

[DESCRIPTION]

[Invention Title]

NOVEL POLYPEPTIDE AND METHOD OF PRODUCING IMP USING THE SAME

[Technical Field]

The present disclosure relates to a novel protein variant having an activity of exporting 5'-inosine monophosphate, a microorganism containing the protein variant, a method for preparing 5'-inosine monophosphate using the microorganism, and a method for increasing export of 5'-inosine monophosphate using the microorganism.

[Background Art]

5'-Inosine monophosphate (hereinafter, IMP), a nucleic acid material, is an intermediate of the nucleic acid metabolic pathway and is used in many fields such as foods, medicines, various medical applications, *etc.* In particular, IMP is widely used as an additive for food seasonings or foods, along with 5'-guanine monophosphate (hereinafter, GMP). Although IMP itself is known to provide a beef taste, it is known to enhance the flavor of monosodium glutamic acid (MSG) and is thus attracting attention as a taste-enhancing nucleic acid-based seasoning.

Examples of methods for producing IMP include a method of enzymatically degrading ribonucleic acid extracted from yeast cells (Japanese Patent Publication No. 1614/1957), a method for chemically phosphorylating inosine produced by fermentation (*Agri. Biol. Chem.*, 36, 1511, (1972), *etc.*), a method for culturing microorganisms which can directly produce IMP and recovering IMP in the culture broth, *etc.* Among these, the method most frequently used at present is a method using microorganisms capable of directly producing IMP.

Meanwhile, since enzymes do not always exhibit optimal properties in nature with respect to activity, stability, substrate specificity for optical isomers, *etc.* required in industrial applications, various attempts have been made to improve enzymes to suit the intended use by a mutation of their amino acid sequences, *etc.* Among these, although rational design and site-directed mutagenesis of enzymes have been applied to improve enzyme function, in many cases, these attempts were shown to be disadvantageous in that information on the structure of target enzymes is not sufficient or the structure-function correlation is not clear,

thus preventing their effective application. Additionally, a method of improving enzyme activity by attempting the enhancement of enzymes through directed evolution, which is for screening enzymes of desired traits from a library of modified enzymes constructed through random mutagenesis of enzyme genes, was previously reported.

[Disclosure]

[Technical Problem]

To produce IMP in high yield by direct IMP production through microbial fermentation, it is essential that the IMP export be smoothly performed. To achieve the object of the present disclosure, the inventors of the present disclosure have conducted extensive studies, and as a result, have identified the proteins involved in the activity of exporting IMP and have also discovered protein variants having higher activity of exporting IMP, thereby completing the present disclosure.

[Technical Solution]

An object of the present disclosure is to provide a protein variant having the activity of exporting IMP.

Another object of the present disclosure is to provide a polynucleotide encoding the protein variant of the present disclosure.

Still another object of the present disclosure is to provide a vector including the polynucleotide of the present disclosure.

Still another object of the present disclosure is to provide a microorganism producing IMP, in which the microorganism includes the protein variant of the present disclosure and the vector of the present disclosure.

Still another object of the present disclosure is to provide a method for preparing IMP, which includes culturing the microorganism of the genus *Corynebacterium* of the present disclosure in a medium, and recovering IMP from the microorganism or the medium.

Still another object of the present disclosure is to provide a method for increasing export of IMP, which includes a step of enhancing an activity of the protein exporting IMP in a microorganism of the genus *Corynebacterium*.

To achieve the above objects, an aspect of the present disclosure provides a protein variant having an activity of exporting IMP.

As used herein, the term “a protein that exports 5'-inosine monophosphate (IMP)” refers to a protein involved in the extracellular export of IMP. For the purpose of the present disclosure, the term may be used interchangeably with a protein having an activity of exporting IMP, an IMP export protein, a protein capable of exporting IMP, an IMP -exporting protein, *etc.*; specifically, the protein may be expressed as ImpE, more specifically, ImpE1 or ImpE2, and even more specifically, the protein that exports of the present disclosure may be expressed as ImpE2, but the expression of the protein is not limited thereto. Additionally, the protein may be derived from a microorganism of the genus *Corynebacterium*, and specifically from *Corynebacterium stationis*, but the microorganism is not limited thereto.

The protein may be a protein which includes the amino acid sequence represented by SEQ ID NO: 2 or one which consists of the amino acid sequence represented by SEQ ID NO: 2, but any sequence having the same activity as the protein can be included without limitation, and one of ordinary skill in the art can obtain sequence information from GenBank of NCBI, a well-known database. Additionally, the protein of the present disclosure that exports IMP may be a protein which includes an amino acid sequence of SEQ ID NO: 2, or an amino acid sequence which has a homology or identity to the sequence of SEQ ID NO: 2 of at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%. Additionally, it is apparent that any protein having an amino acid sequence with deletion, mutation, substitution, or addition in part of the sequence can also be included in the scope of the present disclosure, as long as the amino acid sequence has a homology or identity described above and has an effect corresponding to that of the protein.

That is, although described as “a protein having an amino acid sequence represented by a particular SEQ ID NO” or “a protein consisting of a particular SEQ ID NO” in the present disclosure, it is apparent that a protein having an amino acid sequence with deletion, modification, substitution, conservative substitution, or addition of some amino acids also falls within the scope of the present invention as long as the protein has an activity the same as or equivalent to that of the protein which consists of an amino acid sequence of the corresponding SEQ ID NO. For example, insofar as the protein has an activity the same as or equivalent to that of the protein variant of the present disclosure, the above expression does not exclude a sequence addition upstream or downstream of the amino acid sequence that does not alter the functions of the protein, a naturally occurring mutation therein, a silent mutation therein, or conservative substitution, and even in a case of such a sequence addition or mutation, it is apparent that the protein also belongs to the scope of the present disclosure.

In the present disclosure, “homology” and “identity” refer to a degree of relevance between two given amino acid sequences or nucleotide sequences, and may be expressed as a percentage.

The terms “homology” and “identity” are often used interchangeably with each other.

The sequence homology or identity of conserved polynucleotide or polypeptide sequences may be determined by standard alignment algorithms and may be used with default gap penalty established by the program being used. Substantially, homologous or identical polynucleotides or polypeptides can generally hybridize under moderate or high stringency, along the entire length or at least about 50%, about 60%, about 70%, about 80%, or about 90% or higher of the entire length. In the hybridization, polynucleotides that include degenerate codons instead of codons are also considered.

Whether any two polynucleotide or polypeptide sequences have a homology, similarity, or identity may be determined, for example, using a known computer algorithm such as the “FASTA” program (*e.g.*, Pearson *et al.*, (1988) *Proc. Natl. Acad. Sci. USA* 85: 2444) using default parameters. Alternatively, it may be determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453), which is performed in the Needleman program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277) (version 5.0.0 or versions thereafter) (GCG program package (Devereux, J., *et al.*, *Nucleic Acids Research* 12: 387 (1984)), BLASTP, BLASTN, FASTA (Atschul, [S.] [F.] *et al.*, *J Molec Bio* 215]: 403 (1990); Guide to Huge Computers, Martin J. Bishop, [ED.], Academic Press, San Diego, 1994, and [CARILLO ETA/.] (1988) *SIAM J Applied Math* 48: 1073). For example, the homology, similarity, or identity may be determined using BLAST or ClustalW of the National Center for Biotechnology Information (NCBI).

The homology, similarity, or identity of polynucleotide or polypeptide sequences may be determined by comparing sequence information using, for example, the GAP computer program (*e.g.*, Smith and Waterman, *Adv. Appl. Math* (1981) 2:482) as published. In summary, the GAP program defines the homology or identity as the value obtained by dividing the number of similarly aligned symbols (*i.e.* nucleotides or amino acids) by the total number of the symbols in the shorter of the two sequences. Default parameters for the GAP program may include (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) and the weighted comparison matrix of Gribskov *et al.* (1986), *Nucl. Acids Res.* 14: 6745, as disclosed in Schwartz and Dayhoff, eds., *Atlas of Protein Sequence*

and Structure, National Biomedical Research Foundation, pp. 353-358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap (or a gap opening penalty of 10 and a gap extension penalty of 0.5); and (3) no penalty for end gaps. Accordingly, as used herein, the term “homology” or “identity” refers to relevance between sequences.

As used herein, the term “variant” refers to a polypeptide which, in conservative substitution and/or modification of one or more amino acids, differs from the recited sequence but retains the functions or properties of the protein. A variant differs from identified sequences being distinguished by several amino acid substitutions, deletions, or additions. Such variant may generally be identified by modifying one amino acid of the polypeptide sequence and evaluating the properties of the variant. That is, the abilities of the variant protein may be increased, unaltered, or decreased compared to those of the native protein. In addition, some modified polypeptides may include modified ones in which one or more moieties (*e.g.*, a N-terminal leader sequence, transmembrane domain, *etc.*) are removed. Other variants may include variants in which a small portion has been removed from the N- and/or C-terminal of the mature protein. As used herein, the term “conservative substitution” refers to replacement of an amino acid with another amino acid having similar structural and/or chemical properties. The variant may have, for example, one or more conservative substitutions while still retaining one or more biological activities. Such amino acid substitution may be generally made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or amphipathic nature. For example, positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include glutamic acid and aspartic acid; aromatic amino acids include phenylalanine, tryptophan, and tyrosine; and hydrophobic amino acids include alanine, valine, isoleucine, leucine, methionine, phenylalanine, proline, glycine, and tryptophan.

