

MICROORGANISMS FOR PRODUCING PUTRESCINE OR ORNITHINE AND PROCESS FOR PRODUCING PUTRESCINE OR ORNITHINE USING THEM

TECHNICAL FIELD

The present disclosure relates to a recombinant microorganism producing putrescine or ornithine, and a method for producing putrescine or ornithine using the same.

BACKGROUND ART

Biogenic amines (BAs) are nitrogen compounds that are mainly produced by decarboxylation of amino acids or amination and transamination of aldehydes and ketones. These biogenic amines have low molecular weight and are synthesized during the metabolic processes in microorganisms, plants, and animals thus being known as constituting elements which are frequently discovered in their cells.

Among them, putrescine is discovered in gram negative bacteria or fungi and is present in high concentration in various species, and thus putrescine is expected to play an important role in the metabolism of microorganisms. In general, putrescine is an important raw material for the synthesis of polyamine nylon-4,6 and is produced mainly by chemical synthesis. The chemical synthesis is a 3-step process including a catalytic oxidation reaction, a reaction using a cyanide compound, and a hydrogenation reaction using high-pressure hydrogen. Accordingly, there is a demand for the development of a more environment-friendly and energy-effective method involving biomass utilization.

Under these circumstances, various methods for producing putrescine at high concentration by transforming *E. coli* and a microorganism of the genus *Corynebacterium* were disclosed (International Patent Publication No. WO 06/005603; International Patent Publication No. WO 09/125924; Qian ZD *et al.*, *Biotechnol. Bioeng.* 104 (4): 651 - 662, 2009; Schneider *et al.*, *Appl. Microbiol. Biotechnol.* 88 (4): 859 - 868, 2010; Schneider *et al.*, *Appl. Microbiol. Biotechnol.* 95: 169 - 178, 2012).

On the other hand, ornithine is a material widely discovered in plants, animals, and microorganisms, and serves as a precursor for biosynthesis of arginine, proline, and polyamine. Additionally, ornithine plays an important role in the pathway of producing urea from amino

acids or ammonia and disposing through the ornithine cycle during the *in-vivo* metabolism of higher organisms. Ornithine is effective in muscle production and reduction of body fat, and thus it has been used as a nutrient supplement and also as a pharmaceutical drug for improving liver cirrhosis and hepatic dysfunction. Methods of producing ornithine include a method of using milk casein as a digestive enzyme and a method of using *E. coli* or a microorganism of the genus *Corynebacterium* (Korean Patent No. 10-1372635; T. Gotoh *et al.*, *Bioprocess Biosyst. Eng.*, 33: 773 - 777, 2010).

E. coli and a microorganism of the genus *Corynebacterium* are similar in the biosynthetic pathways for producing putrescine or ornithine, but they also exhibit differences as follows. First, the microorganism of the genus *Corynebacterium* has a “cyclic pathway”, in which glutamic acid is converted into N-acetyl-L-glutamic acid and N-acetyl-L-ornithine is converted into L-ornithine by argJ (bifunctional ornithine acetyltransferase/N-acetylglutamate synthase, EC 2.3.1.35). In contrast, *E. coli* is involved in the biosynthesis of putrescine or ornithine by a “linear pathway”, in which argA (N-acetylglutamate synthase, EC 2.3.1.1) and argE (Acetylornithine deacetylase, EC 3.5.1.16) replace the role of the argJ in the microorganism of the genus *Corynebacterium*.

In the microorganism of the genus *Corynebacterium*, it is known that an acetyl group recycles between ornithine and glutamic acid in ArgJ. However, in *E. coli*, ArgA attaches the acetyl group of acetyl-CoA to glutamate in order to produce N-acetyl-glutamate, and ArgE N-acetyl-ornithine decomposes N-acetyl-ornithine to produce ornithine and acetate (Schneider *et al.*, *Appl. Microbiol. Biotechnol.* 91, 17 - 30., 2011).

In particular, pta-ackA (pta, phosphotransacetylase; ackA, acetate kinase) operon and acetyl-coenzyme A synthetase (acs) are known as genes to synthesize acetyl-CoA using acetate.

SUMMARY

TECHNICAL PROBLEM

The present inventors have made many efforts to improve the ability of a microorganism of the genus *Corynebacterium* to produce ornithine and putrescine, and as a result they have discovered that the introduction of *E. coli*-derived *argA* and *argE* into a microorganism of the genus *Corynebacterium* can improve its ability to produce ornithine and putrescine, thereby completing the present invention.

TECHNICAL SOLUTION

In one aspect, the present invention refers to a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, wherein activities of N-acetylglutamate synthase from *E. coli* and acetylornithine deacetylase from *E. coli* are introduced into a microorganism of the genus *Corynebacterium* having ornithine acetyltransferase (ArgJ) activity.

In another aspect, the present invention refers to a method for producing putrescine or ornithine comprising:

- (i) culturing the modified microorganism of the genus *Corynebacterium* of any one of claims 1 to 15 in a medium; and
- (ii) recovering putrescine or ornithine from the cultured microorganism or the medium..

ADVANTAGEOUS EFFECTS OF THE INVENTION

It was confirmed that the microorganism of the genus *Corynebacterium* of the present disclosure producing putrescine or ornithine can improve the amount of putrescine- or ornithine production when the microorganism is introduced with *E. coli*-derived *argA* and *E. coli*-derived *argE*, and also when the acetate utilization pathway is reinforced. Accordingly, the microorganism of the present disclosure can be widely used for the production of putrescine or ornithine, and also, can be widely used as an effective and desirable means to supply raw materials for the production of various polymer products, in which the putrescine or ornithine is used as a raw material, from the economic and environmental aspects.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram illustrating a biosynthetic pathway (a cyclic pathway) for producing putrescine and ornithine in a microorganism of the genus *Corynebacterium* (A) and a biosynthetic pathway (a linear pathway) for producing putrescine and ornithine in *E. coli* (B).

FIG. 2 is a schematic diagram illustrating the biosynthetic pathway which has improved the ability to produce putrescine and ornithine by introducing *E. coli*-derived *argA* and *E. coli*-derived *argE* into a microorganism of the genus *Corynebacterium*, which is in a state expressing *argJ*.

BEST MODE

An aspect of the present disclosure provides a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which activities of N-acetylglutamate synthase from *E. coli* and acetylornithine deacetylase from *E. coli* are introduced.

An exemplary embodiment of the present disclosure provides a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which the *E. coli*-derived N-acetylglutamate synthase consists of an amino acid sequence of SEQ ID NO: 1.

Another exemplary embodiment of the present disclosure provides a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which the *E. coli*-derived acetylornithine deacetylase consists of an amino acid sequence of SEQ ID NO: 3.

Still another exemplary embodiment of the present disclosure provides a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which the microorganism of the genus *Corynebacterium* is selected from the group consisting of *Corynebacterium glutamicum*, *Corynebacterium ammoniagenes*, *Corynebacterium thermoaminogenes*, *Brevibacterium flavum*, and *Brevibacterium lactofermentum*.

Still another exemplary embodiment of the present disclosure provides a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which an activity of phosphotransacetylase and acetate kinase operon (*pta-ackA* operon) is further enhanced compared to its endogenous activity.

Still another exemplary embodiment of the present disclosure provides a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which the phosphotransacetylase and acetate kinase operon consists of an amino acid sequence of SEQ ID NO: 5 or 7.

Still another exemplary embodiment of the present disclosure provides a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which an activity of *E. coli*-derived acetyl-CoA synthetase (*acs*) is further introduced.

Still another exemplary embodiment of the present disclosure provides a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which the *E. coli*-derived acetyl-CoA synthetase (*acs*) consists of an amino acid sequence of SEQ ID NO: 9.

Still another exemplary embodiment of the present disclosure provides a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which an activity of ornithine decarboxylase (ODC) is further introduced.

Still another exemplary embodiment of the present disclosure provides a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which an activity of i) ornithine carbamoyltransferase (ArgF), ii) glutamate exporter, or iii) ornithine carbamoyltransferase and glutamate exporter is further weakened, compared to its endogenous activity.

Still another exemplary embodiment of the present disclosure provides a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which an

activity of at least one selected from the group consisting of acetyl gamma glutamyl phosphate reductase (ArgC), acetylglutamate synthase or ornithine acetyltransferase (ArgJ), acetylglutamate kinase (ArgB), and acetyl ornithine aminotransferase (ArgD), is further enhanced compared to its endogenous activity.

Still another exemplary embodiment of the present disclosure provides a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which an activity of acetyltransferase is further weakened compared to its endogenous activity.

Still another exemplary embodiment of the present disclosure provides a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which the acetyltransferase consists of the amino acid sequence of SEQ ID NO: 30 or 31.

Still another exemplary embodiment of the present disclosure provides a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which an activity of the putrescine exporter is further enhanced compared to its endogenous activity.

Still another exemplary embodiment of the present disclosure provides a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which the putrescine exporter consists of the amino acid sequence of SEQ ID NO: 26 or 28.

Another aspect of the present disclosure provides a method for producing putrescine or ornithine, including:

- (i) culturing the modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine in a medium; and
- (ii) recovering putrescine or ornithine from the cultured microorganism or the medium.

In an exemplary embodiment of the present disclosure, the modified microorganism of the genus *Corynebacterium* is *Corynebacterium glutamicum*.

Hereinafter, the present disclosure will be described in detail.

An embodiment of the present disclosure provides a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which activities of *E. coli*-derived N-acetylglutamate synthase and *E. coli*-derived acetylornithine deacetylase are introduced.

As used herein, the term “N-acetylglutamate synthase” refers to an enzyme which mediates the reaction producing N-acetylglutamate from glutamate and acetyl-CoA, and the N-acetylglutamate produced thereof may be used as a precursor of ornithine and arginine.

In the present disclosure, N-acetylglutamate synthase may include, for example, the

protein having an amino acid sequence of SEQ ID NO: 1, and any protein, which has a homology of 70% or higher, specifically 80% or higher, more specifically 90% or higher, even more specifically 95% or higher, yet even more specifically 98% or higher, and most specifically 99% or higher, to the amino acid sequence above, as long as the protein has the substantial activity of N-acetylglutamate synthase, without limitation.

Additionally, the proteins exhibiting the activity above may show differences in amino acid sequences, according to the species and strains of the microorganism. Accordingly, the N-acetylglutamate synthase of the present disclosure may be, for example, one from *E. coli*, although it is not limited thereto.

As a sequence having a homology to the sequence above, if the amino acid sequence is one which has substantially the same or corresponding to biological activity of a protein of SEQ ID NO: 1, it is obvious in that amino acid sequences with a deletion, a modification, a substitution, or an addition in part of the sequences should also be included in the scope of the present disclosure.

The polynucleotide encoding the N-acetylglutamate synthase of the present disclosure may include, without limitation, a polynucleotide encoding the protein having an amino acid sequence of SEQ ID NO: 1, and any protein, which has a homology of 70% or higher, specifically 80% or higher, more specifically 90% or higher, even more specifically 95% or higher, yet even more specifically 98% or higher, and most specifically 99% or higher, to the above amino acid sequence, as long as the polynucleotide has an activity similar to that of N-acetylglutamate synthase, and for example, a polynucleotide sequence of SEQ ID NO: 2 may be included.

As used herein, the term “acetylornithine deacetylase” refers to an enzyme which mediates the reaction involved in the production of acetic acid and ornithine by mediating the hydrolysis of acetylornithine.

