

[DESCRIPTION]**[Invention Title]**

PUTRESCINE-PRODUCING MICROORGANISM AND METHOD FOR PRODUCING PUTRESCINE USING THE SAME

[Technical Field]

The present disclosure relates to a putrescine-producing microorganism and a method for producing putrescine using the microorganism.

[Background Art]

Putrescine is known as a raw material for preparing polyamide. Until now, putrescine has been prepared by chemical methods using petroleum compounds as raw materials, and technologies for producing putrescine by fermentation using genetic engineering technology and fermentation technology are currently being studied.

For example, a microorganism capable of producing putrescine is known, in which the metabolic pathway of a microorganism of the genus *Corynebacterium* was manipulated (KR Patent Application Publication No. 2014-0115244, International Publication No. WO 2014-148743).

Meanwhile, formate dehydrogenase is an enzyme that reduces NAD^+ (*i.e.*, the second substrate) by catalyzing the oxidation of formic acid, and as a result, it produces NADH and CO_2 . NADH is known as an important material in the overall metabolism of microorganisms. This is because an increase in NADH can lead to an increase in reducing power in microorganisms, which can be advantageous for the production of a target material.

A method for producing succinic acid and bioalcohol under anaerobic conditions by strengthening NADH using formate dehydrogenase is already known. Succinic acid can be produced by a reductive TCA (reverse TCA) pathway under anaerobic fermentation conditions. The amount of NADH in the reductive TCA pathway is directly related to the production of succinic acid, and two moles of NADH are consumed in the pathway from oxaloacetate to succinic acid. In fact, it has been reported that when succinic acid is produced from glucose under anaerobic conditions, the enhancement of FDH can result in a 20% higher yield of succinic

acid (*Appl Environ Microbiol.*, 2012, 78(9): 3325 to 3337). However, unlike succinic acid, NADH is not used as a direct substrate in the biosynthesis pathway of putrescine, and no association has been reported between formate dehydrogenase and putrescine production.

[Disclosure]

[Technical Problem]

The present inventors have made efforts to increase putrescine production in a putrescine-producing microorganism, and as a result, they have confirmed that the overexpression of formate dehydrogenase can increase the levels of NADH and ATP in a putrescine-producing microorganism, and thus, the putrescine production can be increased, thereby completing the present disclosure.

[Technical Solution]

An object of the present disclosure is to provide a putrescine-producing microorganism of the genus *Corynebacterium* in which the activity of formate dehydrogenase (Fdh) is increased compared to that before modification.

Another object of the present disclosure is to provide a method of producing putrescine using the microorganism.

In one aspect, the present disclosure refers to a putrescine-producing microorganism of the genus *Corynebacterium*, in which the microorganism of the genus *Corynebacterium* comprises a gene encoding a formate dehydrogenase (Fdh) having at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 10 and having the activity of a Fdh consisting of the amino acid sequence of SEQ ID NO: 10. In another aspect, the present disclosure refers to a method of producing putrescine, comprising: (a) culturing the microorganism as disclosed herein in a medium; and (b) recovering putrescine from the microorganism or the cultured medium obtained in step (a).

[Advantageous Effects of the Invention]

The microorganism of the genus *Corynebacterium* with increased putrescine productivity of the present disclosure is modified so that the activity of formate dehydrogenase (FDH) can be increased, which leads to an increase in the production of NADH and ATP. As a result, the microorganism can increase putrescine production and can be effectively used for

large-scale production of putrescine.

[Brief Description of Drawings]

FIG. 1 shows an SDS-PAGE gel image illustrating the results of overexpression of CbFdh using an *Escherichia coli* host, in which Lane 1 represents the result of expression of a protein in a cell lysate which was expressed in *E. coli* BL21 DE3 at 18°C for 24 hours; Lane 2

represents the result of a soluble protein expressed in *E. coli* BL21 DE3 at 18°C for 24 hours; Lane 3 represents the result of expression of a protein in a cell lysate which was expressed in *E. coli* BL21 DE3 at 30°C for 8 hours; Lane 4 represents the result of a soluble protein expressed in *E. coli* BL21 DE3 at 30°C for 8 hours; Lane 5 represents the result of expression of a protein in a cell lysate which was expressed in *E. coli* Rosetta DE3 at 18°C for 24 hours; Lane 6 represents the result of a soluble protein expressed in *E. coli* Rosetta DE3 at 18°C for 24 hours; Lane 7 represents the result of expression of a protein in a cell lysate which was expressed in *E. coli* Rosetta DE3 at 30°C for 8 hours; and Lane 8 represents the result of a soluble protein expressed in *E. coli* Rosetta DE3 at 30°C for 8 hours.

