformed into KCCM11016P-*lysC* of Example 1, in which GTG initiation codon of *lysC* was substituted with ATG, and through second crossover, the initiation codons of *lysC* and *tkt* on the

chromosome were substituted with ATG so as to obtain KCCM11016P-lysC-tkt. The nucleotide substitution of the initiation codon of the gene was finally confirmed by PCR using the primers of SEQ ID NOs. 5 and 8 and then by analyzing the nucleotide sequence of the target site.

In the same manner as in Example 1, the pDZ-pyc(ATG) vector was transformed into KCCM11016P-lysC of Example 1, in which GTG initiation codon of *lysC* was substituted with ATG, and through second crossover, the initiation codons of *lysC* and *pyc* on the chromosome were substituted with ATG so as to obtain KCCM11016P *lysC*-*pyc*. The nucleotide substitution of the initiation codon of the gene was finally confirmed by PCR using the primers of SEQ ID NOs. 9 and 12 and then by analyzing the nucleotide sequence of the target site.

The pDZ-tkt(ATG) vector was transformed into KCCM11016P-lysC-pyc of the present Example, in which GTG initiation codon of *lysC* and *pyc* was substituted with ATG, and through second crossover, the initiation codons of *lysC*, *pyc* and *tkt* on the chromosome were substituted with ATG so as to obtain KCCM11016P-lysC-pyc-tkt. The nucleotide substitution of the initiation codon of the gene was finally confirmed by PCR using the primers of SEQ ID NOs. 5 and 8

and then by analyzing the nucleotide sequence of the target site.

The above combinations of the genes are for illustrative purposes only, and the scope of the gene combinations is not intended to be limited thereto.

Example 8: Production of lysine in strain having substituted ATG initiation codon

KCCM11016P-lysC, KCCM11016P-tkt, KCCM11016P-pyc, KCCM11016P-lysC-tkt, KCCM11016P-lysC-pyc, KCCM11016P-lysC-pyc-tkt finally prepared in Examples 1, 2, 3, and 7 were cultured for L-lysine production by the following method.

The parent strain KCCM11016P and KCCM11016P-lysC, KCCM11016P-tkt, KCCM11016P-pyc, KCCM11016P-lysC-tkt, KCCM11016P-lysC-pyc, KCCM11016P-lysC-pyc-tkt were inoculated in respective 250 ml corner-baffled flasks containing 25 ml of the seed medium described below, and the resultant was cultured at 30°C with shaking at 200 rpm for 20 hours. 1 mL of the resulting seed culture broth was inoculated into a 250 ml corner-baffled flask containing 24 ml of the production medium described below, and cultured at 30°C with shaking (200 rpm) for 120 hours.

After completing the culture, the quantity of produced L-lysine was measured by HPLC. The results of measuring L-

lysine in the culture broths of KCCM11016P and KCCM11016P-lysC, KCCM11016P-tkt, KCCM11016P-pyc, KCCM11016P-lysC-tkt, KCCM11016P-lysC-pyc, KCCM11016P-lysC-pyc-tkt are shown in the following Table 7.

[Table 7]

Strain	Lysine (g/l)		
	Batch 1	Batch 2	Batch 3
KCCM11016P	45.2	44.7	45.6
KCCM11016P-lysC	47.5	46.2	47.9
KCCM11016P-tkt	47.4	48.5	47.0
KCCM11016P-pyc	47.9	46.3	47.8
KCCM11016P-lysC-tkt	49.7	49.3	49.2
KCCM11016P-lysC-pyc	50.1	48.9	49.6
KCCM11016P-lysC-pyc-tkt	50.7	50.2	51.1

Seed medium (pH 7.0)

20 g of raw sugar, 10 g of peptone, 5 g of yeast extract, 1.5 g of urea, 4 g of KH_2PO_4 , 8 g of K_2HPO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 100 μg of biotin, 1000 μg of Thiamine-HCl, 2000 μg of calcium-pantothenate, 2000 μg of nicotine amide (in 1 liter of distilled water)

Production medium (pH 7.0)