Additionally, the variant may also include deletion or addition of amino acids, which have minimal effects on properties and a secondary structure of a polypeptide. For example, the polypeptide may be conjugated to a signal (or leader) sequence at the N-terminus of a protein that is involved in the co-translational or post-translational transfer of the protein. The polypeptide may also be conjugated to another sequence or linker to enable identification, purification, or synthesis of the polypeptide.

Specifically, the protein variant having the activity of exporting IMP of the present

disclosure may be a protein variant having an amino acid sequence, in which, from the N-terminus in the amino acid sequence of SEQ ID NO: 2, at least one amino acid selected from the group consisting of the 123rd amino acid, the 243rd amino acid, the 387th amino acid, the 405th amino acid; the 413th amino acid, and the 458th amino acid is substituted with another amino acid, but the amino acid substitution is not limited thereto.

For example, the protein variant having the activity of exporting IMP of the present disclosure may be a protein variant having the IMP-exporting activity may be one which has, from the N-terminus in the amino acid sequence of SEQ ID NO: 2, a substitution of the 123rd amino acid with cysteine (*i.e.*, F123C); a substitution of the 243rd amino acid with valine (*i.e.*, I243V); a substitution of the 387th amino acid with threonine (*i.e.*, S387T); a substitution of the 405th amino acid with tyrosine (*i.e.*, F405Y); a substitution of the 413th amino acid with threonine (*i.e.*, M413T); a substitution of the 458th amino acid with lysine (*i.e.*, N458K); or a combination thereof, but the amino acid substitution is not limited thereto. More specifically, the protein variant having an activity of exporting IMP may be a protein, which has an amino acid sequence selected from the group consisting of SEQ ID NOS: 73, 74, 75, 76, 77, 78, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, and 155, or an amino acid sequence having a homology of at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% or higher to these amino acid sequences. Additionally, it is apparent that any protein having an amino acid sequence, in which part of the amino acid sequence is deleted, modified, substituted, or added, can also be used as the protein of the present disclosure, as long as the amino acid sequence has the sequence homology described above and shows an effect equivalent to those of the above polypeptides.

Additionally, the protein variant having the activity of exporting IMP of the present disclosure may be a protein variant consisting of an amino acid sequence, which further includes, from the N-terminus in the amino acid sequence of SEQ ID NO: 2, a substitution of the 2nd amino acid with another amino acid, a substitution of the 64th amino acid with another amino acid, or a combination thereof. Specifically, the protein variant having the activity of exporting IMP of the present disclosure may be a protein variant, which further includes, from the N-terminus in the amino acid sequence of SEQ ID NO: 2, a substitution of the 2nd amino acid with isoleucine, a substitution of the 64th amino acid with glutamic acid or aspartate, or a combination thereof.

The “substitution with another amino acid” is not limited as long as the other amino

acid is an amino acid different from the amino acid before the substitution. For example, when the 2nd amino acid from the N-terminus in the amino acid sequence of SEQ ID NO: 2 is substituted with another amino acid, the other amino acid is not limited as long as the other amino acid is an amino acid other than valine, and when the 64th amino acid from the N-terminus in the amino acid sequence of SEQ ID NO: 2 is substituted with another amino acid, the other amino acid is not limited as long as the other amino acid is an amino acid other than glycine.

Another aspect of the present disclosure provides a polynucleotide encoding the protein variant of the present disclosure, or a vector containing the polynucleotide of the present disclosure.

As used herein, the term “polynucleotide” refers to a polymer of nucleotides in which nucleotide monomers are extended in a long chain by covalent bonds and which has a DNA strand or RNA strand longer than a certain length.

With regard to the polynucleotide of the present disclosure, based on codon degeneracy, it is apparent that the proteins which consist of the amino acid sequence of SEQ ID NO: 73, 74, 75, 76, 77, 78, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, or 155, or polynucleotides which can be translated into proteins having a homology to the above proteins, can also be included in the scope of the present disclosure. For example, the polynucleotide of the present disclosure may be a polynucleotide sequence having the base sequence of SEQ ID NO: 79, 80, 81, 82, 83, 84, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, or 156, and more specifically, a polynucleotide consisting of the base sequence of SEQ ID NO: 79, 80, 81, 82, 83, 84, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, or 156. Additionally, any sequence which encodes a protein that has an activity of the protein having an amino acid sequence of SEQ ID NO: 73, 74, 75, 76, 77, 78, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, or 155, determined by hybridization under stringent conditions with a probe that can be prepared from a known gene sequence (*e.g.*, a sequence complementary to all or part of the above nucleotide sequences), can be included without limitation.

The term “stringent conditions” refers to conditions under which specific

hybridization between polynucleotides is made possible. Such conditions are specifically described in references (*e.g.*, J. Sambrook *et al.*, *supra*). For example, the conditions may include performing hybridization between genes having a high homology, a homology of 40% or higher, specifically 90% or higher, more specifically 95% or higher, even more specifically 97% or higher, and most specifically 99% or higher, while not performing hybridization between genes having a homology of lower than the above homologies; or to perform hybridization once, specifically two or three times, under conventional washing conditions for southern hybridization of 60°C, 1× SSC, and 0.1% SDS, specifically at a salt concentration and temperature corresponding to 60°C, 0.1× SSC, and 0.1% SDS, and more specifically 68°C, 0.1× SSC, and 0.1% SDS.

Hybridization requires that two nucleic acids have a complementary sequence, although mismatches between bases may be possible depending on the stringency of the hybridization. The term “complementary” is used to describe the relationship between mutually hybridizable nucleotide bases. For example, with respect to DNA, adenosine is complementary to thymine, and cytosine is complementary to guanine. Accordingly, the present disclosure may also include isolated nucleic acid fragments complementary to the entire sequence as well as substantially similar nucleic acid sequences.

Specifically, polynucleotides having a homology can be detected at a T_m value of 55°C using hybridization conditions that include a hybridization step and using the conditions described above. Additionally, the T_m value may be 60°C, 63°C, or 65°C, but is not limited thereto and may be appropriately adjusted by an ordinary person skilled in the art according to the intended purpose.

The stringency suitable for the hybridization of polynucleotides depends on the length and complementarity of the polynucleotides and the related variables are well known in the art (see Sambrook *et al.*, *supra*, 9.50 to 9.51 and 11.7 to 11.8).

In the present disclosure, the polynucleotide which encodes the amino acid sequence of the protein having an IMP-exporting activity may be *impE2* gene, and the explanation of the polynucleotide is as described above.

In the present disclosure, the explanation of the polynucleotide encoding the protein variant, which has an IMP-exporting activity, is also as described above.

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 the target protein can be expressed in an

appropriate host. The control sequence may include a promoter capable of initiating transcription, any operator sequence for controlling 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 replicable in the host cell, and it may be constructed using any vector known in the art. 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. Specifically, *pDZ*, *pACYC177*, *pACYC184*, *pCL*, *pECCG117*, *pUC19*, *pBR322*, *pMW118*, *pCC1BAC* vectors, *etc.* may be used.

In an embodiment, the polynucleotide encoding the target protein may be replaced with a modified polynucleotide(variant) within the chromosome using a vector for the insertion into the chromosome in a cell. 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. In particular, a selection marker for confirming the insertion into the chromosome may be further included. The selection marker is used for selection of a transformed cell, *i.e.*, in order to confirm whether the target nucleic acid has been inserted, and markers capable of 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 capable of expressing the selection markers can survive or express other phenotypic traits, and thus the transformed cells can be easily selected.

Still another aspect of the present disclosure provides a microorganism which can produce IMP that includes the protein variant of the present disclosure, a polynucleotide encoding the protein variant of the present disclosure, or the vector of the present disclosure. Specifically, the microorganism of the present disclosure may be a microorganism prepared by transformation using a vector containing the polynucleotide encoding the protein variant of the present disclosure, but the microorganism is not limited thereto.

As used herein, the term “transformation” refers to a process of introducing a vector including a polynucleotide encoding a target protein into a host cell, thereby enabling the expression of the protein encoded by the polynucleotide in the host cell. For the transformed polynucleotide, it does not matter whether it is inserted into the chromosome of the host cell and located therein or located outside the chromosome, as long as the transformed polynucleotide can be expressed in the host cell. Additionally, the polynucleotide includes DNA and RNA which encode the target protein. The polynucleotide may be inserted in any form 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 of the 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 a self-replicable expression vector. Additionally, the polynucleotide may be introduced into a host cell as is and operably linked to a sequence essential for its expression in the host cell, but 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, *i.e.*, a conjugate of the present disclosure, and the above gene sequence.