In the present disclosure, acetylornithine deacetylase may include, without limitation, the protein having an amino acid sequence of SEQ ID NO: 3, and any protein, which has a homology of 70% or higher, specifically 80% or higher, more specifically 90% or higher, even more specifically 95% or higher, yet even more specifically 98% or higher, and most specifically 99% or higher, to the above amino acid sequence, as long as the protein has the substantial activity of separating acetyl group and ornithine from acetylornithine.

Additionally, the proteins exhibiting the activity above may show a difference in amino

acid sequences, according to the species and strains of the microorganism. Accordingly, the acetylornithine deacetylase of the present disclosure may be one from *E. coli*, although it is not limited thereto. As a sequence having a homology, if the amino acid sequence is one which has substantially the same or corresponding to biological activity of a protein of SEQ ID NO: 3, it is obvious in that amino acid sequences with a deletion, a modification, a substitution, or an addition in part of the sequences should also be included in the scope of the present disclosure.

The polynucleotide encoding acetylornithine deacetylase of the present disclosure may include, as long as the polynucleotide has an activity similar to that of the acetylornithine deacetylase protein, the protein having an amino acid sequence of SEQ ID NO: 3, or a polynucleotide encoding a protein, which has a homology of 70% or higher, specifically 80% or higher, more specifically 90% or higher, even more specifically 95% or higher, yet even more specifically 98% or higher, and most specifically 99% or higher, to the amino acid sequence above, for example, a polynucleotide sequence of SEQ ID NO: 4.

Additionally, the polynucleotide encoding N-acetylglutamate synthase or acetylornithine deacetylase of the present disclosure may be hybridized with the polynucleotide sequence of SEQ ID NO: 2 or SEQ ID NO: 4 or a probe derived from the polynucleotide sequence under stringent conditions, and it may be a modified type of N-acetylglutamate synthase or acetylornithine deacetylase that functions normally. In the above, the term “stringent conditions” refers to a condition that enables a specific hybridization between polynucleotides. For example, the stringent conditions are specifically described in references (*e.g.*, J. Sambrook et al., *supra*).

In the above, the term “homology” refers to the degree of identity with the given amino acid sequence or a polynucleotide sequence, and may be indicated in percentage. As used herein, the homologous sequence having the same or similar activity with the given polypeptide sequence or polynucleotide sequence may be indicated in terms of “% homology”. For example, the % homology may be confirmed using standard software, *i.e.*, BLAST 2.0, for calculating parameters such as score, identity, and similarity, or by comparing sequences via southern hybridization experiments, and the appropriate hybridization condition to be defined may be determined by a method known to a skilled person in the art (*e.g.*, J. Sambrook et al., *Molecular Cloning, A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory press, Cold Spring Harbor, New York, 1989; F.M. Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., New York).

On the other hand, the microorganism of the present disclosure may include both a natural type and a modified type, *e.g.*, microorganisms that belong to the genus *Escherichia*, the genus *Shigella*, the genus *Citrobacter*, the genus *Salmonella*, the genus *Enterobacter*, the genus *Yersinia*, the genus *Klebsiella*, the genus *Erwinia*, the genus *Corynebacterium*, the genus *Brevibacterium*, the genus *Lactobacillus*, the genus *Selenomonas*, the genus *Vibrio*, the genus *Pseudomonas*, the genus *Streptomyces*, the genus *Arcanobacterium*, the genus *Alcaligenes*, *etc.* Specifically, the microorganism of the present disclosure may be a microorganism belonging to the genus *Corynebacterium*, more specifically, a microorganism selected from the group consisting of *Corynebacterium glutamicum*, *Corynebacterium ammoniagenes*, *Corynebacterium thermoaminogenes*, *Brevibacterium flavum*, and *Brevibacterium lactofermentum*, and more specifically, *Corynebacterium glutamicum*, but it is not limited thereto.

Specifically, as used herein, the term “a microorganism of the genus *Corynebacterium* producing putrescine or ornithine” refers to a microorganism of the genus *Corynebacterium* producing putrescine or ornithine in a natural state; or a microorganism of the genus *Corynebacterium* producing putrescine or ornithine prepared by providing the ability to produce putrescine or ornithine into its parent strain, which cannot produce putrescine or ornithine.

The microorganism, which is provided with the ability to produce putrescine or ornithine or can produce putrescine or ornithine, may have an improved ability to produce ornithine, which is used as a raw material for biosynthesis of putrescine, by modifying the activities of acetylglutamate synthase (which converts glutamate into N-acetylglutamate), ornithine acetyltransferase (ArgJ, which converts acetylornithine into ornithine), acetylglutamate kinase (ArgB, which converts acetylglutamate into N-acetylglutamyl phosphate), gamma glutamyl phosphate reductase (ArgC, which converts N-acetylglutamyl phosphate into N-acetylglutamate semialdehyde), and acetyl ornithine aminotransferase (ArgD, which converts acetylglutamate semialdehyde into N-acetylornithine) to be increased, compared to their endogenous activities, in order to increase the biosynthetic pathway from glutamate to ornithine, although not particularly limited thereto.

Additionally, the microorganism may be modified to weaken the activities of ornithine carbamoyltransferase (ArgF, which is involved in the synthesis arginine from ornithine), a protein(s) involved in the export of glutamate, and/or a protein(s) that acetylates putrescine, compared to their endogenous activities; and/or to introduce the activity of ornithine

decarboxylase (ODC).

As used herein, the term “introduction of activity” may refer to an activity of a protein, which is not present or weak in a microorganism, is newly introduced or enhanced in the corresponding microorganism. Specifically, it may include inserting or delivering a gene encoding a protein, which is not present in the microorganism, into the microorganism to be expressed therein, or inducing a modification of the protein for enhancing the expression of the protein, which is not expressed or almost not expressed in the microorganism, but is not limited thereto.

On the other hand, in the present disclosure, modifications such as introduction of activity, enhancement of activity, weakening of activity, *etc.*, may occur through a process called transformation. As used herein, the term “transformation” refers to a process of introducing a vector, which includes a polynucleotide encoding a particular protein or a promoter sequence with strong or weak activity, *etc.*, into the host cell thereby enabling the expression of the protein encoded by the polynucleotide or inducing a modification of the chromosome in the host cell. Additionally, the polynucleotide includes DNA and RNA which encode the target protein. The polynucleotide may be inserted in any form insofar as it can be introduced into a host cell and expressed or induce a modification therein. For example, the polynucleotide may be introduced into a host cell in the form of an expression cassette, which is a gene construct including all essential elements required for self-expression. The expression cassette may conventionally include a promoter operably connected to the polynucleotide, a transcription termination signal, a ribosome-binding domain, and a translation termination signal, and may be in the form of an expression vector capable of self-replication. Additionally, the polynucleotide may be introduced into a host cell as it is, and operably connected to a sequence essential for its expression in the host cell, but is not limited thereto.

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

As used herein, the term “vector” refers to a DNA construct including the nucleotide sequence of the polynucleotide encoding a protein of interest, in which the protein of interest is operably linked to a suitable regulatory sequence so that the protein of interest can be expressed in an appropriate host. The regulatory sequence includes a promoter capable of initiating

transcription, any operator sequence for regulation of the transcription, a sequence encoding a suitable mRNA ribosome-binding domain, and a sequence for regulating transcription and translation. The vector, after being transformed into a suitable host cell, may be replicated or function irrespective of the host genome, or may be integrated into the host genome itself.

The vector used in the present disclosure may not be particularly limited as long as the vector is replicable in a host cell, and any vector known in the art may be used. Examples of the vector may include natural or recombinant plasmids, cosmids, viruses, and bacteriophages. For example, as a phage vector or cosmid vector, *pWE15*, *M13*, *MBL3*, *MBL4*, *IXII*, *ASHII*, *APII*, *t10*, *t11*, *Charon4A*, *Charon21A*, etc., may be used; and as a plasmid vector, those based on *pBR*, *pUC*, *pBluescriptII*, *pGEM*, *pTZ*, *pCL*, *pET*, etc., may be used. The vector to be used in the present disclosure may not be particularly limited and any vector known in the art may be used. Specifically, *pDZTn*, *pACYC177*, *pACYC184*, *pCL*, *pECCG117*, *pUC19*, *pBR322*, *pMW118*, *pCCIBAC* vectors, etc., may be used.

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

The microorganism of the genus *Corynebacterium* of the present disclosure may be a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which an activity of phosphotransacetylase and acetate kinase operon (*pta-ackA* operon) is further enhanced compared to its endogenous enzyme.

In the present disclosure, the phosphotransacetylase and acetate kinase operon (*pta-ackA* operon) are operons including genes that reversibly mediate the metabolic pathway, in which

acetyl-CoA produced from glucose or pyruvate converts into acetic acid via acetyl phosphate, and the metabolic pathway in the opposite direction.

In the present disclosure, the phosphotransacetylase and acetate kinase operon may include, without limitation, the proteins including an amino acid sequence of SEQ ID NO: 5 or SEQ ID NO: 7, or any protein, which has a homology of 70% or higher, specifically 80% or higher, more specifically 90% or higher, specifically, even more specifically 95% or higher, yet even more specifically 98% or higher, or most specifically 99% or higher, to the above amino acid sequences, as long as the protein substantially mediates the reaction of producing acetyl-CoA from acetic acid.

Additionally, since the amino acid sequences of the proteins exhibiting the activities may vary according to the species or strains of a given microorganism, the phosphotransacetylase and acetate kinase operon in the present disclosure may not be limited to those origins from which they are derived. It is obvious in that any amino acid sequence, which has a homology to the sequences above and has a biological activity substantially the same as or corresponding to the protein of SEQ ID NO: 5 or SEQ ID NO: 7, can also belong to the scope of the present disclosure, although the amino acid sequence may have deletion, modification, substitution, or addition, in part of the sequence.

The polynucleotide encoding the phosphotransacetylase and acetate kinase operon of the present disclosure may include the polynucleotide which encodes the amino acid of SEQ ID NO: 5 or SEQ ID NO: 7, or the polynucleotide which encodes a protein having a homology of 70% or higher, specifically 80% or higher, more specifically 90% or higher, even more specifically 95% or higher, yet even more specifically 98% or higher, and most specifically 99% or higher to the above amino acid sequences, and most specifically may include the polynucleotide sequence of SEQ ID NO: 6 or SEQ ID NO: 8.

As used herein, the term “enhancement of activity” not only includes the drawing of a higher effect than the original function due to the new introduction of an activity or an increase in the activity of a protein itself, but also includes the increase in its activity by an increase in the activity of an endogenous gene, amplification of an endogenous gene from internal or external factor(s), deletion of regulatory factor(s) for inhibiting the gene expression, an increase in gene copy number, introduction of a gene from outside, modification of the expression regulatory sequence, and specifically, an increase in enzyme activity due to replacement or modification of

a promoter and a mutation within the gene, *etc.*

Specifically, in the present disclosure, the increase in activity may be performed by:

- 1) increasing copy number of a polynucleotide encoding the enzyme,
- 2) modifying the expression regulatory sequence for increasing the expression of the polynucleotide,
- 3) modifying the polynucleotide sequence on the chromosome for enhancing the activity of the enzyme, or
- 4) modifying by a combination thereof,

but the method is not limited thereto.