100 g of glucose, 40 g of $(\text{NH}_4)_2\text{SO}_4$, 2.5 g of soybean protein, 5 g of corn steep solids, 3 g of urea, 1 g of KH_2PO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 100 μg of biotin, 1000 μg of Thiamine-HCl, 2000 μg of calcium-pantothenate, 3000 μg of nicotine amide, 30 g of CaCO_3 (in 1 liter of distilled water)

As shown in Table 7, *Corynebacterium glutamicum* KCCM11016P-lysC, KCCM11016P-tkt, KCCM11016P-pyc, KCCM11016P-lysC-tkt, and KCCM11016P-lysC-pyc having substituted ATG initiation codon were found to show 4~9% increase in L-lysine productivity, compared with the parent strain KCCM11016P. In particular, KCCM11016P-lysC-pyc-tkt introduced with all three genes was found to show an increase in L-lysine productivity as high as 12%, compared to the parent strain KCCM11016P.

Example 9: Development of KFCC10750-derived strain having substituted ATG initiation codons in lysC, tkt, and pyc genes and Comparison of lysine productivity

In order to examine whether substitution of the initiation codons with ATG in *lysC*, *pyc*, and *tkt* genes also affects lysine productivity in other lysine-producing strains belonging to *Corynebacterium glutamicum*, the L-lysine-producing strain *Corynebacterium glutamicum* KFCC10750 (Korean Patent No. 10-0073610) was introduced with all three

genes which showed the most excellent effect of increasing lysine productivity in Example 8 so as to prepare a recombinant strain, which was named as KFCC10750-lysC-pyc-tkt. The strain was cultured in the same manner as in Example 8, and the L-lysine concentration was analyzed (Table 8).

[Table 8]

Strain	Lysine (g/l)		
	Batch 1	Batch 2	Batch 3
KFCC10750	38.3	38	38.5
KFCC10750-lysC-pyc-tkt	44.1	43.8	44.5

As shown in Table 8, *Corynebacterium glutamicum* KFCC10750-lysC-pyc-tkt introduced with all three genes showed 15% increase in the lysine productivity, compared to the parent strain KFCC10750.

Example 10: Development of KCCM10770P-derived strain having substituted ATG initiation codons in *lysC*, *tkt*, *pyc* genes and Comparison of lysine productivity

Another L-lysine-producing strain *Corynebacterium glutamicum* KCCM10770P (Korean Patent No. 10-0924065) was introduced with all three genes which showed the most excellent effect of increasing lysine productivity in

Example 8 so as to prepare a recombinant strain, which was named as KCCM10770P-lysC-pyc-tkt. The strain was cultured in the same manner as in Example 8, and the L-lysine concentration was analyzed (Table 9).

[Table 9]

Strain	Lysine (g/l)		
	Batch 1	Batch 2	Batch 3
KCCM10770P	47.8	47.2	47.5
KCCM10770P-lysC-pyc-tkt	52.8	52.8	52.4

As shown in Table 9, *Corynebacterium glutamicum* KCCM10770P-lysC-pyc-tkt introduced with all three genes showed 11% increase in the lysine productivity, compared to the parent strain KCCM10770P.

Example 11: Development of CJ3P-derived strain having substituted ATG initiation codons in *lysC*, *tkt*, *pyc* genes and Comparison of lysine productivity

Still another L-lysine-producing strain *Corynebacterium glutamicum* CJ3P (Binder et al. Genome Biology 2012, 13:R40) was introduced with all three genes which showed the most excellent effect of increasing lysine productivity in Example 8 so as to prepare a recombinant strain, which was named as CJ3P-lysC-pyc-tkt. The strain was cultured in the

same manner as in Example 8, and the L-lysine concentration was analyzed (Table 10).

[Table 10]

Strain	Lysine (g/l)		
	Batch 1	Batch 2	Batch 3
CJ3P	8.3	8	8.4
CJ3P-lysC-pyc-tkt	9.7	9.6	10.0

As shown in Table 10, *Corynebacterium glutamicum* CJ3P-lysC-pyc-tkt introduced with all three genes showed 18% increase in the lysine productivity, compared to the parent strain CJ3P.

Effect of the invention

The present invention provides a *Corynebacterium* sp. microorganism having improved L-lysine productivity, in which initiation codons of one or more genes encoding aspartate kinase, transketolase, or pyruvate carboxylase are substituted to enhance activities of the corresponding enzymes, compared to their endogenous activities in a native microorganism.