As used herein, the term “IMP-producing microorganism” refers to a microorganism which is naturally capable of producing IMP; or a microorganism whose parent strain is not naturally capable of producing and/or exporting IMP which is provided with an ability to produce or export IMP. In the present disclosure, the microorganism producing IMP can be used interchangeably with a microorganism exporting IMP or a microorganism having an activity of exporting IMP.

The IMP-producing microorganism is a host cell or microorganism, which includes a protein variant having an activity of exporting IMP or a polynucleotide encoding the protein variant, or which is transformed with a vector containing the polynucleotide encoding the protein variant, and is thereby capable of expressing the protein variant. Specifically, the microorganism of the present disclosure may be a microorganism of the genus *Escherichia*, a microorganism of the genus *Serratia*, a microorganism of the genus *Erwinia*, a

microorganism of the genus *Enterobacteria*, a microorganism of the genus *Salmonella*, a microorganism of the genus *Streptomyces*, a microorganism of the genus *Pseudomonas*, a microorganism of the genus *Brevibacterium*, a microorganism of the genus *Corynebacterium*, etc., and more specifically, the microorganism of the present disclosure may be a microorganism of the genus *Corynebacterium*.

As used herein, the term “IMP-producing microorganism of the genus *Corynebacterium*” refers to a microorganism of the genus *Corynebacterium* which is naturally capable of producing IMP or capable of producing IMP by mutation. Specifically, as used herein, the microorganism of the genus *Corynebacterium* capable of producing IMP may be a native strain of the microorganism of the genus *Corynebacterium* capable of producing IMP; or a microorganism of the genus *Corynebacterium* with enhanced ability to producing IMP prepared by inserting a gene associated with IMP production or by enhancing or attenuating the endogenous gene associated with IMP production. More specifically, in the present disclosure, the microorganism of the genus *Corynebacterium* capable of producing IMP may be a microorganism of the genus *Corynebacterium* which has improved ability of producing IMP, by including a protein variant having an activity of exporting IMP or a polynucleotide encoding the protein variant, or by being transformed with a vector containing the polynucleotide encoding the protein variant. The “microorganism of the genus *Corynebacterium* with enhanced ability to producing IMP” may be a microorganism of the genus *Corynebacterium* with improved ability to producing IMP compared to that of its parent strain before transformation or that of an unmodified microorganism of the genus *Corynebacterium*. The “unmodified microorganism of the genus *Corynebacterium*” may be a native type of the microorganism of the genus *Corynebacterium*, or a microorganism of the genus *Corynebacterium* which does not contain the protein variant capable of exporting IMP, or a microorganism of the genus *Corynebacterium* which is not transformed with a vector containing a polynucleotide encoding the protein variant capable of exporting IMP.

In an embodiment of the present disclosure, the microorganism of the present disclosure may be a microorganism in which an activity of adenylosuccinate synthetase and/or IMP dehydrogenase is further attenuated.

Specifically, the microorganism of the present disclosure may be *Corynebacterium glutamicum*, *Corynebacterium ammoniagenes*, *Brevibacterium lactofermentum*, *Brevibacterium flavum*, *Corynebacterium thermoaminogenes*, *Corynebacterium efficiens*, or *Corynebacterium stationis*, but the microorganism is not limited thereto.

Still another aspect of the present disclosure provides a method for preparing IMP, which includes culturing the microorganism of the genus *Corynebacterium* producing IMP of the present disclosure in a medium.

Specifically, the method of the present disclosure may further include a step of recovering IMP from the microorganism of the present disclosure or the medium of the present disclosure.

In the above method of the present disclosure, the cultivation of the microorganism may be performed in a batch process, continuous process, fed-batch process, *etc.* known in the art, but the cultivation process is not particularly limited thereto. In particular, with respect to the cultivation conditions, the pH of the culture may be adjusted to a suitable pH (*e.g.*, pH 5 to 9, specifically pH 6 to 8, and most specifically with an appropriate basic compound (*e.g.*, sodium hydroxide, potassium hydroxide, or ammonia) or acidic compound (*e.g.*, phosphoric acid or sulfuric acid), and the aerobic condition of the culture may be maintained by introducing oxygen or an oxygen-containing gas mixture to the culture. The cultivation temperature may generally be in the range of 20°C to 45°C, and specifically 25°C to 40°C for about 10 to 160 hours, but the cultivation conditions are not limited thereto. The IMP produced by the above cultivation may be secreted into the culture or may be retained in the cells.

Additionally, examples of the carbon sources to be used in the culture medium may include sugars and carbohydrates (*e.g.*, glucose, sucrose, lactose, fructose, maltose, molasses, starch, and cellulose); oils and fats (*e.g.*, soybean oil, sunflower 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), but are not limited thereto. These carbon sources may be used alone or in combination, but are not limited thereto. Examples of the nitrogen sources to be used in the culture medium may include nitrogen-containing organic compounds (*e.g.*, peptone, yeast extract, meat gravy, 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), *etc.* These nitrogen sources may be used alone or in combination, but are not limited thereto. Examples of the phosphorus sources to be used in the culture medium may include potassium dihydrogen phosphate, dipotassium hydrogen phosphate, corresponding sodium-containing salts, *etc.*, but are not limited thereto. Additionally, metal salts (*e.g.*, magnesium sulfate or iron sulfate),

amino acids, vitamins, *etc.*, which are essential growth-promoting materials, may be contained in the medium.

In the present disclosure, the method for recovering the IMP produced in the step of cultivation may be performed by collecting the IMP from the culture broth using an appropriate method known in the art. For example, methods such as centrifugation, filtration, anion exchange chromatography, crystallization, HPLC, *etc.* may be used, and the desired IMP can be recovered from a culture or cultured microorganism using an appropriate method known in the art.

Further, the recovery step may include a purification process and may be performed using an appropriate method known in the art. Thus, the IMP to be recovered may be in a purified form or a microorganism fermentation broth containing IMP.

Still another aspect of the present disclosure provides a composition for producing IMP, which contains a protein variant capable of exporting IMP of the present disclosure or a polynucleotide encoding the protein variant.

The composition of the present disclosure may further contain any constitution that is capable of operating the polynucleotide without limitation. In the composition of the present disclosure, the polynucleotide may be in a form in which the polynucleotide is included in a vector such that an operably linked gene can be expressed in a host cell where the polynucleotide is introduced.

Additionally, the composition may further contain any appropriate excipient conventionally used in compositions for producing IMP (*e.g.*, preservatives, humectants, dispersing agents, suspending agents, buffering agents, stabilizing agents, isotonic agents, *etc.*), but the appropriate excipient is not limited thereto.

Still another aspect of the present disclosure provides use of the protein variant of the present disclosure for increasing IMP production in a microorganism of the genus *Corynebacterium*.

Still another aspect of the present disclosure provides a method for increasing export of IMP, which includes enhancing an activity of the protein consisting of SEQ ID NO: 2 in a microorganism of the genus *Corynebacterium*. Specifically, the enhancement of the activity of the protein consisting of SEQ ID NO: 2 may be performed by introducing, applying, or including a protein variant capable of exporting IMP, in which the protein variant consists of

an amino acid sequence which has, from the N-terminus in the amino acid sequence of SEQ ID NO: 2, a substitution of the 123rd amino acid with another amino acid, a substitution of the 243rd amino acid with another amino acid, a substitution of the 387th amino acid with another amino acid, a substitution of the 405th amino acid with another amino acid; a substitution of the 413th amino acid with another amino acid, a substitution of the 458th amino acid with another amino acid, or a combination thereof. The terms, “protein capable of exporting IMP”, “protein variant capable of exporting IMP”, and “microorganism of the genus *Corynebacterium*” are as described above.

Still another aspect of the present disclosure provides use of the protein variant of the present disclosure for increasing export of IMP in a microorganism of the genus *Corynebacterium*.

[Advantageous Effects of the Invention]

IMP can be produced in high yield by culturing a microorganism of the genus *Corynebacterium* producing IMP using a protein variant, which is capable of exporting IMP.

[DETAILED DESCRIPTION OF THE INVENTION]

The present disclosure will be described in detail as follows. Meanwhile, each of the explanations and exemplary embodiments disclosed herein can be applied to other respective explanations and exemplary embodiments. That is, all of the combinations of various factors disclosed herein belong to the scope of the present disclosure. Additionally, the scope of the present disclosure should not be limited by the specific disclosure provided hereinbelow.

Example 1: Discovery of IMP export proteins

A genomic DNA library of *Corynebacterium stationis* ATCC6872 was prepared for the identification of membrane proteins of *Corynebacterium* involved in the export of IMP. Then, since the wild-type strain of *Corynebacterium* cannot produce IMP, or even if it does produce IMP, it produces only a small amount thereof, a strain called *CJI0323*, which is capable of producing IMP, derived from the ATCC6872 strain was prepared for the identification of the ability to produce IMP. The *CJI0323* strain prepared was subjected to screening of membrane proteins involved in IMP export using the genomic DNA library of

the *ATCC6872* strain. The specific details of the experiment are as follows.