The increase of copy number of a polynucleotide (method 1) may be performed in a form in which the polynucleotide is operably linked to a vector, or by inserting the polynucleotide into the chromosome of a host cell, although the method is not particularly limited thereto. Specifically, the copy number of a polynucleotide within the chromosome of the host cell may be increased by introducing a vector which can replicate and function regardless of a host cell and to which the polynucleotide encoding the protein of the present disclosure is operably linked; or may be increased by introducing a vector, which can insert the polynucleotide into the chromosome of a host cell and to which the polynucleotide is operably linked, into a host cell.

Then, the modification of the expression regulatory sequence for increasing the expression of a polynucleotide (method 2) may be performed by inducing a modification on the sequence through deletion, insertion, non-conservative or conservative substitution of the polynucleotide sequence, or a combination thereof to further enhance the activity of expression regulatory sequence, or by replacing the polynucleotide sequence with a polynucleotide sequence having a stronger activity, although the method is not particularly limited thereto. The expression regulatory sequence includes a promoter, an operator sequence, a sequence coding for ribosome-binding site, and a sequence regulating the termination of transcription and translation, although not particularly limited thereto.

A strong exogenous promoter, instead of the original promoter, may be connected to the upstream region of the expression unit of the polynucleotide. Examples of the strong promoter may be *CJ7* promoter, *lysCPI* promoter, *EF-Tu* promoter, *groEL* promoter, *aceA* or *aceB* promoter, *etc.*, and more specifically, the expression rate may be improved by being operably connected to *Corynebacterium*-derived *lysCPI* promoter (WO 2009/096689) or *CJ7* promoter

(Korean Patent No. 10-0620092 and WO 2006/065095), but the strong promoter is not limited thereto.

Furthermore, the modification of a polynucleotide sequence on the chromosome (method 3) may be performed by inducing a modification on the expression regulatory sequence through deletion, insertion, non-conservative or conservative substitution of the polynucleotide sequence, or a combination thereof to further enhance the activity of the polynucleotide sequence, or by replacing the polynucleotide sequence with an improved polynucleotide sequence having a stronger activity, although the method is not particularly limited thereto.

Specifically, in the present disclosure, the activity of the phosphotransacetylase and acetate kinase operon (*pta-ackA* operon) may be enhanced in comparison with its endogenous activity by any one method selected from the group consisting of a method of increasing the copy number of the operon in a cell, a method of introducing a modification on an expression regulatory sequence of the operon, a method of replacing an expression regulatory sequence of a gene on the operon with a sequence having a stronger activity, a method of replacing the genes encoding the enzymes with mutated genes on the chromosome for increasing the activities of the enzymes constituting the operon, and a method of introducing a modification on the gene on the chromosome for increasing the activities of the enzymes constituting the operon. Specifically, the method of replacing an expression regulatory sequence of a gene on the operon with a sequence having a stronger activity may be achieved by replacing an endogenous promoter of the acetylase and acetate kinase operon with *CJ7* promoter, *lysCPI* promoter, *EF-Tu* promoter, *groEL* promoter, *aceA* or *aceB* promoter, *etc.*, but the replacement is not limited thereto.

As used herein, the term “endogenous activity” refers to an active state of an enzyme in a non-modified state originally possessed by a microorganism, and the term “enhancement compared to its endogenous activity” refers to an increased state of the activity of the enzyme possessed by the microorganism after manipulation, such as the introduction of a gene exhibiting an activity or an increase of the copy number of the corresponding gene, deletion of the inhibition-regulatory factor of the expression of the gene, or modification of the expression regulatory sequence, *e.g.*, use of an improved promoter, compared to the activity possessed by the microorganism before manipulation.

In the present disclosure, the microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which an activity of *E. coli*-derived acetyl-CoA synthetase (acs) may be further introduced therein.

In the present disclosure, acetyl-CoA synthetase (acs) is an enzyme which mediates the reaction for producing acetyl-CoA from ATP, acetic acid, and CoA.

In the present disclosure, the acetyl-CoA synthetase may include, without limitation, the proteins having the amino acid sequence of SEQ ID NO: 9, or any protein having a homology of 70% or higher, specifically 80% or higher, more specifically 90% or higher, even more specifically 95% or higher, yet even more specifically 98% or higher, and most specifically 99% or higher, to the amino acid sequence above, as long as the protein has the substantial activity of mediating the synthesis of acetyl-CoA.

Additionally, since the amino acid sequences of the proteins exhibiting the activities may vary according to the species or strains of a given microorganism, the acetyl-CoA synthetase (acs) in the present disclosure may not be limited to the origin from which it is derived, and for example, it may be from *E. coli*. It is obvious in that any amino acid sequence, which has a homology to the sequence above and has a biological activity substantially the same as or corresponding to the protein of SEQ ID NO: 9, can also belong to the scope of the present disclosure, although the amino acid sequence may have deletion, modification, substitution, or addition, in part of the sequence.

The polynucleotide encoding the acetyl-CoA synthetase (acs) of the present disclosure may include the polynucleotide which encodes a protein including the amino acid sequence of SEQ ID NO: 9, or any protein having a homology of 70% or higher, specifically 80% or higher, more specifically 90% or higher, even more specifically 95% or higher, yet even more specifically 98% or higher, and most specifically 99% or higher, to the above amino acid sequence, and most specifically, it may include the polynucleotide sequence of SEQ ID NO: 10.

The microorganism of the genus *Corynebacterium* of the present disclosure may be a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which an activity of ornithine decarboxylase (ODC) is further introduced therein.

As used herein, the term “ornithine decarboxylase” refers to an enzyme which produces putrescine by mediating the decarboxylation of ornithine. Although the microorganism of the genus *Corynebacterium* lacks the putrescine biosynthetic enzyme, when ornithine decarboxylase

(ODC) is introduced from the outside, putrescine is exported outside the cell as putrescine is being synthesized. The ornithine decarboxylase that can be introduced from the outside can be used in the present disclosure as long as it has the activity above, irrespective of the origin from which the microorganism is derived, and specifically, one from *E. coli* may be introduced.

The microorganism of the genus *Corynebacterium* of the present disclosure may be a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which, activities of i) ornithine carbamoyltransferase (ArgF), ii) glutamate exporter, or iii) ornithine carbamoyltransferase and glutamate exporter is further weakened, compared to its endogenous activity. The glutamate exporter of the genus *Corynebacterium* may be NCgl1221.

The microorganism of the genus *Corynebacterium* of the present disclosure may be a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which, an activity of at least one selected from the group consisting of acetyl gamma glutamyl phosphate reductase (ArgC), acetylglutamate synthase or ornithine acetyltransferase (ArgJ), acetylglutamate kinase (ArgB), and acetyl ornithine aminotransferase (ArgD) is further enhanced compared to its endogenous activity.

Additionally, the microorganism of the genus *Corynebacterium* may be a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which, an activity of acetyltransferase, specifically the activity of NCgl1469, is further weakened in comparison with its endogenous activity.

Lastly, the microorganism of the genus *Corynebacterium* may be a modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, in which an activity of a putrescine exporter, specifically the activity of NCgl2522, is further enhanced compared to its endogenous activity

As used herein, “weakening of activity” not only includes the drawing of a lower effect than the original function due to the reduction or inactivation of the activity of a protein itself, but also includes the decrease in its activity by a decrease in the activity of an endogenous gene, activation of regulatory factor(s) for inhibiting gene expression, a decrease in gene copy number, modification of the expression regulatory sequence, and specifically, an inactivation or reduction in enzyme activity due to replacement or modification of a promoter and a mutation within a gene, *etc.*

Specifically, in the present disclosure, the weakening of activity may be performed by:

- 1) deleting a part or an entirety of a polynucleotide encoding the protein,
 - 2) modifying an expression regulatory sequence for reducing an expression of the polynucleotide,
 - 3) modifying a polynucleotide sequence on the chromosomes to weaken an activity of the protein, and
 - 4) a selected method from a combination thereof,
- but the method is not limited thereto.

Specifically, the method of deleting a part or an entirety of a polynucleotide encoding a protein may be performed by replacing a polynucleotide encoding the endogenous target protein on the chromosome with a polynucleotide having a partial deletion in the polynucleotide sequence or a marker gene using a vector for chromosomal insertion within bacteria. As used herein, the term “a part” may vary depending on the kinds of polynucleotides, but it may specifically refer to 1 to 300, more specifically 1 to 100, and even more specifically 1 to 50.

Additionally, the method of modifying the expression regulatory sequence may be performed by inducing a modification on the expression regulatory sequence through deletion, insertion, non-conservative or conservative substitution of a polynucleotide sequence, or a combination thereof to further weaken the activity of the expression regulatory sequence, or by replacing the polynucleotide sequence with a polynucleotide sequence having a weaker activity. The expression regulatory sequence includes a promoter, an operator sequence, a sequence encoding a ribosome-binding site, and a sequence regulating the termination of transcription and translation.

Additionally, the method of modifying a polynucleotide sequence on the chromosome may be performed by inducing a modification on the sequence through deletion, insertion, non-conservative or conservative substitution of the polynucleotide sequence, or a combination thereof to further weaken the activity of the enzyme, or by replacing the polynucleotide sequence with an improved polynucleotide sequence having a stronger activity.

Additionally, the method of deleting the regulatory factor which inhibits the expression of the polynucleotide of the enzyme may be performed by replacing the polynucleotide for the expression inhibiting factor with a polynucleotide having a partial deletion in the polynucleotide sequence or a marker gene. As used herein, the term “a part” may vary depending on the kinds of polynucleotides, but it may specifically refer to 1 to 300, more specifically 1 to 100, and even

more specifically 1 to 50.

In particular, acetyl gamma glutamyl phosphate reductase (ArgC), acetylglutamate synthase or ornithine acetyltransferase (ArgJ), acetylglutamate kinase (ArgB), acetylornithine aminotransferase (ArgD), ornithine carbamoyltransferase (ArgF), proteins involved in the export of glutamate and ornithine decarboxylase (ODC) may respectively include the amino acid sequence of SEQ ID NO: 32, 33, 34, 35, 36, 37, or 38, or any amino acid sequence, which specifically has a homology of 70% or higher, more specifically 80% or higher, and even more specifically 90% or higher, to the above amino acid sequences, although not particularly limited thereto. Additionally, the protein that acetylates putrescine may include an amino acid sequence of SEQ ID NO: 30 or 31, or any amino acid sequence, which specifically has a homology of 70% or higher, more specifically 80% or higher, and even more specifically 90% or higher, to the above amino acid sequences, although the amino acid sequence is not particularly limited thereto.

Additionally, in the present disclosure, the putrescine exporter may include an amino acid sequence of SEQ ID NO: 26 or 28, or any amino acid sequence, which specifically has a homology of 70% or higher, more specifically 80% or higher, and even more specifically 90% or higher, to the above amino acid sequences.

Among the proteins described above, the enhancement of activities of acetyl gamma glutamyl phosphate reductase (ArgC), acetylglutamate synthase or ornithineacetyltransferase (ArgJ), acetylglutamate kinase (ArgB), acetylornithine aminotransferase (ArgD), ornithine decarboxylase (ODC) and putrescine exporter may be achieved, for example, by a method selected from an increase in copy number of the polynucleotides encoding the proteins, modification of the expression regulatory sequence for increasing the expression of the polynucleotides, modification of the polynucleotide sequences on the chromosome for enhancing the activities of the above enzymes, deletion of regulatory factor(s) for inhibiting the expression of the polynucleotides of the above enzymes, or a combination thereof.