FIG. 2 shows a graph illustrating the amount of NADH produced over time. The buffer used was 100 mM phosphate buffer (pH 7.2) and the control group used was a reaction sample excluding the soluble protein. CbFdh is a reaction sample containing 10% of a soluble protein, which is formate dehydrogenase overexpressed at 30°C using *E. coli* BL21 DE3. In the case of CbFdh, it was confirmed that the amount of NADH (*i.e.*, a reactant of CbFdh) continued to increase over time.

FIG. 3 shows a graph illustrating the concentration of formic acid over time. The control group is a strain in which pSCEC_CJ7 vector was inserted into a *Corynebacterium glutamicum* microorganism. CbFdh is a microorganism in which the plasmid pSCEC_CJ7_CbFdh capable of expressing the formate dehydrogenase gene derived from *C. boidinii* is inserted. Formic acid at each concentration of 0 g/L, 2 g/L, and 10 g/L was added to the culture medium, and the changes in the formic acid concentration between the control group and CbFdh were observed.

[Best Mode]

To achieve the above objects, in an aspect, the present disclosure provides a putrescine-producing microorganism of the genus *Corynebacterium* in which the activity of formate dehydrogenase is increased.

As used herein, the term “formate dehydrogenase” (hereinafter, “Fdh”) collectively refers to an enzyme which catalyzes an oxidation reaction using formic acid as a substrate and thereby reduces NAD^+ and produces NADH and CO_2 .

Since the amino sequence of a given protein showing an activity may vary depending on the species or strain of the microorganism, the origin or sequences of the Fdh are not limited thereto.

Specifically, the Fdh may be derived from *Ceriporiopsis subvermispota*, *Methylobacterium extorquens*, *Methylosinus trichosporium*, *Cupriavidus oxalaticus*, *Candida methylica*, *Methylotrophic bacterium*, *Ancylobacter aquaticus*, *Komagataella pastoris*, *Mycobacterium vaccae*, *Arabidopsis thaliana*, etc. and may be derived from *Corynebacterium glutamicum* (*Microbiology* (2012), 158, 2428 to 2439), which was recently disclosed. Specifically, the Fdh may be derived from *Candida boidinii*, but the origin of the Fdh is not limited thereto.

Additionally, in the present disclosure, the Fdh may include without limitation any protein that has the amino acid sequence of SEQ ID NO: 10, or any protein which, being a protein substantially having the activity of the Fdh, has an amino acid sequence having a homology to the amino acid sequence of SEQ ID NO: 10 of at least 70%, specifically at least 80%, more specifically at least 90%, even more specifically at least 95%, and most specifically at least 99%.

It is apparent that any amino acid sequence which is the same as that of SEQ ID NO: 10 and has a biological activity substantially the same as or equivalent to the protein of SEQ ID NO: 10 can belong to the scope of the present disclosure, even if the amino acid sequence has a partial deletion, modification, substitution, or addition.

The polynucleotide encoding the Fdh of the present disclosure may include a polynucleotide which has a sequence homology to the amino acid sequence of SEQ ID NO: 10 of at least 70%, specifically at least 80%, more specifically at least 90%, even more specifically at least 95%, and most specifically at least 99%, as long as the polynucleotide has an activity similar to that of the Fdh. For example, the polynucleotide may include the nucleotide sequence of SEQ ID NO: 9.

Additionally, the polynucleotide encoding the Fdh of the present disclosure can be hybridized with the nucleotide sequence of SEQ ID NO: 9 or a probe derived from the nucleotide sequence of SEQ ID NO: 9 under stringent conditions, and it may be a modified form encoding the Fdh that functions normally.

As used herein, the term “homology” refers to a degree of identity to a given amino acid sequence or nucleotide sequence and may be expressed as a percentage. In the present specification, a homologous sequence of the given amino acid sequence or nucleotide sequence having the same or similar activity with the given amino acid sequence or nucleotide sequence may be indicated in terms of “% homology”. For example, the homology may be confirmed using standard software for calculating parameters such as score, identity, and similarity, specifically, BLAST 2.0, or by comparing sequences by southern hybridization experiments under defined strict conditions, and the defined stringent hybridization conditions are within the scope of the technology, and may be determined by a method known to one of ordinary skill 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). As used herein, the term “stringent conditions” refers to conditions that are designed to permit specific hybridization between polynucleotides. For example, these conditions are specifically described in a literature (*e.g.*, J. Sambrook *et al.*, *supra*).