WHAT IS CLAIMED IS:

1. A modified polynucleotide encoding aspartate kinase (LysC), transketolase (Tkt), or pyruvate carboxylase (Pyc), wherein an initiation codon of the polynucleotide is substituted with ATG.

2. The modified polynucleotide according to claim 1, wherein the initiation codon of the polynucleotide encoding aspartate kinase (LysC) or pyruvate carboxylase (Pyc) is GTG and the initiation codon of the polynucleotide encoding transketolase (Tkt) is TTG.

3. The modified polynucleotide according to claim 1, wherein the modified polynucleotide is represented by a nucleotide sequence of SEQ ID NO. 16, 17 or 18.

4. A vector comprising one or more modified polynucleotides selected from the modified polynucleotides encoding aspartate kinase (LysC), transketolase (Tkt), and pyruvate carboxylase (Pyc), wherein an initiation codon of the polynucleotide is substituted with ATG..

5. A modified microorganism that has enhanced activities of one or more enzymes compared to endogenous activities thereof, wherein the enzymes are selected from aspartate kinase, transketolase and pyruvate carboxylase, and the initiation codon of polynucleotides encoding the enzymes are substituted with ATG.

6. The microorganism according to claim 5, wherein the microorganism is transformed with the vector of claim 4.

7. The microorganism according to claim 5, wherein the microorganism is *Corynebacterium* sp.

8. The microorganism according to claim 7, wherein the *Corynebacterium* sp. microorganism is *Corynebacterium glutamicum*.

9. A method for producing L-lysine, comprising the steps of culturing the microorganism of any one of claims 5 to 8; and recovering L-lysine from the cultured microorganism or the culture broth.

청구범위

- [청구항 1] 아스파테이트 키나제(LysC), 트랜스케토라제(Tkt), 또는 파이루베이트 카르복실라제(Pyc)를 코딩하는 각각의 폴리뉴클레오티드의 개시코돈을 ATG로 치환한 변이형 폴리뉴클레오티드.
- [청구항 2] 제1항에 있어서, 상기 아스파테이트 키나제(LysC) 또는 파이루베이트 카르복실라제(Pyc)를 코딩하는 폴리뉴클레오티드의 개시코돈은 GTG이고, 상기 트랜스케토라제(Tkt)를 코딩하는 폴리뉴클레오티드의 개시코돈은 TTG인 변이형 폴리뉴클레오티드.
- [청구항 3] 제1항에 있어서, 상기 변이형 폴리뉴클레오티드는 서열번호 16, 17 또는 18의 염기서열로 표시되는 변이형 폴리뉴클레오티드.
- [청구항 4] ATG로 치환된 개시코돈을 가지는, 아스파테이트 키나제, 트랜스케토라제 및 파이루베이트 카르복실라제를 코딩하는 변이형 폴리뉴클레오티드로 이루어진 군 중에서 선택된 하나 이상의 변이형 폴리뉴클레오티드를 포함하는 벡터.
- [청구항 5] ATG로 치환된 개시코돈을 가지는, 아스파테이트 키나제, 트랜스케토라제 및 파이루베이트 카르복실라제로 이루어진 군에서 선택된 하나 이상의 효소를 코딩하는 변이형 폴리뉴클레오티드를 포함함으로써, 상기 효소 중 하나 이상의 효소의 활성이 내재적 활성보다 증가된 미생물.
- [청구항 6] 제5항에 있어서, 상기 미생물은 제4항의 벡터로 형질전환된 미생물.
- [청구항 7] 제5항에 있어서, 상기 미생물은 코리네박테리움 속 미생물인 미생물.
- [청구항 8] 제7항에 있어서, 상기 코리네박테리움 속 미생물은 코리네박테리움 글루타미쿰(*Corynebacterium glutamicum*)인 미생물.
- [청구항 9] 제5항 내지 제8항 중 어느 한 항에 따른 미생물을 배양하는 단계; 및 상기 배양된 미생물 또는 배양 배지로부터 L-라이신을 회수하는 단계를 포함하는, L-라이신을 생산하는 방법.