Additionally, the weakening of ornithine carbamoyltransferase (ArgF), proteins involved in the export of glutamate, and the proteins that acetylate putrescine may be achieved by a method selected from deletion of a part or the entirety of the polynucleotides encoding the

proteins, modification of the expression regulatory sequence to reduce the expression of the polynucleotides, modification of the polynucleotide sequences on the chromosome to weaken the activities of the proteins, and a combination thereof.

Another aspect of the present disclosure provides a method for producing putrescine or ornithine, including:

- (i) culturing the microorganism of the genus *Corynebacterium* producing putrescine or ornithine in a medium; and
- (ii) recovering putrescine or ornithine from the cultured microorganism or the medium.

In the above method, the microorganism may be cultured in batch culture, continuous culture, fed-batch culture, *etc.*, known in the art, although not particularly limited thereto. In particular, regarding the culturing condition, proper pH (*i.e.*, an optimal pH of 5 to 9, specifically pH 6 to 8, and most specifically pH 6.8) can be maintained using a basic compound (*e.g.*, sodium hydroxide, potassium hydroxide, or ammonia) or an acidic compound (*e.g.*, phosphoric acid or sulfuric acid), although not particularly limited thereto. Additionally, an aerobic condition can be maintained by adding oxygen or an oxygen-containing gas mixture to a cell culture. The culture temperature may be maintained at 20°C to 45°C, and specifically at 25°C to 40°C, and the microorganism may be cultured for about 10 hours to 160 hours. The putrescine or ornithine produced by the culturing above may be secreted to a culture medium or remain in the cells.

Additionally, in the culture medium, carbon sources, such as sugars and carbohydrates (*e.g.*, glucose, sucrose, lactose, fructose, maltose, molasses, starch, and cellulose), oils and fats (*e.g.*, soybean oil, sunflower seed oil, peanut oil, and coconut oil), fatty acids (*e.g.*, palmitic acid, stearic acid, and linoleic acid), alcohols (*e.g.*, glycerol and ethanol), and organic acids (*e.g.*, acetic acid), may be used individually or in combination, but are not limited thereto; nitrogen sources, such as nitrogen-containing organic compounds (*e.g.*, peptone, yeast extract, meat juice, malt extract, corn steep liquor, soybean flour, and urea), or inorganic compounds (*e.g.*, ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate, and ammonium nitrate), may be used individually or in combination, but are not limited thereto; and potassium sources, such as potassium dihydrogen phosphate, dipotassium hydrogen phosphate, or sodium-containing salts corresponding thereto, may be used individually or in combination,

but are not limited thereto. Additionally, other essential growth-stimulating substances including metal salts (*e.g.*, magnesium sulfate or iron sulfate), amino acids, and vitamins may be further contained in the medium, but are not limited thereto.

The method of recovering the putrescine or ornithine produced during the culturing of the present disclosure may be performed by an appropriate culture method known in the art, for example, such as batch culture, continuous culture, or fed-batch culture, and thereby the target amino acid can be recovered from the culture.

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: Introduction of *E. coli*-derived *argA* and *E. coli*-derived *argE* into a strain producing putrescine and confirmation of putrescine-producing ability of the strain

1-1. Preparation of a strain simultaneously introduced with *E. coli*-derived *argA* and *E. coli*-derived *argE* into a transposon gene of ATCC13032-based strain producing putrescine

In order to confirm whether the introduction of the *E. coli*-derived *argA* gene and the *E. coli*-derived *argE* gene into an ATCC13032-based strain producing putrescine can improve putrescine-producing ability, *argA* and *argE* genes were introduced into the transposon gene of the strain.

As the vector for transformation enabling the introduction of the transposon gene region of a microorganism of the genus *Corynebacterium* within the chromosome, *pDZTn* (WO 2009/125992) was used, and *lysCPI* promoter (International Patent Publication No. WO 2009/096689, SEQ ID NO: 39) was used as the promoter.

Specifically, a primer pair of SEQ ID NOS: 11 and 12 for obtaining the homologous recombinant fragments in the *argA* ORF region was prepared based on the polynucleotide sequence (SEQ ID NO: 2) of the *E. coli*-derived *argA* gene, which encodes N-acetylglutamate synthase. Additionally, a primer pair of SEQ ID NOS: 15 and 16 for obtaining the homologous recombinant fragments in the *argE* ORF region was prepared based on the polynucleotide

sequence (SEQ ID NO: 4) of the *E. coli*-derived *argE* gene, which encodes the acetylornithine deacetylase, and a primer pair of SEQ ID NOS: 13 and 14 for obtaining the homologous recombinant fragments in the *lysCPI* region was prepared based on the polynucleotide sequence (SEQ ID NO: 39) of the *lysCPI* (Table 1).

[Table 1]

Primer	Sequence (5' → 3')
PlysC-argA-F (SEQ ID NO:11)	GAAAGGTGCACAAAGATGGTAAAGGAACGTAAAACCG
Tn-argA-RXh (SEQ ID NO:12)	GCCCACTAGTCTCGAGCATGCGGCGTTGATTTTG
Tn-PlysC-FXh (SEQ ID NO:13)	GAATGAGTTCCTCGAGCCGATGCTAGGGCGAAAA
PlysC-R (SEQ ID NO:14)	CTTTGTGCACCTTTCGATCTACGTGCTGACAGTTAC
PlysC-argE-F (SEQ ID NO:15)	GAAAGGTGCACAAAGATGAAAAACAAATTACCGCC
Tn-argE-RXh (SEQ ID NO:16)	GCCCACTAGTCTCGAGGTTTGAGTCACTGTCGGTCG

First, a gene fragment with a size of about 1.6 kb was amplified using the chromosome of *E. coli* *W3110* strain as the template along with a primer pair of SEQ ID NOS: 11 and 12, in order to obtain the *argA* gene. In particular, PCR was performed by repeating 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute and 30 seconds. The thus-obtained fragments were subjected to electrophoresis in a 0.8% agarose gel, and the bands of desired sizes were eluted and purified.

Additionally, the *lysCPI* promoter region was by performing PCR using the chromosome of the *KCCM10919P* (International Patent Publication No. WO 2009/096689) strain as the template along with a primer pair of SEQ ID NOS: 13 and 14, which was performed by repeating 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds.

The *pDZTn* vector was treated with *XhoI* and then each of the PCR products obtained

thereof was subjected to fusion cloning. The fusion cloning was performed using the In-Fusion® HD Cloning Kit (Clontech) and the thus-obtained plasmid was named as *pDZTn-lysCPI-argA*.

Then, for obtaining the *argE* gene, PCR products were obtained by amplifying the gene fragment with a size of about 1.4 kb in the same manner as described above, using the chromosome of the *E. coli* W3110 strain as the template along with a primer pair of SEQ ID NOS: 15 and 16, and was subjected to fusion cloning with the *lysCPI* promoter region. The thus-obtained plasmid was named as *pDZTn-lysCPI-argE*.

Then, the plasmid *pDZTn-lysCPI-argA* was introduced into the *KCCM11240P* (Korean Patent Application Publication No. 10-2013-0082478) strain by electroporation to obtain transformants, and the transformants were plated on BHIS plate media (Braine heart infusion (37 g/L), sorbitol (91 g/L), and agar (2%)) containing kanamycin (25 µg/mL) and X-gal (5-bromo-4-chloro-3-indolin-*D*-galactoside) and cultured to form colonies. Among the colonies, blue colonies were selected and thereby the transformed strains introduced with the plasmid *pDZTn-lysCPI-argA* were selected.

The selected strains were cultured with shaking (30°C, 8 hours) in CM media (glucose (10 g/L), polypeptone (10 g/L), yeast extract (5 g/L), beef extract (5 g/L), NaCl (2.5 g/L), urea (2 g/L), pH 6.8) and sequentially diluted from 10⁻⁴ to 10⁻¹⁰, plated on solid media containing X-gal, and cultured to form colonies. Among the thus-formed colonies, white colonies which appeared at a relatively low rate were selected and the strain introduced with the *argA*-encoding gene by a secondary crossover was finally selected. The finally selected strain was subjected to PCR using a primer pair of SEQ ID NOS: 12 and 13 and it was confirmed that the *argA*-encoding gene was introduced, and the modified strain of *Corynebacterium glutamicum* was named as *KCCM11240P Tn:lysCPI-argA*.

For the introduction of the strain introduced with *argA* prepared above, the *pDZTn-lysCPI-argE* prepared above was transformed into the *KCCM11240P Tn:lysCPI-argA* in the same manner as described above, and the introduction of *argE* into the transposon was confirmed in the finally selected strain by performing PCR using a primer pair of SEQ ID NOS: 13 and 16. The thus-selected modified strain of *Corynebacterium glutamicum* was named as

KCCM11240P Tn:lysCP1-argA Tn:lysCP1-argE.

1-2. Preparation of a strain simultaneously introduced with *E. coli*-derived *argA* and *E. coli*-derived *argE* into a transposon gene of *ATCC13869*-based strain producing putrescine

The *DAB12-a ΔNCgl1469* (Korean Patent Application Publication No. 10-2013-0082478), which is a *Corynebacterium glutamicum ATCC13869*-based strain producing putrescine, was named as *DAB12-b*, and *argA* and *argE* were introduced into the transposon gene in order to confirm whether the introduction of the *E. coli*-derived *argA* and *E. coli*-derived *argE* genes can be associated with the improvement of the putrescine-producing ability of the resulting strain.

First, the *pDZTn-lysCP1-argA*, which was previously prepared, was transformed into the *Corynebacterium glutamicum DAB12-b* in the same manner as in Example 1-1, and the introduction of *argA* into the transposon was confirmed. The thus-selected modified strain of *Corynebacterium glutamicum* was named as *DAB12-b Tn:lysCP1-argA*.

Then, for the introduction of *argE* into the strain, which is already introduced with *argA*, the *pDZTn-lysCP1-argE*, which was previously prepared, was transformed into the *DAB12-b Tn:lysCP1-argA* in the same manner as in Example 1-1, and the introduction of *argE* into the transposon was confirmed. The thus-selected modified strain of *Corynebacterium glutamicum* was named as *DAB12-b Tn:lysCP1-argE*.

1-3. Evaluation of putrescine-producing ability of a *Corynebacterium* strain producing putrescine introduced with *E. coli*-derived *argA* gene and *E. coli*-derived *argE* gene

The putrescine-producing ability was compared among the modified strains of *Corynebacterium glutamicum* prepared in Examples 1-1 and 1-2, in order to confirm the effect of the introduction of the *E. coli*-derived *argA* and the *E. coli*-derived *argE* into a strain producing putrescine on putrescin production.

Specifically, two different kinds of modified strains of *Corynebacterium glutamicum*, i.e., (*KCCM11240P Tn:lysCP1-argA Tn:lysCP1-argE*; *DAB12-b Tn:lysCP1-argA*

Tn:lysCPI-argE) prepared in Examples 1-1 and 1-2, and two different kinds of parent strains (*i.e.*, *KCCM11240P* and *DAB12-b*) were respectively plated on 1 mM arginine-containing CM plate media (1% glucose, 1% polypeptone, 0.5% yeast extract, 0.5% beef extract, 0.25% NaCl, 0.2% urea, 100 μ L of 50% NaOH, 2% agar, pH 6.8, based on 1 L), and cultured at 30°C for 24 hours.