As used herein, the term “increase of activity” means that the activity is increased compared to the endogenous activity possessed by a microorganism or its activity before modification. The increase of activity may include both introducing an exogenous Fdh and enhancing the activity of the endogenous Fdh. Specifically, the increase of activity may mean that the activity of the Fdh is increased and thereby putrescine-producing ability is increased.

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

- (1) increasing the copy number of a polynucleotide encoding the enzyme;
- (2) modifying the expression control sequence for increasing the expression of the polynucleotide;
- (3) modifying the polynucleotide sequence on the chromosome for enhancing the activity of the enzyme; and
- (4) modifying the polynucleotide sequence to enhance the enzyme activity by a combination of Methods (1) to (3), *etc.*, but the methods are not limited thereto.

The increase of the copy number of a polynucleotide of Method (1) may be performed in a form where the polynucleotide is operably linked to a vector or by inserting the polynucleotide into the chromosome of a host cell, but the method is not particularly limited thereto. Specifically, the increase of the copy number of a polynucleotide may be performed by introducing into a host cell a vector, to which the polynucleotide encoding the enzyme of the present disclosure is operably linked, that can replicate and function regardless of a host. Alternatively, the increase of the copy number of a polynucleotide may be performed by introducing into a host cell a vector, to which the polynucleotide is operably linked, that can insert the polynucleotide into the chromosome of the host cell, thereby increasing the copy number of the polynucleotide in the chromosome of the host cell.

Additionally, in an aspect, the increase of the copy number may be performed by introducing an exogenous polynucleotide or the polynucleotide in a codon-optimized modified form. The introduction of an exogenous polynucleotide sequence may be performed by introducing into a host cell an exogenous polynucleotide encoding an enzyme that exhibits the same or similar activity to the enzyme. The exogenous polynucleotide may be used without limitation regardless of its origin or sequence as long as it exhibits the same or similar activity to the above enzyme. Additionally, for the optimized transcription and translation of the exogenous polynucleotide in a host cell, its codon may be optimized and introduced into a host cell. The introduction may be performed by one of ordinary skill in the art by selecting a suitable transformation method known in the art, and the expression of the introduced polynucleotide in the host cell can produce the enzyme, thereby increasing its activity.

Then, the modification of the expression control sequence for increasing the expression of a polynucleotide of Method (2) may be performed by inducing a modification of the expression control sequence by deletion, insertion, non-conservative or conservative substitution, or a combination thereof to further enhance the activity of the expression control sequence; or by substituting with a nucleic acid sequence having much stronger activity, although the method is not particularly limited thereto. The expression control sequence may include a promoter, an operator sequence, a sequence encoding a ribosome-binding region, sequences controlling the termination of transcription and translation, *etc.*, but the expression control sequence is not particularly limited thereto.

Specifically, instead of the original promoter, a strong heterologous promoter may be linked upstream of a unit for the polynucleotide expression, and examples of the strong promoter may include CJ7 promoter, lysCP1 promoter, EF-Tu promoter, groEL promoter, aceA promoter, aceB promoter, *etc.* More specifically, a *Corynebacterium*-derived promoter (*e.g.*, lysCP1 promoter: WO 2009/096689) or CJ7 promoter (Korean Patent No. 10-0620092 and International Publication No. WO 2006/065095) may be operably linked to the unit for the polynucleotide expression so as to increase the expression rate of the polynucleotide encoding the enzyme, but the promoter is not limited thereto.

Furthermore, the modification of the polynucleotide sequence on the chromosome of Method (3) may be performed by inducing a mutation on the expression control sequence by deletion, insertion, non-conservative or conservative substitution of the polynucleotide sequence, or a combination thereof, or by replacing the sequence with a polynucleotide sequence modified to have a further enhanced activity, but the method is not particularly limited thereto.

Finally, Method (4), which relates to modification for enhancing the enzyme activity by a combination of Methods (1) to (3), may be performed by applying a combination of at least one method among the following methods: increasing the copy number of the polynucleotide encoding the enzyme, modifying the expression control sequence to increase the expression of the polynucleotide, modifying the polynucleotide sequence on the chromosome, and modifying an exogenous polynucleotide exhibiting the activity of the enzyme or a codon-optimized modified polynucleotide thereof.

As used herein, the term “vector” refers to a DNA construct including the nucleotide sequence of the polynucleotide encoding a target protein, in which the target protein is operably linked to a suitable control sequence so that it can be expressed in an appropriate host. The control sequence includes a promoter capable of initiating transcription, any operator sequence for the control of the transcription, a sequence encoding an appropriate mRNA ribosome-binding domain, and a sequence controlling the termination of transcription and translation. The vector, after being transformed into a suitable host cell, may be replicated or function irrespective of the host genome, or may be integrated into the host genome itself.