Example 1-1: Selection of IMP-producing strain, *CJI0323*

The *ATCC6872* cells were suspended in a phosphate buffer (pH 7.0) or citrate buffer (pH 5.5) at a concentration of 10^7 cells/mL to 10^8 cells/mL to prepare an *ATCC6872*-derived IMP-producing strain, and the cells were subjected to UV treatment and placed at room temperature or 32°C for 20 to 40 minutes to induce mutation. The resulting cells were washed twice with a 0.85% saline solution, and then diluted and plated on a medium, which was prepared by adding a resistance-providing material at an appropriate concentration to a minimal medium containing 1.7% agar, and colonies were obtained thereafter. Each colony was cultured in a nutrient medium and cultured in a seed medium for 24 hours. After culturing the colonies for 3 to 4 days in a fermentation medium, the colony with the highest amount of IMP produced accumulated in the culture medium was selected. In the course of preparing a strain capable of producing IMP at high concentration, in order to provide adenine auxotrophy, guanine leakage, lysozyme susceptibility, 3,4-dihydroproline resistance, streptomycin resistance, azetidine carboxylic acid resistance, thiaproline resistance, azaserine resistance, sulfaguanidine resistance, norvaline resistance, and trimethoprim resistance, the procedures above were performed sequentially for each material. As a result, *CJI0323*, which showed resistance to the above materials and excellent ability to produce IMP, was finally selected. The degree of resistance between *ATCC6872* and *CJI0323* was compared and the results are shown in Table 1 below.

[Table 1]

Characteristics	<i>ATCC6872</i>	<i>CJI0323</i>
Adenine auxotrophy	Non-auxotrophy	Auxotrophy
Guanine leakage	Non-auxotrophy	Leaky auxotrophy
Lysozyme susceptibility	80 µg/mL	8 µg/mL
3,4-Dihydroproline resistance	1000 µg/mL	3500 µg/mL
Streptomycin resistance	500 µg/mL	2000 µg/mL
Azetidine carboxylic acid	5 mg/mL	30 mg/mL

resistance		
Thiaproline resistance	10 µg/mL	100 µg/mL
Azaserine resistance	25 µg/mL	100 µg/mL
Sulfaguanidine resistance	50 µg/mL	200 µg/mL
Norvaline resistance	0.2 mg/mL	2 mg/mL
Trimethoprim resistance	20 µg/mL	100 µg/mL

- Minimal medium: 2% glucose, 0.3% sodium sulfate, 0.1% KH₂SO₄, 0.3% K₂HPO₄, 0.3% magnesium sulfate, calcium chloride (10 mg/L), iron sulfate (10 mg/L), zinc sulfate (1 mg/L), manganese chloride (3.6 mg/L), L-cysteine (20 mg/L), calcium pantothenate (10 mg/L), thiamine hydrochloride (5 mg/L), biotin (30 µg/L), adenine (20 mg/L), guanine (20 mg/L), pH 7.3

- Nutrient medium: 1% peptone, 1% meat juice, 0.25% sodium chloride, 1% yeast extract, 2% agar, pH 7.2

- Seed medium: 1% glucose, 1% peptone, 1% meat juice, 1% yeast extract, 0.25% sodium chloride, adenine (100 mg/L), guanine (100 mg/L), pH 7.5

- Fermentation medium: 0.1% sodium glutamate, 1% ammonium chloride, 1.2% magnesium sulfate, 0.01% calcium chloride, iron sulfate (20 mg/L), manganese sulfate (20 mg/L), zinc sulfate (20 mg/L), copper sulfate (5 mg/L), L-cysteine (23 mg/L), alanine (24 mg/L), nicotinic acid (8 mg/L), biotin (45 µg/L), thiamine hydrochloride (5 mg/L), adenine (30 mg/L), 1.9% phosphoric acid (85%), 2.55% glucose, 1.45% fructose

Example 1-2: Experiments on fermentation titer of *CJI0323*

The seed medium (2 mL) was dispensed into test tubes (diameter: 18 mm), which were then autoclaved and each inoculated with *ATCC6872* and *CJI0323*. Thereafter, the resultants were shake-cultured at 30°C for 24 hours and then used as a seed culture solution.

The fermentation medium (29 mL) was dispensed into Erlenmeyer flasks (250 mL) for shaking, autoclaved at 121°C for 15 minutes, and the seed culture solution (2 mL) was inoculated thereto and cultured for 3 days. The culture conditions were set to 170 rpm, 30°C, and a pH of 7.5.

Upon completion of the culture, the amount of IMP produced was measured by HPLC (SHIMAZDU LC20A) and the results of the culture are shown in Table 2 below.

[Table 2]

Strain	IMP (g/L)
<i>ATCC6872</i>	0
<i>CJI0323</i>	9.52

The *CJI0323* strain was named *Corynebacterium stationis* CN01-0323, deposited on November 7, 2017, to the Korean Culture Center of Microorganisms (KCCM), an international depositary authority under the Budapest Treaty, and assigned Accession Number KCCM12151P.

Example 1-3: Discovery of exporting proteins

Screening conditions showing growth inhibition of the *CJI0323* strain were established by additionally adding IMP to the minimal medium containing 1.7% agar. The plasmids of the genomic library of the *ATCC6872* strain were transformed into the *CJI0323* strain by electroporation (van der Rest *et al.* 1999), and those colonies in which the growth inhibition was released under the medium conditions supplemented with an excess amount of IMP were selected. Plasmids were obtained from the selected colonies and analyzed by a sequencing technique. As a result, one kind of membrane protein involved in the release of the growth inhibition was identified under the condition where an excess amount of IMP was added.

The one kind of membrane protein from *Corynebacterium* was identified based on the amino acid sequence of SEQ ID NO: 2 and the nucleotide sequence of SEQ ID NO: 4 (NCBI GenBank: NZ_CP014279, WP_066795121, MFS transporter). The membrane protein is known as the MFS transporter, but its specific function has not been confirmed, and further, its function regarding the IMP export is still unknown. In the present disclosure, the membrane protein was named ImpE2(WT).

Example 2: Identification of ImpE1 and ImpE2

Example 2-1: Confirmation of *impE1* and *impE2*

In order to examine the functions of the membrane protein, ImpE2, the gene structure of SEQ ID NO: 4 was confirmed in the NCBI (NCBI GenBank: NZ_CP014279, WP_066795121, MFS transporter). As a result, it was confirmed that the 7 bp starting portion of the ORF of SEQ ID NO: 4 (*impE2*) overlaps in 7 nucleotide bases with a different gene (NCBI GenBank: NZ_CP014279, WP_066795119, transcriptional regulator), which is located upstream of *impE2*. Since the functions of the gene located upstream of *impE2* and the protein encoded by the gene have not been confirmed, in the present disclosure, the protein was named ImpE1(WT) (the amino acid sequence of SEQ ID NO: 1 and the nucleotide sequence of SEQ ID NO: 3).

Example 2-2: Preparation of *impE1*- or *impE2*-deficient vector

In order to confirm whether the deletion of *ImpE1* or *ImpE2*, which are involved in releasing the growth inhibition caused by IMP as identified in Examples 1 and 2-1, in an IMP-producing strain can reduce its IMP-exporting ability, attempts were made to prepare vectors deficient in each of the genes.

The gene fragments for preparing the vectors were obtained by PCR using the genomic DNA of the *ATCC6872* strain as a template.

Specifically, the PCR for *impE1* was performed using primers of SEQ ID NOS: 5 and 6 and primers of SEQ ID NOS: 7 and 8; and the PCR for *impE2* was performed using the primers of SEQ ID NOS: 9 and 10 and primers of SEQ ID NOS: 11 and 12 (Table 3).

[Table 3]

SEQ ID NO	Primer	Sequence (5' – 3')
5	<i>impE1</i> kop-1	GCTCTAGACGAGAAAGCTAAAGCCGGTGA
6	<i>impE1</i> kop-2	GTTTTTAGCTACCATTTGTTACACCCCGTGCAAGTTT
7	<i>impE1</i> kop-3	GCACGGGGTGTAACAATGGTAGCTAAAACTCCACC
8	<i>impE1</i> kop-4	GCTCTAGAAATAGTTGGGGAAGTCCACTC
9	<i>impE2</i> kop-1	GCTCTAGACTTGGATGACCTGGTGGAAAA
10	<i>impE2</i> kop-2	CTTGGAGAAAATTCCTACCATTCCAGTCCTTTCGT

11	impE2 kop-3	GGACTGGAATGGTAGGAAATTTTCTCCAAGGGAAAT
12	impE2 kop-4	GGACTAGTGGATTGTGTTGACGCACGATG
65	impE1E2 kop-2	CTTGGAGAAAATTTCTGTTACACCCCGTGCAAGTTT
66	impE1E2 kop-3	GCACGGGGTGTAACAGAAATTTTCTCCAAGGGAAAT

In particular, the primers used were prepared based on information on a gene of *Corynebacterium stationis* (ATCC6872) (NCBI Genbank: NZ_CP014279) registered in NIH GenBank and the nucleotide sequences adjacent thereto.