Each of the strains cultured therefrom in an amount of about one platinum loop was inoculated into 25 mL of titer media (8% glucose, 0.25% soybean protein, 0.50% corn steep solids, 4% (NH₄)₂SO₄, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.15% urea, biotin (100 μ g), thiamine HCl (3 mg), calcium-pantothenic acid (3 mg), nicotinamide (3 mg), 5% CaCO₃, based on 1 L), and cultured with shaking at 30°C at a rate of 200 rpm for 98 hours. In all cultures of the strains, 1 mM arginine was added to the media. Upon completion of culture, the concentration of putrescine produced in each culture broth was measured and the results are shown in Table 2 below.

[Table 2]

Strains	Putrescine (g/L)
<i>KCCM 11240P</i>	12.2
<i>KCCM11240P Tn:lysCPI-argA Tn:lysCPI-argE</i>	13.4
<i>DAB12-b</i>	13.3
<i>DAB12-b Tn:lysCPI-argA Tn:lysCPI-argE</i>	14.6

As shown in Table 2 above, both of the two modified strains of *Corynebacterium glutamicum* simultaneously introduced with *E. coli*-derived *argA* and *E. coli*-derived *argE* genes showed an increase of putrescine production by 9.8% or higher.

Example 2: Enhancement of *pta-ackA* in the strain producing putrescine introduced with *E. coli*-derived *argA* and *E. coli*-derived *argE* and confirmation of putrescine-producing ability of the strain

2-1. Preparation of a strain having a substitution of the *pta-ackA* promoter from an ATCC13032-based *Corynebacterium* strain producing putrescine

The strain producing putrescine introduced with *E. coli*-derived *argA* and *E. coli*-derived *argE* genes, prepared in Example 1, was further enhanced in its activity of phosphotransacetylase and acetate kinase (*pta-ackA*) and the effect of the enhancement on the putrescine-producing ability of the strain was examined.

For this purpose, the promoter of the *pta-ackA* operon within the chromosome was substituted with a promoter having a stronger activity in comparison with its endogenous promoter, specifically, the *lysCPI* promoter (International Patent Publication No. WO 2009/096689) was introduced to the upstream of the initiation codon of the *pta-ackA* operon.

First, a homologous recombinant fragment, which includes the *lysCPI* promoter and both ends of the promoter have the original *pta-ackA* sequence on the chromosome, was obtained. Specifically, the 5'-end region of the *lysCPI* promoter was obtained by performing PCR using the genomic DNA of the *Corynebacterium glutamicum ATCC13032* along with a primer pair of SEQ ID NOS: 17 and 18. In particular, PCR reaction was performed by repeating 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds.

Additionally, the *lysCPI* promoter region was obtained by performing PCR in the same condition using a primer pair of SEQ ID NOS: 14 and 19, and the 3'-end region of the *lysCPI* promoter was obtained by performing PCR using the genomic DNA of the *Corynebacterium glutamicum ATCC13032* as a template along with a primer pair of SEQ ID NOS: 20 and 21. The primers used in obtaining the *lysCPI* promoter are shown in Table 1 above and Table 3 below.

[Table 3]

Primer	Sequence (5' -> 3')
Pro-pta-FX (SEQ ID NO: 17)	CCGGGGATCCTCTAGAGGGGTCTAAAAAATGTGGAGT
pta-PlysC-R (SEQ ID NO: 18)	GCCGTGCTTTTCGCCCTAGCATCGGACATCGCCTTTCTAAT TT
PlysC-F (SEQ ID NO: 19)	CCGATGCTAGGGCGAAAAGCACGGC

PlysC-pta-ackA-F (SEQ ID NO: 20)	GAAAGGTGCACAAAGATGTCTGACACACCGACCTCAGCTC
Pro-pta-RX (SEQ ID NO: 21)	GCAGGTCGACTCTAGATTATCCGGCATTGGCTCT

Each of the PCR products obtained above was subjected to fusion cloning using the *pDZ* vector treated with *Xba*I. The fusion cloning was performed using the In-Fusion® HD Cloning Kit (Clontech) and the thus-obtained plasmid was named as *pDZ-lysCPI-1'pta-ackA*.

The plasmid *pDZ-lysCPI-1'pta-ackA* prepared from the above was respectively introduced into the *KCCM11240P* and *KCCM11240P Tn:lysCPI-argA Tn:lysCPI-argE* strains, which is a modified strain of *Corynebacterium glutamicum* prepared in Example 1-1, by electroporation to obtain transformants, and the transformants were plated on BHIS plate media (Braine heart infusion (37 g/L), sorbitol (91 g/L), and agar (2%)) containing kanamycin (25 µg/mL) and X-gal (5-bromo-4-chloro-3-indolin-*D*-galactoside) and cultured to form colonies. Among the colonies, blue colonies were selected and thereby the transformed strains introduced with the plasmid *pDZ-lysCPI-1'pta-ackA* were selected.

The selected strains were cultured with shaking (30°C, 8 hours) in CM media (glucose (10 g/L), polypeptone (10 g/L), yeast extract (5 g/L), beef extract (5 g/L), NaCl (2.5 g/L), urea (2 g/L), pH 6.8) and sequentially diluted from 10⁻⁴ to 10⁻¹⁰, plated on solid media containing X-gal, and cultured to form colonies. Among the thus-formed colonies, white colonies which appeared at a relatively low rate were selected and the strain, in which the *pta-ackA* promoter was substituted with the *lysCPI* promoter by a secondary crossover, was finally selected.

The finally selected strain was subjected to PCR using a primer pair of SEQ ID NOS: 19 and 21 and was confirmed that the *lysCPI* promoter was introduced to the upstream of the initiation codon of *pta-ackA* within the chromosome. In particular, the PCR reaction was performed by repeating 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute.

The thus-selected modified strains of *Corynebacterium glutamicum* were named as *KCCM11240P lysCPI-1'pta-ackA* and *KCCM11240P Tn:lysCPI-argA Tn:lysCPI-argE lysCPI-1'pta-ackA*, respectively.

2-2. Preparation of a strain having a substitution of the *pta-ackA* promoter from an *ATCC13869*-based *Corynebacterium* strain producing putrescine

In order to confirm the sequence of the gene encoding the *pta-ackA* derived from *Corynebacterium glutamicum ATCC13869* and the protein expressed therefrom by the method disclosed in Example 2-1, PCR was performed using the genomic DNA of *Corynebacterium glutamicum ATCC13869* as a template along with a primer pair of SEQ ID NOS: 17 and 22 (Tables 3 and 4). In particular, the PCR reaction was performed by repeating 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 3 minutes.

The thus-obtained PCR products were separated by electrophoresis and the sequences were analyzed. As a result, it was confirmed that the gene encoding the *pta-ackA* derived from *Corynebacterium glutamicum ATCC13869* includes a polynucleotide sequence described by SEQ ID NO: 8 and that the protein encoded by the gene includes an amino acid sequence described by SEQ ID NO: 7.

On the other hand, as a result of the comparison between the amino acid sequence of *pta-ackA* derived from *Corynebacterium glutamicum ATCC13032* (SEQ ID NO: 5) and the amino acid sequence of *pta-ackA* derived from *Corynebacterium glutamicum ATCC13869*, it was confirmed that they have a sequence homology of 99.4%.

[Table 4]

Primer	Sequence (5' → 3')
Pta-ackA-R (SEQ ID NO: 22)	TGCAGTTTCACCCCTTAA
13869_pta-PlysC-R (SEQ ID NO: 23)	GCCGTGCTTTTCGCCCTAGCATCGGACATCGCCTTT CTAGTTT

First, a homologous recombinant fragment, which includes the *lysCPI* promoter and both ends of the promoter have the original *pta-ackA* sequence on the chromosome, was obtained. Specifically, the 5'-end region of the *lysCPI* promoter was obtained by performing PCR using the genomic DNA of the *Corynebacterium glutamicum ATCC13869* along with a primer pair of

SEQ ID NOS: 17 and 23. In particular, PCR reaction was performed by repeating 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. Additionally, the *lysCPI* promoter region was obtained by performing PCR in the same condition using a primer pair of SEQ ID NOS: 14 and 19, and the 3'-end region of the *lysCPI* promoter was obtained by performing PCR using the genomic DNA of the *Corynebacterium glutamicum ATCC13869* as a template along with a primer pair of SEQ ID NOS: 20 and 21. The primers used in the promoter substitution are shown in Tables 1, 3 and 4.

Each of the PCR products obtained thereof was subjected to fusion cloning using the *pDZTn* vector treated with *XhoI*. The fusion cloning was performed using the In-Fusion® HD Cloning Kit (Clontech) and the thus-obtained plasmid was named as *pDZ-lysCPI-2'pta-ackA*.

The plasmid *pDZ-lysCPI-2'pta-ackA* prepared from the above was respectively transformed into *DAB12-b* and *DAB12-b Tn:lysCPI-argA Tn:lysCPI-argE*, which is a modified strain of *Corynebacterium glutamicum* prepared in Example 1-2, in the same manner as in Example 2-1. As a result, it was confirmed that the *lysCPI* promoter was introduced to the upstream of the initiation codon of *pta-ackA* within the chromosome. The modified strains of *Corynebacterium glutamicum* were named as *DAB12-b lysCPI-2'pta-ackA* and *DAB12-b Tn:lysCPI-argA Tn:lysCPI-argE lysCPI-2'pta-ackA*, respectively.

2-3. Evaluation of putrescine-producing ability of a strain with enhanced *pta-ackA*

In order to confirm the effect of the enhancement of *pta-ackA* in a strain producing putrescine introduced with *E. coli*-derived *argA* and *E. coli*-derived *argE*, the putrescine-producing ability was compared among the modified strains of *Corynebacterium glutamicum* prepared in Examples 2-1 and 2-2.

Specifically, four kinds of modified strains of *Corynebacterium glutamicum* (*KCCM11240P lysCPI-1'pta-ackA*; *KCCM11240P Tn:lysCPI-argA Tn:lysCPI-argE lysCPI-1'pta-ackA*; *DAB12-b lysCPI-2'pta-ackA*; and *DAB12-b Tn:lysCPI-argA Tn:lysCPI-argE lysCPI-2'pta-ackA*) and four kinds of parent strains (*KCCM11240P*; *KCCM11240P Tn:lysCPI-argA Tn:lysCPI-argE*; *DAB12-b*; and *DAB12-b Tn:lysCPI-argA Tn:lysCPI-argE*) were respectively plated on 1 mM arginine-containing CM plate media (1% glucose, 1% polypeptone, 0.5% yeast extract, 0.5% beef extract, 0.25% NaCl, 0.2% urea, 100 µL of 50% NaOH, 2% agar, pH 6.8, based on 1 L), and cultured at 30°C for 24 hours. Each of the

strains cultured therefrom in an amount of about one platinum loop was inoculated into 25 mL of titer media (8% glucose, 0.25% soybean protein, 0.50% corn steep solids, 4% (NH₄)₂SO₄, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.15% urea, biotin (100 µg), thiamine HCl (3 mg), calcium-pantothenic acid (3 mg), nicotinamide (3 mg), 5% CaCO₃, based on 1 L), and cultured with shaking at 30°C at a rate of 200 rpm for 98 hours. In all cultures of the strains, 1 mM arginine was added to the media. Upon completion of culture, the concentration of putrescine produced in each culture broth was measured and the results are shown in Table 5 below.