The vector used in the present disclosure may not be particularly limited as long as the vector is able to replicate 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, and specifically, pDZ, pACYC177, pACYC184, pCL, pECCG117, pUC19, pBR322, pMW118, pCC1BAC, *etc.* may be used.

In an embodiment, a polynucleotide encoding a target protein in the chromosome may be replaced with a modified polynucleotide through a vector for chromosomal insertion. The insertion of the polynucleotide into the chromosome may be performed by any method known in the art (*e.g.*, homologous recombination), but the method is not limited thereto.

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

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

As used herein, the term “putrescine-producing microorganism” or “microorganism having putrescine productivity” refers to a microorganism naturally having a putrescine-producing ability or a microorganism, in which a putrescine-producing ability is provided to its parent strain having no putrescine-producing ability.

The putrescine-producing microorganism may be a microorganism having increased productivity of ornithine (*i.e.*, a raw material for the biosynthesis of putrescine), in which the microorganism is modified to have higher activities of acetylglutamate synthase, converting glutamate to *N*-acetylglutamate, or ornithine acetyltransferase (ArgJ), converting acetyl ornithine to ornithine, acetylglutamate kinase (ArgB), converting acetyl glutamate to *N*-acetylglutamyl phosphate, acetyl *gamma* glutamyl phosphate reductase (ArgC), converting acetyl glutamyl phosphate to *N*-acetylglutamate semialdehyde, or acetylornithine aminotransferase (ArgD), converting acetylglutamate semialdehyde to *N*-acetylornithine, compared to their endogenous activities, in order to enhance the biosynthesis pathway from glutamate to ornithine glutamate, but is not particularly limited thereto.

Additionally, the microorganism may be a microorganism which is modified to inactivate endogenous activity of ornithine carbamoyltransferase (ArgF), involved in the synthesis of arginine from ornithine, a protein exhibiting the activity of a glutamate exporter, and/or acetyltransferase, which acetylates putrescine, and/or is modified to introduce the activity of ornithine decarboxylase (ODC).

In particular, the ornithine carbamoyltransferase (ArgF), a protein exhibiting the activity of a glutamate exporter, ornithine decarboxylase (ODC), ornithine acetyltransferase (ArgJ), acetylglutamate kinase (ArgB), acetyl *gamma* glutamyl phosphate reductase (ArgC), and acetylornithine aminotransferase (ArgD) may specifically include an amino acid sequence represented by each of SEQ ID NOS: 11, 12, 13, 14, 15, 16, and 17, or an amino acid sequence having a homology to the above sequences of at least 70%, specifically at least 80%, more specifically at least 90%, even more specifically at least 95%, and most specifically at least 99%, but the amino acid sequences are not particularly limited thereto.

Additionally, the acetyltransferase that acetylates putrescine may specifically include an amino acid sequence represented by SEQ ID NO: 18 or 19 or an amino acid sequence having a homology to the above sequences of at least 70%, specifically at least 80%, more specifically at

least 90%, even more specifically at least 95%, and most specifically at least 99%, but the amino acid sequences are not particularly limited thereto.

Additionally, the microorganism may be one in which the activity of the protein exhibiting putrescine export is increased compared to its endogenous activity, but the microorganism is not limited thereto. The protein exhibiting the activity of putrescine export may include an amino acid sequence represented by SEQ ID NO: 20 or 21, and an amino acid sequence having a homology to the above sequences of at least 70%, specifically at least 80%, more specifically at least 90%, even more specifically at least 95%, and most specifically at least 99%, but the amino acid sequences are not particularly limited thereto.

Meanwhile, the microorganism of the present disclosure may be a microorganism having putrescine productivity and it may include prokaryotic microorganisms expressing the Fdh protein (e.g., the microorganisms of the genus *Escherichia*, *Shigella*, *Citrobacter*, *Salmonella*, *Enterobacter*, *Yersinia*, *Klebsiella*, *Erwinia*, *Corynebacterium*, *Brevibacterium*, *Lactobacillus*, *Selenomanas*, *Vibrio*, *Pseudomonas*, *Streptomyces*, *Arcanobacterium*, *Alcaligenes*, etc.). Specifically, the microorganism of the present disclosure may be a microorganism of genus *Corynebacterium* or genus *Escherichia*, and more specifically *Corynebacterium glutamicum*, but the microorganism is not limited thereto.

In another aspect, the present disclosure provides the use of the *Corynebacterium* microorganism for producing putrescine. The *Corynebacterium* microorganism may be a microorganism in which the activity of formate dehydrogenase (Fdh) is increased compared to that before its modification, and the use may be to produce putrescine.