PCR was performed by initial denaturation at 94°C for 5 minutes; 25 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 52°C for 3 minutes, and polymerization at 72°C for 1 minute; and final polymerization at 72°C for 5 minutes.

Overlapping PCR was performed using two fragments of the *impE1* gene, which were amplified using the primers of SEQ ID NOS: 5 and 6 and the primers of SEQ ID NOS: 7 and 8, as templates, and as a result, a polynucleotide template (1.8 kbp) was obtained. The obtained gene fragment was cloned into a linearized *pDZ* vector (Korean Patent No. 10-0924065 and International Patent Publication No. 2008-033001), which was digested with the restriction enzyme (*Xba*I), and ligated using T4 ligase, and thereby the *pDZ-ΔimpE1* vector was prepared. Additionally, overlapping polymerase chain reaction was performed using a fragment of the *impE2* gene, amplified using the primers of SEQ ID NOS: 9 and 10, and two fragments of the *impE2* gene, amplified using the primers of SEQ ID NOS: 11 and 12, as templates, and as a result, a polynucleotide template (1.7 kbp) was obtained. The obtained gene fragment was digested with restriction enzymes, *Xba*I and *Spe*I. The gene fragment was cloned using T4 ligase into a linearized *pDZ* vector, which had already been digested with the restriction enzyme (*Xba*I), and thereby the *pDZ-ΔimpE2* vector was prepared.

Example 2-3: Preparation of *impE1*- and *impE2*-integration-deficient vectors

Since the *impE1* and *impE2* genes, which encode proteins involved in releasing the growth inhibition caused by IMP, are overlapped, there is a need to regulate both genes simultaneously. Therefore, attempts were made to prepare a vector in which both *impE1* and *impE2* are deficient.

For the PCR of *impE1* and *impE2* genes, primers of SEQ ID NOS: 5 and 65 and primers of SEQ ID NOS: 66 and 12 were used. The primers used were prepared based on information on a gene of *Corynebacterium stationis* (ATCC6872) (NCBI Genbank: NZ_CP014279) registered in NIH GenBank and the nucleotide sequences adjacent thereto. Overlapping PCR was performed using a fragment of the *impE1* gene, amplified using the primers of SEQ ID NOS: 5 and 65, and two fragments of the *impE2* gene, amplified using the primers of SEQ ID NOS: 66 and 12, as templates, and as a result, a polynucleotide template (2.0 kbp) was obtained. The obtained gene fragments were digested with *Xba*I and *Spe*I, respectively. The gene fragments were cloned using T4 ligase into a linearized *pDZ* vector, which had already been digested with the restriction enzyme (*Xba*I), and thereby the *pDZ-ΔimpE1E2* vector was prepared.

Example 2-4: Preparation of *impE1*- and *impE2*-deficient strains

The two kinds of plasmids prepared in Example 2-2 and one kind of plasmid prepared in Example 2-3 were each transformed into the *CJI0323* strain by electroporation (using the transformation method disclosed in *Appl. Microbiol. Biotechnol.* (1999) 52: 541 to 545). The strains in which the vector was inserted into the chromosome by recombination of the homologous sequences were selected on a medium containing kanamycin (25 mg/L). The selected primary strains were subjected to a second cross-over. The genetic deficiency in the finally transformed strains was confirmed by performing PCR using the primer pairs of SEQ ID NOS: 5 and 8, SEQ ID NOS: 9 and 12, and SEQ ID NOS: 5 and 12.

The selected strains were named *CJI0323_ΔimpE1*, *CJI0323_ΔimpE2*, and *CJI0323_ΔimpE1E2*. Additionally, the ability to produce IMP of these strains was evaluated.

The seed medium (2 mL) was dispensed into test tubes (diameter: 18 mm), which were then autoclaved, each inoculated with *CJI0323*, *CJI0323_ΔimpE1*, *CJI0323_ΔimpE2*, and *CJI0323_ΔimpE1E2*, shake-cultured at 30°C for 24 hours, and used as seed culture solutions. The fermentation medium (29 mL) was dispensed into Erlenmeyer flasks (250 mL) for shaking and autoclaved at 121°C for 15 minutes. Then, the seed culture solution (2 mL) was inoculated thereto and the resultant was cultured for 3 days. The culture conditions were set to 170 rpm, 30°C, and a pH of 7.5.

Upon completion of the culture, the amount of IMP produced was measured by HPLC,

and the results of the culture are shown in Table 4 below.

[Table 4]

Strain	IMP (g/L)
<i>CJI0323</i>	9.52
<i>CJI0323_ΔimpE1</i>	1.92
<i>CJI0323_ΔimpE2</i>	1.88
<i>CJI0323_ΔimpE1E2</i>	1.80

The IMP amount accumulated in each strain was compared with that of the parent strain, *Corynebacterium stationis* *CJI0323*. As a result, it was found that, as shown in Table 4 above, the IMP concentrations of the strains *CJI0323_ΔimpE1*, *CJI0323_ΔimpE2*, and *CJI0323_ΔimpE1E2* were reduced by about 8 g/L under the same conditions compared to the *CJI0323* strain, confirming that ImpE1 and ImpE2 are proteins involved in the IMP export.

Example 3: Confirmation of nucleotide sequences of *impE1* and *impE2* of IMP-producing strain, *CJI0323*

In the case of the *CJI0323* strain producing IMP at high concentration in Example 1, it is possible that the strain has an improved IMP-exporting ability so as to produce IMP at high concentration. Accordingly, an attempt was made to confirm the presence of any mutation in *impE1* and *impE2* of the *CJI0323* strain.

The chromosomal DNA of the *CJI0323* strain was amplified by polymerase chain reaction (hereinafter, “PCR”). Specifically, first, PCR was performed by repeating 28 cycles consisting of denaturation at 94°C for 1 minute, annealing at 58°C for 30 seconds, and polymerization at 72°C for 2 minutes using the chromosomal DNA of the *CJI0323* strain as a template along with the primers of SEQ ID NOS: 13 and 14 (Table 5), and thereby a fragment of about 2.8 kbp was amplified.

[Table 5]

SEQ ID NO	Primer	Sequence (5' to 3')
13	impE1E2 seqF	GAACGGAGTCATCTCCTTTGC
14	impE1E2 seqR	CCAAACGCTCTGCAAGAACTG

Upon analysis of the nucleotide sequence using the same primers, it was confirmed that the 490th nucleotide of the *impE1* gene (*i.e.*, g) was substituted with ‘a’, compared to the nucleotide sequence of the wild-type strain, *ATCC6872*. This substitution indicates that there was a mutation in which the 164th amino acid of the ImpE1 protein (*i.e.*, glutamic acid) was substituted with lysine.

Additionally, it was confirmed that the 4th nucleotide of the *impE2* gene (*i.e.*, g) was substituted with ‘a’ (this means that the 666th nucleotide of the *impE1* gene (*i.e.*, g) was substituted with ‘a’) and the 191st nucleotide of the *impE1* gene (*i.e.*, g) was substituted with ‘a’. These substitutions indicate that there were mutations in which the 2nd amino acid of the ImpE2 protein (*i.e.*, valine), which corresponds to the 222nd amino acid of the ImpE1 protein, was substituted with isoleucine; and the 64th amino acid of the ImpE2 protein (*i.e.*, glycine) was substituted with glutamic acid.

The *impE1* nucleotide of the *CJI0323* strain was named *impE1_CJI0323* (SEQ ID NO: 87) and the protein thereof was named ImpE1_*CJI0323* (SEQ ID NO: 85), whereas the *impE2* nucleotide of the *CJI0323* strain was named *impE2_CJI0323* (SEQ ID NO: 88) and the protein thereof was named ImpE2_*CJI0323* (SEQ ID NO: 86).

Example 4: Recovery of mutations in *impE1* and *impE2*

Example 4-1: Preparation of vectors for recovering mutations in *impE1* or *impE2*

In Example 3, the presence of any mutation in *impE1* and *impE2* of the IMP-producing strain *CJI0323* was examined. As a result, it was confirmed that *impE1* had one mutation and *impE2* had two mutations. Since the *CJI0323* strain produces IMP at a high concentration, it is highly likely that the mutation is one that can improve the ability to export IMP. Accordingly, after recovering the mutated *impE1* and *impE2* to the native wild-type *ImpE* without mutation, the following experiment was performed to confirm whether additionally discovered protein variants have improved IMP-exporting ability.

To prepare a recovery vector, PCR was performed using *Corynebacterium stationis* *ATCC6872* as a template.

The *impE1impE2* gene fragment amplified using the primers of SEQ ID NOS: 89 and 90 was treated with a restriction enzyme, *XbaI*, and cloned into the *XbaI* restriction site on the *pDZ* vector, and thereby the *pDZ-impE1E2(WT)* was prepared.

Example 4-2: Preparation of vectors with single mutation in *impE1* or *impE2*

The plasmid prepared in Example 4-1 was transformed into the *CJI0323* strain by electroporation (using the transformation method disclosed in *Appl. Microbiol. Biotechnol.* (1999) 52: 541 to 545). The strains in which the vector was inserted into the chromosome by recombination of the homologous sequences were selected on a medium containing kanamycin (25 mg/L). The selected primary strains were subjected to a second cross-over. The recovery of the mutation in the finally transformed strains was confirmed by performing PCR using the primer pair of SEQ ID NOS: 89 and 90, followed by nucleotide sequencing analysis. The prepared strain was named *CJI0323_impE1E2(WT)*.