[Table 5]

Strains	Putrescine (g/L)
<i>KCCM 11240P</i>	12.2
<i>KCCM 11240P lysCP1-1'pta-ackA</i>	12.3
<i>KCCM11240P Tn:lysCP1-argA Tn:lysCP1-argE</i>	13.4
<i>KCCM11240P Tn:lysCP1-argA Tn:lysCP1-argE lysCP1-1'pta-ackA</i>	14.1
<i>DAB12-b</i>	13.3
<i>DAB12-b lysCP1-2'pta-ackA</i>	13.4
<i>DAB12-b Tn:lysCP1-argA Tn:lysCP1-argE</i>	14.6
<i>DAB12-b Tn:lysCP1-argA Tn:lysCP1-argE lysCP1-2'pta-ackA</i>	15.2

As shown in Table 5, when *pta-ackA* was enhanced in *KCCM 11240P* and *DAB12-b*, respectively, the amount of putrescine production was at the same level. However, when *pta-ackA* was enhanced in the two different kinds of modified strains of *Corynebacterium glutamicum* simultaneously introduced with *E. coli*-derived *argA* and *E. coli*-derived *argE* genes (*KCCM11240P Tn:lysCP1-argA Tn:lysCP1-argE*; *DAB12-b Tn:lysCP1-argA Tn:lysCP1-argE*), respectively, the amount of putrescine production was increased by 14.3% or higher, compared to the parent strain. Additionally, the amount of putrescine production was increased by 4% or higher, based on the modified strains.

As such, the present inventors named the microorganism of the genus *Corynebacterium* (*Corynebacterium glutamicum KCCM11240P Tn:lysCP1-argA Tn:lysCP1-argE :lysCP1-1'pta-ackA*), which has an improved ability to produce putrescine, prepared from the *Corynebacterium glutamicum KCCM 11240P* strain producing putrescine by

introducing the activities of *E. coli*-derived *argA* and *E. coli*-derived *argE* and enhancing the activity of *pta-ackA* to the *Corynebacterium glutamicum* KCCM 11240P strain, as CC01-1145, and deposited in the Korean Culture Center of Microorganisms (KCCM) on November 21, 2014, with the accession number KCCM11606P under the Budapest Treaty.

Example 3: Introduction of *E. coli*-derived *acs* into a strain producing putrescine introduced with *E. coli*-derived *argA* and *E. coli*-derived *argE* and confirmation of the putrescine-producing ability of the resulting strain

3-1. Preparation of a strain introduced with *E. coli*-derived *acs* into a transposon gene of an *ATCC13032*-based strain producing putrescine

The *acs* was introduced into the transposon gene using the *lysCPI* promoter in order to confirm whether the introduction of *E. coli*-derived acetyl-CoA synthetase (*acs*) gene into an *ATCC13032*-based strain producing putrescine, which is already introduced with *E. coli*-derived *argA* and *E. coli*-derived *argE*, can improve the putrescine-producing ability.

Specifically, a primer pair of SEQ ID NOS: 24 and 25 for obtaining the homologous recombinant fragment around the *acs* ORF region and a primer pair of SEQ ID NOS: 13 and 14 for obtaining the homologous recombinant fragment around the *lysCPI* promoter region were prepared as shown in Table 1 above and Table 6 below, based on the polynucleotide sequence described by SEQ ID NO: 10 of the gene encoding the *acs*.

[Table 6]

Primer	Sequence (5' →3')
PlyC-acs-F (SEQ ID NO: 24)	GAAAGGTGCACAAAGATGAGCCAAATTCACAAA
Tn-acs-RXh (SEQ ID NO: 25)	GCCCACTAGTCTCGAGAAGGCGTTTACGCCGCATCC

Specifically, for obtaining the *acs* gene, the gene fragment with a size of about 2 kb was amplified using the chromosome of the *E. coli* *W3110* strain as a template along with a primer pair of SEQ ID NOS: 24 and 25. In particular, PCR reaction was performed by repeating 30

cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute and 30 seconds. Then, the thus-obtained PCR products were subjected to electrophoresis in a 0.8% agarose gel and the bands of desired sizes were eluted and purified.

Additionally, the *lysCPI* promoter region was obtained by performing PCR using the chromosome of the *KCCM10919P* (International Patent Publication No. WO 2009/096689) strain as the template along with a primer pair of SEQ ID NOS: 13 and 14, which was performed by repeating 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds.

The *pDZ* vector was treated with *XhoI* and each of the thus-obtained PCR products was subjected to fusion cloning. The fusion cloning was performed using the In-Fusion® HD Cloning Kit (Clontech). The thus-obtained plasmid was named as *pDZTn-lysCPI-acs*.

Then, the plasmid *pDZTn-lysCPI-acs* was introduced into the *KCCM11240P* and *KCCM11240P Tn:lysCPI-argA Tn:lysCPI-argE*, which is a modified strain of *Corynebacterium glutamicum* prepared in Example 1-1, respectively, by electroporation to obtain transformants, and the transformants were plated on BHIS plate media (Braine heart infusion (37 g/L), sorbitol (91 g/L), and agar (2%)) containing kanamycin (25 µg/mL) and X-gal (5-bromo-4-chloro-3-indolin-*D*-galactoside) and cultured to form colonies. Among the colonies, blue colonies were selected and thereby the transformed strains introduced with the plasmid *pDZTn-lysCPI-acs* were selected.

The selected strains were cultured with shaking (30°C, 8 hours) in CM media (glucose (10 g/L), polypeptone (10 g/L), yeast extract (5 g/L), beef extract (5 g/L), NaCl (2.5 g/L), urea (2 g/L), pH 6.8) and sequentially diluted from 10⁻⁴ to 10⁻¹⁰, plated on solid media containing X-gal, and cultured to form colonies. Among the thus-formed colonies, white colonies which appeared at a relatively low rate were selected and the strains introduced with the *acs*-encoding gene by a secondary crossover were finally selected. The finally selected strains were subjected to PCR using a primer pair of SEQ ID NOS: 13 and 25 and confirmed that the *acs*-encoding gene was introduced, and the modified strains of *Corynebacterium glutamicum* were named as *KCCM11240P Tn:lysCPI-acs* and *KCCM11240P Tn:lysCPI-argA Tn:lysCPI-argE Tn:lysCPI-acs*, respectively.

3-2. Preparatioin of a strain introduced with *E. coli*-derived *acs* into a transposon gene of

ATCC13869-based strain producing putrescine

As in Example 3-1, the *pDZTn-lysCPI-acs* prepared from the above was transformed into *DAB12-b* and the *DAB12-b Tn:lysCPI-argA Tn:lysCPI-argE*, which is a modified strain of *Corynebacterium glutamicum* prepared in Example 1-2, respectively, in the same manner as in Example 3-1, and it was confirmed that the *acs* was introduced into the transposon gene.

The thus-selected modified strains of *Corynebacterium glutamicum* were named as *DAB12-b Tn:lysCPI-acs* and *DAB12-b Tn:lysCPI-argA Tn:lysCPI-argE Tn:lysCPI-acs*, respectively.

3-3. Evaluation of putrescine-producing ability of a strain introduced with *E. coli*-derived *acs*

In order to confirm the effect of the introduction of *acs* in a strain producing putrescine, which is already introduced with *E. coli*-derived *argA* and *E. coli*-derived *argE*, putrescine-producing ability was compared among the modified strains of *Corynebacterium glutamicum* prepared in Examples 3-1 and 3-2.

Specifically, four kinds of modified strains of *Corynebacterium glutamicum* (*KCCM11240P Tn:lysCPI-acs*; *KCCM11240P Tn:lysCPI-argA Tn:lysCPI-argE Tn:lysCPI-acs*; *DAB12-b Tn:lysCPI-acs*; and *DAB12-b Tn:lysCPI-argA Tn:lysCPI-argE Tn:lysCPI-acs*) and four kinds of parent strains (*KCCM11240P*; *KCCM11240P Tn:lysCPI-argA Tn:lysCPI-argE*; *DAB12-b*; and *DAB12-b Tn:lysCPI-argA Tn:lysCPI-argE*) were respectively plated on 1 mM arginine-containing CM plate media (1% glucose, 1% polypeptone, 0.5% yeast extract, 0.5% beef extract, 0.25% NaCl, 0.2% urea, 100 μ L of 50% NaOH, 2% agar, pH 6.8, based on 1 L), and cultured at 30°C for 24 hours. Each of the strains cultured therefrom in an amount of about one platinum loop was inoculated into 25 mL of titer media (8% glucose, 0.25% soybean protein, 0.50% corn steep solids, 4% (NH₄)₂SO₄, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.15% urea, biotin (100 μ g), thiamine HCl (3 mg), calcium-pantothenic acid (3 mg), nicotinamide (3 mg), 5% CaCO₃, based on 1 L), and cultured with shaking at 30°C at a rate of 200 rpm for 98 hours. In all cultures of the strains, 1 mM arginine was added to the media. Upon completion of culture, the concentration of putrescine produced in each culture broth was measured and the results are

shown in Table 7 below.

[Table 7]

Strains	Putrescine (g/L)
<i>KCCM 11240P</i>	12.2
<i>KCCM 11240P Tn:lysCPI-acs</i>	12.2
<i>KCCM11240P Tn:lysCPI-argA Tn:lysCPI-argE</i>	13.4
<i>KCCM11240P Tn:lysCPI-argA Tn:lysCPI-argE Tn:lysCPI-acs</i>	13.9
<i>DAB12-b</i>	13.3
<i>DAB12-b Tn:lysCPI-acs</i>	13.2
<i>DAB12-b Tn:lysCPI-argA Tn:lysCPI-argE</i>	14.6
<i>DAB12-b Tn:lysCPI-argA Tn:lysCPI-argE Tn:lysCPI-acs</i>	15.1

As shown in Table 7, when *acs* was introduced into *KCCM 11240P* and *DAB12-b*, respectively, the amount of putrescine production was at the same level. However, when *acs* was introduced in the two different kinds of modified strains of *Corynebacterium glutamicum* simultaneously introduced with *E. coli*-derived *argA* and *E. coli*-derived *argE* genes (*KCCM11240P Tn:lysCPI-argA Tn:lysCPI-argE*; *DAB12-b Tn:lysCPI-argA Tn:lysCPI-argE*), respectively, the amount of putrescine production was increased by 13.5% or higher, compared to the parent strain. Additionally, the amount of putrescine production was increased by 3.4% or higher, compared to the above modified strains.

Example 4: A strain having introduction of *E. coli*-derived *argA*, *E. coli*-derived *argE*, and substitution of *pta-ackA* promoter from a strain producing putrescine with improved putrescine export ability, and the putrescine-producing ability of the strain

4-1. Preparation of a strain having introduction of *E. coli*-derived *argA*, *-argE* and substitution of *pta-ackA* promoter from a strain having improved putrescine export ability

A strain was prepared to examine whether the introduction of *E. coli*-derived *argA* and *E. coli*-derived *argE* and the enhancement of the activity of the *Corynebacterium* *pta-ackA* can improve the putrescine-producing ability, based on the KCCM11401P (Korean Patent Application Publication No. 10-2014-0115244) strain with improved putrescine export ability.

Specifically, the *pDZTn-lysCPI-argA* prepared in Example 1-1 was transformed into the *KCCM11401P* in the same manner as in Example 1-1, and as a result, it was confirmed that *argA* was introduced into the transposon gene. The thus-selected modified strain of *Corynebacterium glutamicum* was named as *KCCM11401P Tn:lysCPI-argA*.