In still another aspect, the present disclosure provides a method of producing putrescine, which includes (a) culturing a putrescine-producing microorganism of the genus *Corynebacterium* in which the activity of formate dehydrogenase (Fdh) is increased in a medium; and (b) recovering putrescine from the microorganism or the cultured medium obtained in step (a).

The explanations of formate dehydrogenase and the microorganism with enhanced putrescine productivity are the same as described above.

In the above method, culturing a microorganism of the genus *Corynebacterium* may be

performed by a known batch culture, continuous culture, fed-batch culture, *etc.*, but the method is not particularly limited thereto. In particular, for the culture conditions, an appropriate pH (*e.g.*, a pH of 5 to 9, specifically a pH of 6 to 8, and most specifically a pH of 6.8) may be adjusted using a basic compound (*e.g.*, sodium hydroxide, potassium hydroxide, or ammonia) or an acidic compound (*e.g.*, phosphoric acid or sulfuric acid), but the pH adjustment is not particularly limited thereto. Additionally, oxygen or an oxygen-containing gas mixture may be introduced into the culture to maintain aerobic conditions. The temperature of the culture may be maintained at 20°C to 45°C, specifically, 25°C to 40°C, and may be cultured for 10 hours to 160 hours, but the cultivation conditions are not limited thereto. The produced putrescine may be secreted into the medium or may remain in the cells.

Additionally, as a carbon source for a culture medium used, 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), organic acids (*e.g.*, acetic acid), *etc.* may be used alone or in combination, but the carbon source is not limited thereto. As a nitrogen source, nitrogen-containing organic compounds (*e.g.*, peptone, yeast extract, meat juice, malt extract, corn steep liquor, soybean meal powder, and urea), inorganic compounds (*e.g.*, ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate, and ammonium nitrate), *etc.* may be used alone or in combination, but the nitrogen source is not limited thereto. As a phosphorous source, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium-containing salts corresponding thereto, *etc.* may be used alone or in combination, but the phosphorous source is not limited thereto. Additionally, essential growth-promoting materials such as metal salts (*e.g.*, magnesium sulfate and iron sulfate), amino acids, and vitamins may be contained in the culture medium.

With regard to recovery of the putrescine produced during the cultivation of the present disclosure, desired amino acids may be collected from the culture broth by a suitable method known in the art (*e.g.*, centrifugation, filtration, anion exchange chromatography, crystallization, HPLC, *etc.*), and the putrescine may be recovered from the cultured medium or microorganism using a suitable method known in the art.

[DETAILED DESCRIPTION OF THE 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 scope of the invention is not limited by these Examples.

Example 1: Expression of *CbFdh* in *E. coli* and evaluation of reactivity**1) Expression of *CbFdh* gene in *E. coli***

For the overexpression of *Candida boidinii* formate dehydrogenase (*CbFdh*) in *E. coli*, *Candida boidinii* KCTC17776 strain was cultured and its genomic DNA was obtained. The formate dehydrogenase gene (*CbFdh*) (SEQ ID NO: 9) was inserted into pET28a vector using primers of SEQ ID NOS: 1 and 2.

Specifically, PCR was performed under the following conditions: 30 cycles, each consisting of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. The PCR product was electrophoresed on 1.0% agarose gel and a 1.1 kb band was eluted and purified. A sample containing the purified PCR product and a pET28a vector solution was treated with restriction enzymes, *Nco*I and *Xho*I, at 37°C for 4 hours, electrophoresed on 1.5% agarose gel, and the nucleic acid fragments with a size of the *CbFdh* and the vector were each cut out, and the purified nucleic acid fragments were obtained using the Gel prep kit (GeneAll, Korea). The *CbFdh* fragment and the vector fragment, in an amount of 1 mg each, were ligated using T4 ligase and electroporated into *E. coli* DH5 α strain at 2,500 V. After the electroporation, the recovered strain was plated on LB plate medium containing spectinomycin (50 μ g/L), cultured overnight at 37°C for one day, and the resistant strains were selected therefrom. The recovered strain was plated on LB plate medium containing kanamycin (50 μ g/L), cultured overnight at 37°C for one day, and the resistant strains were selected. The selected strain was subjected to PCR under the same conditions as described above using T7 promoter and primers of SEQ ID NOS: 3 and 4 of the terminator sequence, electrophoresed on 1.0% agarose gel, and the insertion of *CbFdh* was confirmed by observing a 1.3 kb band.