Example 5: Discovery of mutations in *impE2*

Among the three kinds of mutations discovered through the results in Example 3, the one having the highest IMP-exporting ability was selected and the following experiment was performed to discover mutations having a higher IMP-exporting ability compared to the same.

Example 5-1: Selection of mutations having the highest IMP-exporting ability among *impE1E2* mutations

A vector with a single E164K mutation in the *ImpE1* gene was prepared using the native wild-type strain, *Corynebacterium stationis* ATCC6872, as a template along with the primers of SEQ ID NOS: 91 and 92 and primers of SEQ ID NOS: 93 and 94. Overlapping PCR was performed using an E164K-1 gene fragment amplified using the primers of SEQ ID NOS: 91 and 92 and two E164K-2 gene fragments amplified using the primers of SEQ ID NOS: 93 and 94, and thereby a template with a 1.8 kbp polynucleotide was obtained. The obtained gene fragments were digested with *XbaI* and cloned into a linearized *pDZ* vector, which had already been digested with *XbaI*, using T4 ligase, and thereby the *pDZ-impE1(E164K)* vector was prepared.

A vector with a single V2I mutation in the *ImpE2* gene was prepared using the ATCC6872 strain as a template along with the primers of SEQ ID NOS: 91 and 95 and primers of SEQ ID NOS: 96 and 94. Overlapping PCR was performed using a V2I-1 gene fragment amplified using the primers of SEQ ID NOS: 91 and 95 and two V2I-2 gene fragments amplified using the primers of SEQ ID NOS: 96 and 94, and thereby a template with a 1.8 kbp polynucleotide was obtained. The obtained gene fragments were digested

with *Xba*I and cloned into a linearized *pDZ* vector, which had already been digested with *Xba*I, using T4 ligase, and thereby the *pDZ-impE2(V2I)* vector was prepared.

A vector with a single G64E mutation in the *ImpE2* gene was prepared using the *ATCC6872* strain as a template along with the primers of SEQ ID NOS: 91 and 97 and primers of SEQ ID NOS: 98 and 94. Overlapping PCR was performed using a G64E-1 gene fragment amplified using the primers of SEQ ID NOS: 91 and 97 and two G64E-2 gene fragments amplified using the primers of SEQ ID NOS: 98 and 94, and thereby a template with a 1.8 kbp polynucleotide was obtained. The obtained gene fragments were digested with *Xba*I and cloned into a linearized *pDZ* vector, which had already been digested with *Xba*I, using T4 ligase, and thereby the *pDZ-impE2(G64E)* vector was prepared.

[Table 6]

SEQ ID NO	Primer	Sequence (5' to 3')
89	impE1E2 WT F	GCTCTAGAGAACGGAGTCATCTCCTTTGC
90	impE1E2 WT R	GCTCTAGACCAAACGCTCTGCAAGAACTG
91	impE1 164K-1	GCTCTAGACTTGGATGACCTGGTGGAAAA
92	impE1 164K-2	CTGGGGCGCGTTGTTTTTCAGGATGCTCCCGAAGACG
93	impE1 164K-3	AACAACGCGCCCCAGAATTGG
94	impE1 164K-4	GCTCTAGAAATAGTTGGGGAAGTCCACTC
95	impE2 V2I-2	TGGAGTTTTTAGCTATCATTCCAGTCCTTTCGTGTAA
96	impE2 V2I-3	TAGCTAAAAACTCCACCCCAA
97	impE2 G64E-2	CCGAAAATCATCTGCTCCAAAGAGCTCATCAGCATGG
98	impE2 G64E-3	GCAGATGATTTTCGTTCCGC

The three kinds of plasmids prepared in Example 4-2 were transformed into the *CJI0323_impE1E2(WT)* strain (using the transformation method disclosed in *Appl. Microbiol. Biotechnol.* (1999) 52: 541 to 545). The strains in which the vector was inserted into the chromosome by recombination of the homologous sequences were selected on a medium containing kanamycin (25 mg/L). The selected primary strains were subjected to a second cross-over. The introduction of the mutation in the finally transformed strains was confirmed by performing PCR using the primer pair of SEQ ID NOS: 13 and 14, followed by nucleotide sequencing analysis. The selected strains were named *CJI0323_impE1(E164K)*,

CJI0323_impE2(V2I), and *CJI0323_impE2(G64E)*.

The *CJI0323_impE1(E164K)*, *Corynebacterium stationis CJI0323_impE2(V2I)*, and *Corynebacterium stationis CJI0323_impE2(G64E)* strains were deposited on November 2, 2018, to the Korean Culture Center of Microorganisms (KCCM), an international depositary authority under the Budapest Treaty, and assigned Accession Numbers KCCM12359P, KCCM12360P, and KCCM12361P, respectively.

The seed medium (2 mL) was dispensed into test tubes (diameter: 18 mm), which were then autoclaved, each inoculated with *CJI0323_impE1E2(WT)*, *CJI0323_impE1(E164K)*, *CJI0323_impE2(V2I)*, and *CJI0323_impE2(G64E)*, shake-cultured at 30°C for 24 hours, and used as seed culture solutions. The fermentation medium (29 mL) was dispensed into Erlenmeyer flasks (250 mL) for shaking and autoclaved at 121°C for 15 minutes. Then, the seed culture solutions (2 mL) were inoculated thereto and the resultants were cultured for 3 days. The culture conditions were set to 170 rpm, 30°C, and a pH of 7.5.

Upon completion of the culture, the amount of IMP produced was measured by HPLC, and the results of the culture are shown in Table 7 below.

[Table 7]

Strain	IMP (g/L)
<i>CJI0323</i>	9.52
<i>CJI0323_impE1E2(WT)</i>	2.32
<i>CJI0323_impE1(E164K)</i>	2.57
<i>CJI0323_impE2(V2I)</i>	3.11
<i>CJI0323_impE2(G64E)</i>	3.27

As shown above, it was confirmed that each of the three kinds of mutations is involved in IMP export, and that the *CJI0323_impE2(G64E)* strain had the greatest amount of IMP production among the three kinds of mutations.

Example 5-2: Preparation of vectors for substitutional insertion of amino acids in *impE2* mutation

To confirm the positional importance of the *impE2(G64E)* mutation among the

representative three kinds of mutations with enhanced ability to produce IMP as identified in the results above, a vector for introducing a mutation of substituting the 64th amino acid in the amino acid sequence of impE2 with a different amino acid was prepared.

The procedure of preparing the vector for the introduction of the ImpE2(G64E) mutation is as follows.

Based on the reported polynucleotide sequences, the chromosomal genes of *Corynebacterium stationis* CJI0323 were isolated, and gene fragments were obtained by performing PCR using the chromosomal DNA of *Corynebacterium stationis* CJI0323 as a template along with primer pairs between the primer of SEQ ID NO: 15 and each of SEQ ID NOS: 16 to 33. PCR was performed by initial denaturation at 94°C for 5 minutes; 20 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and polymerization at 72°C for 1 minute; and final polymerization at 72°C for 5 minutes. As a result, 18 kinds of 1 kbp polynucleotides were obtained.

Then, the chromosomal genes of *Corynebacterium stationis* CJI0323 were isolated, and gene fragments were obtained by performing PCR using the chromosomal DNA of *Corynebacterium stationis* CJI0323 as a template along with primer pairs between the primer of SEQ ID NO: 34 and each of SEQ ID NOS: 35 to 52. PCR was performed by initial denaturation at 94°C for 5 minutes; 20 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and polymerization at 72°C for 1 minute; and final polymerization at 72°C for 5 minutes. As a result, 18 kinds of 1 kbp polynucleotides were obtained.

Overlapping PCR was performed using two fragments obtained from the above results as a template, and thereby 18 kinds of 2 kbp polynucleotides to be used as templates were obtained. The obtained gene fragments were digested with a restriction enzyme, *Xba*I, ligated to the linearized *pDZ* vector, which had already been digested with a restriction enzyme, *Xba*I, transformed into *E. coli* DH5 α , and the transformants were plated on a solid LB medium containing kanamycin (25 mg/L).

The sequence information on the primers used for the preparation of the vector is shown in Table 8 below.