Additionally, for introducing *argE* into the strain, which is already introduced with *argA* as prepared in Example 1-1, the *pDZTn-lysCPI-argE* prepared in Example 1-1 was transformed into the *KCCM11401P Tn:lysCPI-argA* in the same manner as in Example 1-1 and it was confirmed that *argE* was introduced into the transposon gene. The thus-selected modified strain was named as *KCCM11401P Tn:lysCPI-argA Tn:lysCPI-argE*.

Then, the *pDZ-lysCPI-1'pta-ackA* prepared in Example 2-1 was transformed into the *KCCM11401P Tn:lysCPI-argA Tn:lysCPI-argE* in the same manner as in Example 2-1, and it was confirmed that the *lysCPI* promoter was introduced to the upstream of the initiation codon of *pta-ackA* within the chromosome. The above modified strain of *Corynebacterium glutamicum* was named as *KCCM11401P Tn:lysCPI-argA Tn:lysCPI-argE lysCPI-1'pta-ackA*.

4-2. Evaluation of a strain having introduction of *E. coli*-derived *argA*, *E. coli*-derived *argE* and substitution of *pta-ackA* promoter from a strain having improved putrescine export ability

In order to confirm the effect of the introduction of *E. coli*-derived *argA* and *E. coli*-derived *argE* and the enhancement of *pta-ackA* activity on a strain of *Corynebacterium glutamicum* producing putrescine with improved putrescine export ability, the putrescine-producing ability was compared among the modified strains of *Corynebacterium glutamicum* prepared in Example 4-1.

Specifically, the modified strains of *Corynebacterium glutamicum* (*KCCM11401P Tn:lysCPI-argA Tn:lysCPI-argE*, *KCCM11401P Tn:lysCPI-argA Tn:lysCPI-argE lysCPI-1'pta-ackA*) and the parent strain (*KCCM11401P*) were respectively plated on 1 mM arginine-containing CM plate media (1% glucose, 1% polypeptone, 0.5% yeast extract, 0.5% beef extract, 0.25% NaCl, 0.2% urea, 100 μ L of 50% NaOH, 2% agar, pH 6.8, based on 1 L), and cultured at 30°C for 24 hours. Each of the strains cultured therefrom in an amount of about one platinum loop was inoculated into 25 mL of titer media (8% glucose, 0.25% soybean protein, 0.50% corn steep solids, 4% (NH₄)₂SO₄, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.15% urea,

biotin (100 µg), thiamine HCl (3 mg), calcium-pantothenic acid (3 mg), nicotinamide (3 mg), 5% CaCO₃, based on 1 L), and cultured with shaking at 30°C at a rate of 200 rpm for 98 hours. In all cultures of the strains, 1 mM arginine was added to the media. Upon completion of culture, the concentration of putrescine produced in each culture broth was measured and the results are shown in Table 8 below.

[Table 8]

Strains	Putrescine (g/L)
<i>KCCM11401P</i>	11.8
<i>KCCM11401P Tn:lysCPI-argA Tn:lysCPI-argE</i>	13.2
<i>KCCM11401P Tn:lysCPI-argA Tn:lysCPI-argE lysCPI-1'pta-ackA</i>	13.7

As shown in Table 8, it was confirmed that when the *KCCM11401P* having enhanced putrescine export ability was introduced with *E. coli*-derived *argA* gene and *E. coli*-derived *argE* gene, the amount of putrescine production was increased by 11.9% compared to that of the parent strain, and when the strain was further enhanced with *pta-ackA*, the amount of putrescine production was increased by 16.1% compared to that of the parent strain.

Example 5: Introduction of *E. coli*-derived *argA* and *E. coli*-derived *argE* in a strain producing ornithine and confirmation of the ornithine-producing ability of the strain

5-1. Preparation of a strain simultaneously introduced with *E. coli*-derived *argA* and *E. coli*-derived *argE* into a transposon gene of *KCCM11137P*-based strain producing ornithine

In order to confirm whether the introduction of *E. coli*-derived *argA* gene and *E. coli*-derived *argE* gene into the *KCCM11137P* (Korean Patent Application Publication No. 10-1372635) strain, which is a *Corynebacterium glutamicum ATCC13032*-based strain producing ornithine, can improve ornithine-producing ability, *argA* gene and *argE* gene were introduced into a transposon gene of the strain using the vector prepared in Example 1-1.

First, the plasmid *pDZTn-lysCPI-argA* was introduced into the *KCCM11137P* strain by electroporation to obtain transformants, and the transformants were plated on BHIS plate media (Brain heart infusion (37 g/L), sorbitol (91 g/L), and agar (2%)) containing kanamycin (25

μg/mL) and X-gal (5-bromo-4-chloro-3-indolin-*D*-galactoside) and cultured to form colonies. Among the colonies, blue colonies were selected and thereby the strains introduced with the plasmid *pDZTn-lysCPI-argA* were selected.

The selected strains were cultured with shaking (30°C, 8 hours) in CM media (glucose (10 g/L), polypeptone (10 g/L), yeast extract (5 g/L), beef extract (5 g/L), NaCl (2.5 g/L), urea (2 g/L), pH 6.8) and sequentially diluted from 10⁻⁴ to 10⁻¹⁰, plated on solid media containing X-gal, and cultured to form colonies. Among the thus-formed colonies, white colonies which appeared at a relatively low rate were selected and the strain introduced with the *argA*-encoding gene by a secondary crossover was finally selected. The finally selected strain was subjected to PCR using a primer pair of SEQ ID NOS: 12 and 13 and confirmed that the *argA*-encoding gene was introduced, and the modified strain of *Corynebacterium glutamicum* was named as *KCCM11137P Tn:lysCPI-argA*.

For the introduction of *argE* into the strain, which is already introduced with *argA* as prepared above, the *pDZTn-lysCPI-argE* prepared in Example 1-1 was transformed into the *KCCM11137P Tn:lysCPI-argA* in the same manner as in Example 1-1, and thereby it was confirmed that the *argE* was introduced within the transposon gene.

The thus-selected modified strain of *Corynebacterium glutamicum* was named as *KCCM11137P Tn:lysCPI-argA Tn:lysCPI-argE*.

5-2. Evaluation of ornithine-producing ability of a *Corynebacterium* strain producing ornithine introduced with *E. coli*-derived *argA* and *E. coli*-derived *argE*

In order to confirm the effect of the introduction of *E. coli*-derived *argA* and *E. coli*-derived *argE* on ornithine production in a strain producing ornithine, the ornithine-producing ability was compared among the modified strains of *Corynebacterium glutamicum* prepared in Example 5-1.

Specifically, one kind of a modified strain of *Corynebacterium glutamicum* (*KCCM11137P Tn:lysCPI-argA Tn:lysCPI-argE*) and one kind of a parent strain (*KCCM11137P*) were respectively plated on 1 mM arginine-containing CM plate media (1% glucose, 1% polypeptone, 0.5% yeast extract, 0.5% beef extract, 0.25% NaCl, 0.2% urea, 100 μL of 50% NaOH, 2% agar, pH 6.8, based on 1 L), and cultured at 30°C for 24 hours. Each of the strains cultured therefrom in an amount of about one platinum loop was inoculated into 25 mL of

titer media (8% glucose, 0.25% soybean protein, 0.50% corn steep solids, 4% (NH₄)₂SO₄, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.15% urea, biotin (100 µg), thiamine HCl (3 mg), calcium-pantothenic acid (3 mg), nicotinamide (3 mg), 5% CaCO₃, based on 1 L), and cultured with shaking at 30°C at a rate of 200 rpm for 98 hours. In all cultures of the strains, 1 mM arginine was added to the media. Upon completion of culture, the concentration of putrescine produced in each culture broth was measured and the results are shown in Table 9 below.

[Table 9]

Strains	Ornithine (g/L)
<i>KCCM11137P</i>	7.8
<i>KCCM11137P Tn:lysCPI-argA Tn:lysCPI-argE</i>	8.9

As shown in Table 9, it was confirmed that when the modified strain of *Corynebacterium glutamicum* introduced with *E. coli*-derived *argA* gene and *E. coli*-derived *argE* gene showed an increase in the amount of ornithine production by 14.1% compared to that of the parent strain.

Example 6: Enhancement of *pta-ackA* in a strain introduced with *E. coli*-derived *argA* and *E. coli*-derived *argE* and confirmation of ornithine-producing ability of the strain

6-1. Preparation of a strain having a substitution of *pta-ackA* promoter from an ATCC13032-based strain producing ornithine

In order to confirm whether the enhancement of *pta-ackA* activity into the ATCC13032-based strain producing ornithine introduced with *E. coli*-derived *argA* and *E. coli*-derived *argE* can improve the ornithine-producing ability, the *lysCPI* promoter (WO 2009/096689) was introduced to the upstream of the initiation codon of *pta-ackA* operon within the chromosome.

First, the plasmid *pDZ-lysCPI-1'pta-ackA* prepared in Example 2-1 was introduced into *KCCM11137P* and *KCCM11137P Tn:lysCPI-argA Tn:lysCPI-argE* strains, respectively, by electroporation to obtain transformants and the transformants were plated on BHIS plate media (Braine heart infusion (37 g/L), sorbitol (91 g/L), and agar (2%)) containing kanamycin (25 µg/mL) and X-gal (5-bromo-4-chloro-3-indolin-*D*-galactoside) and cultured to form colonies.

Among the colonies, blue colonies were selected and thereby the transformed strains introduced with the plasmid *pDZ-lysCPI-1'pta-ackA* were selected.

The selected strains were cultured with shaking (30°C, 8 hours) in CM media (glucose (10 g/L), polypeptone (10 g/L), yeast extract (5 g/L), beef extract (5 g/L), NaCl (2.5 g/L), urea (2 g/L), pH 6.8) and sequentially diluted from 10^{-4} to 10^{-10} , plated on solid media containing X-gal, and cultured to form colonies. Among the thus-formed colonies, white colonies which appeared at a relatively low rate were selected and the strain, in which the *pta-ackA* promoter was substituted with the *lysCPI* promoter by a secondary crossover, was finally selected. The finally selected strain was subjected to PCR using a primer pair of SEQ ID NOS: 19 and 21 and confirmed that the *lysCPI* promoter was introduced to the upstream of the initiation codon of *pta-ackA* operon within the chromosome. In particular, PCR reaction was performed by repeating 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute.

The thus-selected modified strains of *Corynebacterium glutamicum* were named as *KCCM11137P lysCPI-1'pta-ackA* and *KCCM11137P Tn:lysCPI-argA Tn:lysCPI-argE lysCPI-1'pta-ackA*, respectively.

6-2. Evaluation of ornithine-producing ability of a strain with enhanced pta-ackA activity

In order to confirm the effect of the enhancement of *pta-ackA* activity on a strain producing ornithine introduced with *E. coli*-derived *argA* and *E. coli*-derived *argE*, the ornithine-producing ability was compared among the modified strains of *Corynebacterium glutamicum* prepared in Example 6-1.