The strain, in which the insertion of *CbFdh* was confirmed, was cultured in LB medium

(3 mL) at 37°C for 12 hours after adding ampicillin (50 mg/mL) thereto. The cultured strain was added into LB medium (50 mL) containing an antibiotic and cultured at 37°C. When the absorbance at 600 nm reached 0.8, 0.2 mM IPTG was added thereto and expression was induced under various temperature/time conditions. The cultured strain was washed and the cells were lysed using a sonicator. After the cell lysis, CbFdh (41 kDa, SEQ ID NO: 10) was confirmed to be overexpressed through the results of SDS-PAGE gel electrophoresis (FIG. 1).

2) Evaluation of activity of expressed *CbFdh* gene

For the evaluation of the activity of *CbFdh*, 100 mM phosphate buffer (pH 7.2) was used as a reaction buffer. The solution which was prepared by adding 10 mM NAD⁺ and 0.1% sodium formate to the buffer was used as the control group. Meanwhile, the lysate of the cells, in which the overexpression of CbFdh was confirmed in Example 1-1, was added to the control group to a concentration of 10% and the activity of *CbFdh* was evaluated. The changes in value of the reaction solution were confirmed at the wavelength of 339 nm using a 96-well plate reader. The light at the wavelength of 340 nm is known to be selectively absorbed by NADH.

As a result, it was confirmed that NADH was continuously produced for several minutes (FIG. 2). Through this Example, it was evaluated that CbFdh can be overexpressed in *E. coli* and that the expressed protein has its unique activity.

Example 2: Preparation of *Corynebacterium* microorganism expressing *CbFdh*

Then, an attempt was made to confirm whether the putrescine-producing ability can be increased by enhancing the function of *CbFdh* to a putrescine-producing microorganism of the genus *Corynebacterium*. To express *CbFdh* in the microorganism of the genus *Corynebacterium* and confirm its activity, CJ7 promoter (KCCM10617, KR Pat. No. 10-0620092) was introduced in a region upstream of the initiation codon of the *CbFdh* gene.

First, PCR was performed using the genomic DNA of *Corynebacterium glutamicum* ATCC13032 as a template along with a pair of primers of SEQ ID NOS: 5 and 6 so as to obtain the gene including the CJ7 promoter sequence. PCR was performed under the following conditions: 30 cycles, each consisting of denaturation at 95°C for 30 seconds, annealing at 55°C

for 30 seconds, and extension at 72°C for 30 seconds. The electrophoresis was performed on 1.5% agarose gel and the presence of a nucleic acid as the PCR product with a size of 400 base pairs (bp) was confirmed. A purified nucleic acid fragment of CJ7 promoter was secured from the obtained PCR product using the PCR prep kit (GeneAll, Korea). A sample containing the purified nucleic acid fragment of CJ7 promoter and a pSCEC vector solution was treated with restriction enzymes, *Bam*HI and *Xba*I, at 37°C for 4 hours, electrophoresed on 1.5% agarose gel, and the nucleic acid fragments with a size of 400 bp were cut out, and the fragment of CJ7 promoter and nucleic acid fragments of the pSCEC vector were obtained using the Gel prep kit (GeneAll, Korea). The fragment of CJ7 promoter and the pSCEC vector, in an amount of 1 mg each, were ligated using T4 ligase and electroporated into *E. coli* DH5 α strain at 2,500 V. After the electroporation, the recovered strain was plated on LB plate medium containing spectinomycin (50 μ g/L), cultured overnight at 37°C for one day, and 18 different types of resistant strains were selected therefrom. The selected 18 different types of strains were subjected to colony PCR using the primers of SEQ ID NOS: 5 and 6, and the presence of a PCR product with a 400 bp size was confirmed. From the results of the colony PCR, the preparation of pSCEC_CJ7 having CJ7 promoter was confirmed.

In the same conditions to obtain the PCR product of CbFdh as in Example 1, the PCR product of CbFdh that can be inserted into pSCEC_CJ7 using the primers of SEQ ID NOS: 7 and 8 was obtained. The pSCEC_CJ7, which was treated with restriction enzymes (*Xba*I and *Sal*I) and the PCR product of CbFdh were ligated and then inserted into *E. coli* DH5 α . The pSCEC_CJ7_CbFdh was obtained from the selected strains and electroporated into putrescine-producing microorganisms of the genus *Corynebacterium* (i.e., KCCM11240P (KR Pat. Application Publication No. 2013-0082478) and KCCM11401P (KR Pat. Application Publication No. 2014-0017243)) at 2,500 V.