[Table 8]

SEQ ID NO	Primer	Sequence (5' to 3')
15	XbaI-impE2 64 1F	GGGTCTAGAAAAGAGCTTAAGGCAGCTGCT

16	impE2 64-R 1R	GAAAATCATCTGGCGCAAAGAGCTCAT
17	impE2 64-H 1R	GAAAATCATCTGGTGCAAAGAGCTCAT
18	impE2 64-D 1R	GAAAATCATCTGGTCCAAAGAGCTCAT
19	impE2 64-K 1R	GAAAATCATCTGCTTCAAAGAGCTCAT
20	impE2 64-S 1R	GAAAATCATCTGGGACAAAGAGCTCAT
21	impE2 64-T 1R	GAAAATCATCTGGGTCAAAGAGCTCAT
22	impE2 64-N 1R	GAAAATCATCTGGTTCAAAGAGCTCAT
23	impE2 64-Q 1R	GAAAATCATCTGCTGCAAAGAGCTCAT
24	impE2 64-C 1R	GAAAATCATCTGGCACAAAGAGCTCAT
25	impE2 64-P 1R	GAAAATCATCTGTGGCAAAGAGCTCAT
26	impE2 64-A 1R	GAAAATCATCTGAGCCAAAGAGCTCAT
27	impE2 64-V 1R	GAAAATCATCTGGACCAAAGAGCTCAT
28	impE2 64-I 1R	GAAAATCATCTGGATCAAAGAGCTCAT
29	impE2 64-L 1R	GAAAATCATCTGCAGCAAAGAGCTCAT
30	impE2 64-M 1R	GAAAATCATCTGCATCAAAGAGCTCAT
31	impE2 64-F 1R	GAAAATCATCTGGAACAAAGAGCTCAT
32	impE2 64-Y 1R	GAAAATCATCTGGTACAAAGAGCTCAT
33	impE2 64-W 1R	GAAAATCATCTGCCACAAAGAGCTCAT
34	XbaI-impE2 64 2R	GGGTCTAGACGGTCAATGAAGTCTCAACGG
35	impE2 64-R 2F	ATGAGCTCTTTGCGCCAGATGATTTTC
36	impE2 64-H 2F	ATGAGCTCTTTGCACCAGATGATTTTC
37	impE2 64-D 2F	ATGAGCTCTTTGGACCAGATGATTTTC
38	impE2 64-K 2F	ATGAGCTCTTTGAAGCAGATGATTTTC
39	impE2 64-S 2F	ATGAGCTCTTTGTCCCAGATGATTTTC
40	impE2 64-T 2F	ATGAGCTCTTTGACCCAGATGATTTTC
41	impE2 64-N 2F	ATGAGCTCTTTGAACCAGATGATTTTC
42	impE2 64-Q 2F	ATGAGCTCTTTGCAGCAGATGATTTTC
43	impE2 64-C 2F	ATGAGCTCTTTGTGCCAGATGATTTTC
44	impE2 64-P 2F	ATGAGCTCTTTGCCACAGATGATTTTC
45	impE2 64-A 2F	ATGAGCTCTTTGGCTCAGATGATTTTC
46	impE2 64-V 2F	ATGAGCTCTTTGGTCCAGATGATTTTC

47	impE2 64-I 2F	ATGAGCTCTTTGATCCAGATGATTTTC
48	impE2 64-L 2F	ATGAGCTCTTTGCTGCAGATGATTTTC
49	impE2 64-M 2F	ATGAGCTCTTTGATGCAGATGATTTTC
50	impE2 64-F 2F	ATGAGCTCTTTGTTCCAGATGATTTTC
51	impE2 64-Y 2F	ATGAGCTCTTTGTACCAGATGATTTTC
52	impE2 64-W 2F	ATGAGCTCTTTGTGGCAGATGATTTTC

After selecting the colonies transformed with the vector into which the target gene was inserted, the plasmids were obtained using a conventionally known plasmid extraction method. The information on the obtained plasmids is shown in Table 9 below.

[Table 9]

No.	Plasmid
1	<i>pDZ-impE2 64R</i>
2	<i>pDZ-impE2 64H</i>
3	<i>pDZ-impE2 64D</i>
4	<i>pDZ-impE2 64K</i>
5	<i>pDZ-impE2 64S</i>
6	<i>pDZ-impE2 64T</i>
7	<i>pDZ-impE2 64N</i>
8	<i>pDZ-impE2 64Q</i>
9	<i>pDZ-impE2 64C</i>
10	<i>pDZ-impE2 64P</i>
11	<i>pDZ-impE2 64A</i>
12	<i>pDZ-impE2 64V</i>
13	<i>pDZ-impE2 64I</i>
14	<i>pDZ-impE2 64L</i>
15	<i>pDZ-impE2 64M</i>
16	<i>pDZ-impE2 64F</i>
17	<i>pDZ-impE2 64Y</i>
18	<i>pDZ-impE2 64W</i>

Example 5-3: Preparation of strains where the amino acid at position 64 of variant

(ImpE2) is substituted with another amino acid, and comparison of ability to produce IMP

The 18 kinds of plasmids prepared in Example 3-1 were transformed into the *CJI0323* strain. The strains in which the vector was inserted into the chromosome by recombination of the homologous sequences were selected on a medium containing kanamycin (25 mg/L). The selected primary strains were subjected to a second cross-over. The introduction of the mutation in the finally transformed strains was confirmed by performing PCR using the primer pair of SEQ ID NOS: 13 and 14, followed by nucleotide sequencing analysis. The strain names according to the inserted mutations are shown in Table 10 below.

[Table 10]

No.	Strain
1	<i>CJI0323::impE2(G64R)</i>
2	<i>CJI0323::impE2(G64H)</i>
3	<i>CJI0323::impE2(G64D)</i>
4	<i>CJI0323::impE2(G64K)</i>
5	<i>CJI0323::impE2(G64S)</i>
6	<i>CJI0323::impE2(G64T)</i>
7	<i>CJI0323::impE2(G64N)</i>
8	<i>CJI0323::impE2(G64Q)</i>
9	<i>CJI0323::impE2(G64C)</i>
10	<i>CJI0323::impE2(G64P)</i>
11	<i>CJI0323::impE2(G64A)</i>
12	<i>CJI0323::impE2(G64V)</i>
13	<i>CJI0323::impE2(G64I)</i>
14	<i>CJI0323::impE2(G64L)</i>
15	<i>CJI0323::impE2(G64M)</i>
16	<i>CJI0323::impE2(G64F)</i>
17	<i>CJI0323::impE2(G64Y)</i>
18	<i>CJI0323::impE2(G64W)</i>

Strains were cultured in the same manner as in Example 2 and the concentration of IMP therefrom was analyzed (Table 11).

[Table 11]

Concentration of IMP Production by <i>impE2</i> Mutation (g/L)	
Strain	Average IMP
<i>CJI0323_impE1E2(WT)</i>	2.32
<i>CJI0323_impE1(E164K)_impE2(V2I)</i>	4.24
<i>CJI0323::impE2(G64R)</i>	4.42
<i>CJI0323::impE2(G64H)</i>	5.14
<i>CJI0323::impE2(G64D)</i>	11.53
<i>CJI0323::impE2(G64K)</i>	8
<i>CJI0323::impE2(G64S)</i>	5.7
<i>CJI0323::impE2(G64T)</i>	5.52
<i>CJI0323::impE2(G64N)</i>	5.9
<i>CJI0323::impE2(G64Q)</i>	4.8
<i>CJI0323::impE2(G64C)</i>	5.9
<i>CJI0323::impE2(G64P)</i>	4.75
<i>CJI0323::impE2(G64A)</i>	4.58
<i>CJI0323::impE2(G64V)</i>	4.56
<i>CJI0323::impE2(G64I)</i>	5.89
<i>CJI0323::impE2(G64L)</i>	5.6
<i>CJI0323::impE2(G64M)</i>	4.3
<i>CJI0323::impE2(G64F)</i>	5.89
<i>CJI0323::impE2(G64Y)</i>	4.6
<i>CJI0323::impE2(G64W)</i>	4.76

As shown above, all of the modified strains showed an increase in the ability to produce IMP compared to the *CJI0323_impE1E2(WT)* strain, and thus, it was reconfirmed that the 64th amino acid mutation of the *impE2* is an important site that has a significant effect on the increase of the ability of the ImpE protein with respect to IMP export. In particular, in the case where the 64th amino acid (*i.e.*, glycine) is substituted with a different amino acid (*i.e.*, aspartate), the ability to export IMP was increased by 172% compared to that of the *CJI0323_impE1(E164K)_impE2(V2I)* strain, which has no mutation in the 64th amino acid. Additionally, it was confirmed that in the case where the 64th amino acid (*i.e.*, glycine) is

substituted with a different amino acid (*i.e.*, aspartate), the ability to produce IMP was improved by 397% compared to the *CJI0323_impE1E2(WT)* strain, which is the strain recovered to a wild-type strain, and by 20% compared to the *CJI0323* strain.

Example 6: Library of *impE* mutation using artificial mutagenesis

To obtain a protein variant having an improved ability to export IMP, a vector library for a first cross-over insertion within the chromosome was prepared by the following method.

In this regard, an attempt was made to perform error-prone PCR with respect to *impE2* of the *CJI0323::impE2(G64D)* strain, which was confirmed to have the greatest ability to export IMP by the results of Example 5-3. To introduce a mutation in the amino acid sequence possessed by the *CJI0323::G64D* strain at a position downstream of the 64th amino acid thereof, *impE* gene variants (1.6 kbp) where nucleotide substitutions are randomly introduced from the 193rd nucleotide of the *impE2* to about 130 bp downstream therefrom were obtained. Error-prone PCR was performed using the Diversify PCR Random Mutagenesis Kit (Clontech), and gene fragments were obtained by PCR using the genomic DNA of the *CJI0323::impE2(G64D)* strain as a template along with a primer pair of SEQ ID NO: 53 and SEQ ID NO: 54 (Table 12).