Specifically, two different kinds of modified strains of *Corynebacterium glutamicum*, (*KCCM11137P lysCPI-1'pta-ackA*; *KCCM11137P Tn:lysCPI-argA Tn:lysCPI-argE lysCPI-1'pta-ackA*) and two different kinds of parent strains (*KCCM11137P*; *KCCM11137P Tn:lysCPI-argA Tn:lysCPI-argE*) were respectively plated on 1 mM arginine-containing CM plate media (1% glucose, 1% polypeptone, 0.5% yeast extract, 0.5% beef extract, 0.25% NaCl, 0.2% urea, 100 μ L of 50% NaOH, 2% agar, pH 6.8, based on 1 L), and cultured at 30°C for 24 hours. Each of the strains cultured therefrom in an amount of about one platinum loop was inoculated into 25 mL of titer media (8% glucose, 0.25% soybean protein, 0.50% corn steep

solids, 4% (NH₄)₂SO₄, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.15% urea, biotin (100 µg), thiamine HCl (3 mg), calcium-pantothenic acid (3 mg), nicotinamide (3 mg), 5% CaCO₃, based on 1 L), and cultured with shaking at 30°C at a rate of 200 rpm for 98 hours. In all cultures of the strains, 1 mM arginine was added to the media. Upon completion of culture, the concentration of ornithine produced in each culture broth was measured and the results are shown in Table 10 below.

[Table 10]

Strains	Ornithine (g/L)
<i>KCCM11137P</i>	7.8
<i>KCCM11137P lysCP1-1'pta-ackA</i>	7.7
<i>KCCM11137P Tn:lysCP1-argA Tn:lysCP1-argE</i>	8.9
<i>KCCM11137P Tn:lysCP1-argA Tn:lysCP1-argE lysCP1-1'pta-ackA</i>	9.4

As shown in Table 10, it was confirmed that when the *KCCM11137P* strain was enhanced with the *pta-ackA* activity, the amount of ornithine production was not increased, whereas when the *KCCM11137P Tn:lysCP1-argA Tn:lysCP1-argE* strain, which is the modified strain of *Corynebacterium glutamicum* simultaneously introduced with *E. coli*-derived *argA* gene and *E. coli*-derived *argE* gene, the amount of ornithine production was increased by 20.5% compared to that of the *KCCM11137P* strain, and also increased by 5.6% compared to the *KCCM11137P Tn:lysCP1-argA Tn:lysCP1-argE* strain.

Example 7: Introduction of *E. coli*-derived *acs* in a strain introduced with *E. coli*-derived *argA* and *E. coli*-derived *argE* and confirmation of ornithine-producing ability of the strain

7-1. Preparation of a strain introduced with *E. coli*-derived *acs* into a transposon gene from *KCCM11137*-based strain producing ornithine

The *acs* was introduced into the transposon gene using the *lysCP1* promoter in order to confirm whether the introduction of *E. coli*-derived *acs* into the *KCCM11137P* (Korean Patent No. 10-1372635) strain, which is a *Corynebacterium glutamicum* ATCC13032-based strain

producing ornithine, can improve the ornithine-producing ability.

First, the plasmid *pDZTn-lysCP1-acs* prepared in Example 3-1 was introduced into *KCCM11137P* and *KCCM11137P Tn:lysCP1-argA Tn:lysCP1-argE* strains, respectively, by electroporation to obtain transformants, and the transformants were plated on BHIS plate media (Brain heart infusion (37 g/L), sorbitol (91 g/L), and agar (2%)) containing kanamycin (25 µg/mL) and X-gal (5-bromo-4-chloro-3-indolin-*D*-galactoside) and cultured to form colonies. Among the colonies, blue colonies were selected and thereby the transformed strains introduced with the plasmid *pDZTn-lysCP1-acs* were selected.

The selected strains were cultured with shaking (30°C, 8 hours) in CM media (glucose (10 g/L), polypeptone (10 g/L), yeast extract (5 g/L), beef extract (5 g/L), NaCl (2.5 g/L), urea (2 g/L), pH 6.8) and sequentially diluted from 10⁻⁴ to 10⁻¹⁰, plated on solid media containing X-gal, and cultured to form colonies. Among the thus-formed colonies, white colonies which appeared at a relatively low rate were selected and the strain introduced with *acs*-encoding gene by a secondary crossover was finally selected.

The finally selected strains were subjected to PCR using a primer pair of SEQ ID NOS: 13 and 25 and confirmed that the *acs*-encoding gene was introduced. The thus-selected modified strains of *Corynebacterium glutamicum* were named as *KCCM11137P Tn:lysCP1-acs* and *KCCM11137P Tn:lysCP1-argA Tn:lysCP1-argE Tn:lysCP1-acs*, respectively.

7-2. Evaluation of ornithine-producing ability of a strain introduced with *E. coli*-derived *acs*

In order to confirm the effect of the introduction of *acs* on a strain producing ornithine introduced with *E. coli*-derived *argA* and *E. coli*-derived *argE*, the ornithine-producing ability was compared among the modified strains of *Corynebacterium glutamicum* prepared in Example 7-1.

Specifically, two different kinds of modified strains of *Corynebacterium glutamicum*, (*KCCM11137P Tn:lysCP1-acs*; *KCCM11137P Tn:lysCP1-argA Tn:lysCP1-argE Tn:lysCP1-acs*) and two different kinds of parent strains (*KCCM11137P*; *KCCM11137P Tn:lysCP1-argA Tn:lysCP1-argE*) were respectively plated on 1 mM arginine-containing CM plate media (1% glucose, 1% polypeptone, 0.5% yeast extract, 0.5% beef extract, 0.25% NaCl, 0.2% urea, 100 µL of 50% NaOH, 2% agar, pH 6.8, based on 1 L), and cultured at 30°C for 24 hours. Each of the

strains cultured therefrom in an amount of about one platinum loop was inoculated into 25 mL of titer media (8% glucose, 0.25% soybean protein, 0.50% corn steep solids, 4% (NH₄)₂SO₄, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.15% urea, biotin (100 µg), thiamine HCl (3 mg), calcium-pantothenic acid (3 mg), nicotinamide (3 mg), 5% CaCO₃, based on 1 L), and cultured with shaking at 30°C at a rate of 200 rpm for 98 hours. In all cultures of the strains, 1 mM arginine was added to the media. Upon completion of culture, the concentration of ornithine produced in each culture broth was measured and the results are shown in Table 11 below.

[Table 11]

Strains	Ornithine (g/L)
<i>KCCM11137P</i>	7.8
<i>KCCM11137P Tn:lysCPI-acs</i>	7.8
<i>KCCM11137P Tn:lysCPI-argA Tn:lysCPI-argE</i>	8.9
<i>KCCM11137P Tn:lysCPI-argA Tn:lysCPI-argE Tn:lysCPI-acs</i>	9.2

As shown in Table 11, it was confirmed that when the *KCCM11137P* strain was introduced with *acs*, the amount of ornithine production was not increased, whereas when the *KCCM11137P Tn:lysCPI-argA Tn:lysCPI-argE* strain, which is the modified strain of *Corynebacterium glutamicum* simultaneously introduced with *E. coli*-derived *argA* gene and *E. coli*-derived *argE* gene, the amount of ornithine production was increased by 17.9% compared to that of the *KCCM11137P* strain, and also increased by 3.4% compared to the *KCCM11137P Tn:lysCPI-argA Tn:lysCPI-argE* strain.

Summarizing the foregoing, it was confirmed that the introduction of *E. coli*-derived *argA* and *E. coli*-derived *argE* into a strain of *Corynebacterium* can increase the amount of putrescine- and ornithine production, and additionally, it was confirmed that the enhancement of the activity of *pta-ackA* gene within a strain of *Corynebacterium* or the introduction of *E. coli*-derived *acs* can further increase the amount of putrescine- and ornithine production.

From the foregoing, a skilled person in the art to which the present invention pertains will be able to understand that the present invention may be embodied in other specific forms without modifying the technical concepts or essential characteristics of the present invention. In this regard, the exemplary embodiments disclosed herein are only for illustrative purposes and should

not be construed as limiting the scope of the present invention. On the contrary, the present invention is intended to cover not only the exemplary embodiments but also various alternatives, modifications, equivalents and other embodiments that may be included within the spirit and scope of the present invention as defined by the appended claims.

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

INTERNATIONAL FORM

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

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

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : <i>Corynebacterium glutamicum</i> CC01-1145	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM11606P
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: ☐ a scientific description ☐ a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on November. 21. 2014. (date of the original deposit) ¹	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : Yurim B/D 45, Hongjenae-2ga-gil Seodaemun-gu SEOUL 120-861 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: November. 21. 2014.

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

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CLAIMS

1. A modified microorganism of the genus *Corynebacterium* producing putrescine or ornithine, wherein activities of N-acetylglutamate synthase from *E. coli* and acetylornithine deacetylase from *E. coli* are introduced into a microorganism of the genus *Corynebacterium* having ornithine acetyltransferase (ArgJ) activity.

2. The modified microorganism according to claim 1, wherein the N-acetylglutamate synthase from *E. coli* consists of an amino acid sequence of SEQ ID NO: 1.

3. The modified microorganism according to claim 1, wherein the acetylornithine deacetylase from *E. coli* consists of an amino acid sequence of SEQ ID NO: 3.

4. The modified microorganism according to claim 1, wherein the microorganism of the genus *Corynebacterium* is *Corynebacterium glutamicum*.

5. The modified microorganism according to claim 1, wherein an activity of phosphotransacetylase and acetate kinase operon (*pta-ackA* operon) is further enhanced compared to its endogenous activity

6. The modified microorganism according to claim 5, wherein the phosphotransacetylase and acetate kinase operon consists of an amino acid sequence of SEQ ID NO: 5 or SEQ ID NO: 7.

7. The modified microorganism according to claim 1, wherein an activity of acetyl-CoA synthetase (*acs*) from *E. coli* is further introduced.

8. The modified microorganism according to claim 7, wherein the acetyl-CoA synthetase consists of an amino acid sequence of SEQ ID NO: 9.

9. The modified microorganism according to claim 1, wherein an activity of ornithine decarboxylase (ODC) is further introduced.

10. The modified microorganism according to claim 1, wherein an activity of i) ornithine carbamoyltransferase (ArgF), ii) glutamate exporter, or iii) ornithine carbamoyltransferase and glutamate exporter is further weakened compared to its endogenous activity.

11. The modified microorganism according to claim 1, wherein an activity of at least one selected from the group consisting of acetyl gamma glutamyl phosphate reductase (ArgC), acetylglutamate synthase or ornithine acetyltransferase (ArgJ), acetylglutamate kinase (ArgB), and acetyl ornithine aminotransferase (ArgD) is further enhanced compared to its endogenous activity.

12. The modified microorganism according to claim 1, wherein an activity of acetyltransferase is further weakened compared to its endogenous activity.

13. The modified microorganism according to claim 12, wherein the acetyltransferase consists of an amino acid sequence of SEQ ID NO: 30 or SEQ ID NO: 31.

14. The modified microorganism according to claim 1, wherein an activity of putrescine exporter is further enhanced compared to its endogenous activity.

15. The modified microorganism according to claim 14, wherein the putrescine exporter consists of an amino acid sequence of SEQ ID NO: 26 or SEQ ID NO: 28.

16. A method for producing putrescine or ornithine, comprising:

- (i) culturing the modified microorganism of the genus *Corynebacterium* of any one of claims 1 to 15 in a medium; and
- (ii) recovering putrescine or ornithine from the cultured microorganism or the medium.

17. The method according to claim 16, wherein the microorganism of the genus *Corynebacterium* is *Corynebacterium glutamicum*.