The strains obtained by electroporation were cultured by plating on BHIS plate medium (brain heart infusion 37 g/L, sorbitol 91 g/L, and agar 2%) containing spectinomycin (50 μ g/L) and thereby colonies were formed. The selected strains were cultured in a shaking incubator in CM medium (glucose 10 g/L, polypeptone 10 g/L, yeast extract 5 g/L, beef extract 5 g/L, NaCl 2.5 g/L, and urea 2 g/L (pH 6.8)) containing spectinomycin (50 μ g/L) and thereby finally selected. The KCCM11240P strain in which pSCEC_CJ7_CbFdh is inserted was named as

KCCM11240P/pSCEC_CJ7_CbFdh (CC04-0081), and the KCCM11240P in which pSCEC_CJ7 is inserted was named as KCCM11240P/pSCEC_CJ7. Likewise, the KCCM11401P strain in which pSCEC_CJ7_CbFdh is inserted was named as KCCM11401P/pSCEC_CJ7_CbFdh, and the KCCM11401P strain in which pSCEC_CJ7 is inserted was named as KCCM11401P/pSCEC_CJ7.

Among these, the CC04-0081 strain was deposited at Korean Culture Center of Microorganisms (KCCM), which is an international depositary authority under the Budapest Treaty, on January 8, 2016 (Accession No. KCCM 11798P).

Example 3: Evaluation of CbFdh activity in *Corynebacterium* microorganism

To confirm the activity of formate decarboxylase in a microorganism of the genus *Corynebacterium* where *CbFdh* is inserted, the changes in formic acid concentration in a medium where formic acid was added were analyzed (FIG. 3). Formic acid at each concentration of 0 g/L, 2 g/L, and 10 g/L was added to a culture broth of a *Corynebacterium* strain where CbFdh activity was enhanced and a culture broth of a *Corynebacterium* strain where an empty vector was inserted. As the strain with enhanced CbFdh activity and the strain with an empty vector, KCCM11240P/pSCEC_CJ7_CbFdh and KCCM11240P/pSCEC_CJ7 were used, respectively.

As a result of the cultivation, it was confirmed that in the case of the strain with an empty vector, formic acid remained in the culture broth when formic acid was added to the culture broth at each concentration of 2 g/L and 10 g/L. In contrast, in the case of the strain with enhanced CbFdh activity, the formic acid added at a concentration of 2 g/L was all decomposed within 24 hours, and additionally, the formic acid added at a concentration of 10 g/L was not all decomposed within 32 hours, but the formic acid level continued to decrease. Comparing with the control group strain at the time point of 32 hours, it was confirmed that about 80% of formic acid was converted.

From the analysis of the changes in the amount of formic acid, it was confirmed that the strain with enhanced CbFdh activity decomposes formic acid. As a result, it was confirmed that the *CbFdh* introduced to a microorganism of the genus *Corynebacterium* was normally

expressed and its function was maintained.

Example 4: Evaluation of productivity of putrescine-producing *Corynebacterium* microorganism with enhanced CbFdh activity

Each of the four types of modified *Corynebacterium glutamicum* strains (*i.e.*, KCCM11240P/pSCEC_CJ7_CbFdh, KCCM11240P/pSCEC_CJ7, KCCM11401P/pSCEC_CJ7_CbFdh, and KCCM11401P/pSCEC_CJ7) prepared for the evaluation of productivity of putrescine-producing *Corynebacterium* microorganism with enhanced CbFdh activity was spread on CM plate medium (glucose (1%), polypeptone (1%), yeast extract (0.5%), beef extract (0.5%), NaCl (0.25%), urea (0.2%), 50% NaOH (100 μ L), spectinomycin (50 μ g), agar (2%), pH 6.8, based on 1 L) containing arginine (1 mM), and cultured at 30°C for 24 hours. A platinum loop of each strain cultured therefrom was inoculated into 25 mL of a titer medium (glucose (8%), soybean protein (0.25%), corn steep solids (0.50%), (NH₄)₂SO₄ (4%), KH₂PO₄ (0.1%), MgSO₄·7H₂O (0.05%), urea (0.15%), biotin (100 μ g), thiamine·HCl (3 mg), calcium-pantothenic acid (3 mg), nicotinamide (3 mg), CaCO₃ 5%, spectinomycin (50 μ g), based on 1 L), and then cultured in a shaking incubator 30°C at 200 rpm for 98 hours in the case of KCCM11240P/pSCEC_CJ7_CbFdh and KCCM11240P/pSCEC_CJ7 strains, and 104 hours in the case of KCCM11401P/pSCEC_CJ7_CbFdh and KCCM11401P/pSCEC_CJ7 strains.

The concentrations of putrescine produced from each culture product were measured and the results are shown in Table 1 below.

[Table 1]

Strain	Addition of Formic Acid (g/L)	Putrescine (g/L)
KCCM11240P/pSCEC_CJ7	0	12.2
KCCM11240P/pSCEC_CJ7	5	12.3
KCCM11240P/pSCEC_CJ7_CbFdh	0	13.4
KCCM11240P/pSCEC_CJ7_CbFdh	5	13.1

KCCM11401P/pSCEC_CJ7	0	11.4
KCCM11401P/pSCEC_CJ7	5	10.7
KCCM11401P/pSCEC_CJ7_CbFdh	0	12.0
KCCM11401P/pSCEC_CJ7_CbFdh	5	12.0

The putrescine concentration in the culture was analyzed by HPLC. As shown in Table 1 above, in the case of the KCCM11240P/pSCEC_CJ7 strain, there was no significant change in the amount of putrescine production according to the presence/absence of formic acid (5 g/L). In contrast, the strain in which KCCM11240P/pSCEC_CJ7_CbFdh was introduced showed an increase in the amount of putrescine production by more than 7% compared to the amount of production by the KCCM11240P/pSCEC_CJ7 strain, regardless of the presence/absence of formic acid (5 g/L). It was confirmed that the amount of putrescine production of the strain in which the Fdh activity was enhanced was increased regardless of the presence/absence of formic acid.

Additionally, in the case of the KCCM11401P/pSCEC_CJ7 strain, which was evaluated in the same medium without the addition of formic acid, the amount of putrescine produced was 11.4 g/L and the KCCM11401P/pSCEC_CJ7 strain cultured in a medium where formic acid (5 g/L) was added, the yield was decreased by about 6% (10.7 g/L). In contrast, in the case of the KCCM11401P/pSCEC_CJ7_CbFdh strain where the CbFdh activity was enhanced, the same amount of putrescine (12.0 g/L) was produced regardless of the presence/absence of formic acid.

As a result of the analysis of the putrescine produced from the KCCM11401P/pSCEC_CJ7_CbFdh strain and the KCCM11401P/pSCEC_CJ7 strain, it was confirmed that the strain where the CbFdh activity was enhanced showed an increase in putrescine production by at least 5% compared to the KCCM11401P/pSCEC_CJ7 strain. It was confirmed that the strain where the CbFdh activity was enhanced showed an increase in putrescine productivity regardless of the presence/absence of formic acid.

Summarizing the above results, it was confirmed that the strain where formate dehydrogenase (CbFdh) is introduced in a putrescine-producing microorganism showed a further

increase in the amount of putrescine production, and this is an effect that appears regardless of the addition of formic acid. Accordingly, it is expected that the present disclosure enables efficient production of putrescine on a large scale.

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



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

INTERNATIONAL FORM

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

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

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : <i>Corynebacterium glutamicum</i> CC04-0081	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM11798P
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 January, 8, 2016. (date of the original deposit) ¹	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : Yurim B/D 45, Hongjeonae-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: January, 8, 2016.

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

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한국미생물보존센터

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KOREAN CULTURE CENTER OF MICROORGANISMS

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[Claims]**[Claim 1]**

A putrescine-producing microorganism of the genus *Corynebacterium*, in which the microorganism of the genus *Corynebacterium* comprises a gene encoding a formate dehydrogenase (Fdh) having at least 90% sequence identity to the amino acid sequence of SEQ ID NO: 10 and having the activity of a Fdh consisting of the amino acid sequence of SEQ ID NO: 10.

[Claim 2]

The microorganism according to claim 1, wherein the Fdh is derived from *Candida boidinii*.

[Claim 3]

The microorganism according to claim 1, wherein the Fdh consists of the amino acid sequence of SEQ ID NO: 10.

[Claim 4]

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

[Claim 5]

The microorganism according to claim 1, wherein the activity of acetyltransferase which acetylates putrescine is further weakened compared to its endogenous activity.

[Claim 6]

The microorganism according to claim 1, wherein the activity of the protein that exhibits the activity of exporting putrescine is increased compared to its endogenous activity.

[Claim 7]

The microorganism according to claim 1, wherein the microorganism is *Corynebacterium glutamicum*.

[Claim 8]

A method of producing putrescine, comprising:

- (a) culturing the microorganism of any one of claims 1 to 7 in a medium; and
- (b) recovering putrescine from the microorganism or the cultured medium obtained in step (a).

[Claim 9]

The method according to claim 8, wherein the microorganism is cultured in a medium not containing formic acid.

[Claim 10]

The method according to claim 8, wherein the microorganism is cultured under an aerobic condition.