[Table 12]

SEQ ID NO	Primer	Sequence (5' to 3')
53	impE lib F	CAGATGATTTTCGGTTCCGCTC
54	impE lib R	GACCGAGACAAAAACGCCAAACG

Mutations were introduced to the amplified gene fragments in an amount of 0 to 3.5 mutations per 1 kb of each gene fragment. PCR was performed by initial denaturation at 94°C for 5 minutes; 30 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and polymerization at 72°C for 1 minute and 36 seconds; and final polymerization at 72°C for 5 minutes. As a result, a 1.6 kbp polynucleotide was obtained.

The amplified gene fragment was ligated to the *pCR2.1-TOPO* vector using the pCR2.1-TOPO TA cloning kit (Invitrogen), transformed into *E. coli DH5α*, and the transformants were plated on a solid LB medium containing kanamycin (25 mg/L). Twenty kinds of the transformed colonies were selected and plasmids were obtained therefrom.

Upon analysis of the polynucleotide sequences of these plasmids, it was confirmed that mutations were introduced at different positions at a frequency of 3.5 mutations/kb. About 20,000 transformed *E. coli* colonies were selected and their plasmids were extracted. The resulting library was named pTOPO_impE library.

Example 7: Selection of strains where *impE* library vectors are inserted

The pTOPO_impE library vectors prepared in Example 6 were transformed by electroporation into the *CJI0323::impE2(G64D)* strain capable of producing IMP at high concentration and the transformants were plated on a nutrient medium containing kanamycin (25 mg/L). As a result, 10,000 colonies of strains, in which modified genes were inserted, were obtained and these colonies were named *CJI0323::impE2(G64D)/pTOPO_impE(mt)1* to *CJI0323::impE2(G64D)/pTOPO_impE(mt)10000*.

- Nutrient medium: 1% peptone, 1% meat juice, 0.25% sodium chloride, 1% yeast extract, 2% agar, pH 7.2

- Seed medium: 1% glucose, 1% peptone, 1% meat juice, 1% yeast extract, 0.25% sodium chloride, adenine (100 mg/L), guanine (100 mg/L), pH 7.5

- Fermentation medium: 0.1% sodium glutamate, 1% ammonium chloride, 1.2% magnesium sulfate, 0.01% calcium chloride, iron sulfate (20 mg/L), manganese sulfate (20 mg/L), zinc sulfate (20 mg/L), copper sulfate (5 mg/L), L-cysteine (23 mg/L), alanine (24 mg/L), nicotinic acid (8 mg/L), biotin (45 µg/L), thiamine hydrochloride (5 mg/L), adenine (30 mg/L), 1.9% phosphoric acid (85%), 2.55% glucose, 1.45% fructose

Each of the obtained 10,000 colonies was inoculated with an autoclaved seed medium (200 µL), shake-cultured in a 96-deepwell plate of the Microplate shaker (TAITEC) at 1,200 rpm at 30°C for 24 hours, and used as a seed culture solution. An autoclaved fermentation medium (290 µL) was dispensed into a 96-deepwell plate and the seed culture solution (200 µL) was inoculated thereto, and the resultant was shake-cultured for 72 hours under the same conditions as described above.

To analyze the amount of IMP produced in the culture solution, upon completion of

culture, the supernatant of the culture solution (3 μ L) was transferred into a 96-well UV-plate, in which distilled water (197 μ L) had been dispensed into each well. Then, the resultant was shaken at 25°C for 30 seconds using the Microplate shaker (TAITEC) and the absorbance at 270 nm was measured using the spectrophotometer. Upon comparison of the above absorbance with that of the *CJI0323::impE2(G64D)* strain, 50 colonies of the strains showing an increase in the absorbance by 10% or higher were selected. Other colonies showed a similar or decreased absorbance compared to that of the control.

The amount of IMP produced in the selected 50 strains was repeatedly confirmed by measuring their absorbance using the same method, and as a result, the top four strains with improved ability to produce IMP compared to that of the *CJI0323::impE2(G64D)* strain were selected.

Example 8: Confirmation of ability to produce IMP of strains selected from *impE2* mutation library

To compare the ability of producing IMP of the four strains selected in Example 7, these four strains were cultured by the following method and the components of the resulting culture solutions were analyzed.

A seed medium (5 mL), which is the same as in Example 2, was dispensed into autoclaved test tubes (diameter: 18 mm) and shake-cultured at 30°C for 24 hours to be used as seed culture solutions. A fermentation medium (29 mL), which is the same as in Example 2, was dispensed into Erlenmeyer flasks (250 mL) for shaking and autoclaved at 121°C for 15 minutes. Then, the seed culture solutions (2 mL) were inoculated thereto and the resultants were cultured for 4 to 5 days. The culture conditions were set to 170 rpm, 30°C, and a pH of 7.5. Upon completion of the culture, the amount of IMP produced was measured by HPLC.

Among these fifty strains, the top four strains with respect to the ability to produce IMP were selected and the cultivation and analysis were performed repeatedly. The concentrations of IMP analyzed are shown in Table 13 below.

[Table 13]

Concentration of IMP produced by selected strains <i>CJI0323::impE2(G64D)/pTOPO_impE(mt)</i> (g/L)

Strain	Average IMP
<i>CJI0323::impE2(G64D)</i>	11.53
<i>CJI0323::impE2(G64D)/pTOPO_impE(mt)-627</i>	13.47
<i>CJI0323::impE2(G64D)/pTOPO_impE(mt)-3605</i>	12.96
<i>CJI0323::impE2(G64D)/pTOPO_impE(mt)-6765</i>	13.17
<i>CJI0323::impE2(G64D)/pTOPO_impE(mt)-9997</i>	12.70

As a result of the IMP concentration analysis, it was confirmed that the concentrations of the IMP of the four selected strains showed a maximum increase of 17% compared to that of the control strain, *CJI0323::impE2(G64D)*.

Example 9: Confirmation of *impE2* gene mutation in selected strains with *impE2* mutation

To confirm the mutations introduced to the *impE2* gene of the four strains selected in Example 8, the polynucleotide sequences of *impE2* mutations were analyzed. To determine these polynucleotide sequences, PCR was performed using a primer pair of SEQ ID NO: 13 and SEQ ID NO: 14.

Analysis was performed for each of the polynucleotide sequences of the modified *impE2* gene fragments obtained above. These polynucleotide sequences were compared to SEQ ID NO: 4 of *impE2* (WT) or SEQ ID NO: 100 of *impE2* (*CJHBI01::G64D*), and as a result, the amino acid sequences of the modified *impE2* were confirmed. The information on the mutations of the amino acid sequences of *impE2* in the selected strains is shown in Table 14 below.

[Table 14]

Amino acid mutations of <i>impE2</i> in selected four strains	
Strain	Amino acid mutations of <i>impE2</i>
<i>CJI0323::impE2(G64D)/pTOPO_impE(mt)-627</i>	<i>impE2</i> (S387T, M413T, N458K)
<i>CJI0323::impE2(G64D)/pTOPO_impE(mt)-3605</i>	<i>impE2</i> (F123C)
<i>CJI0323::impE2(G64D)/pTOPO_impE(mt)-6765</i>	<i>impE2</i> (I243V)
<i>CJI0323::impE2(G64D)/pTOPO_impE(mt)-9997</i>	<i>impE2</i> (F405Y)

Example 10: Preparation of vectors for insertion of chromosome with *impE2* mutations

To confirm the effects of application of *impE2* mutations, which were identified in Example 9, vectors capable of introducing these *impE2* mutations into the chromosome were prepared. The vector preparation process is as follows.

Only the vectors including the library mutations shown in Table 14, excluding the *impE2*(G64D) mutations, were prepared. Specifically, the chromosomal genes of *Corynebacterium stationis* ATCC6872 were isolated, and gene fragments were obtained by PCR using the primer pairs between SEQ ID NO: 56 and each of SEQ ID NOS: 57, 59, 61, and 63. PCR was performed by initial denaturation at 94°C for 5 minutes; 20 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and polymerization at 72°C for 1 minute; and final polymerization at 72°C for 5 minutes to obtain PCR fragments.

Gene fragments were obtained by PCR using each of the chromosomes of the four selected strains as a template along with primer pairs between SEQ ID NO: 55 and each of SEQ ID NOS: 58, 60, 62, and 64. PCR was performed by initial denaturation at 94°C for 5 minutes; 20 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and polymerization at 72°C for 1 minute and 30 seconds; and final polymerization at 72°C for 5 minutes to obtain PCR fragments. Overlapping PCR was performed using two fragments, and the obtained gene fragments were digested with a restriction enzyme (*Xba*I). The resulting gene fragments were ligated using T4 ligase to a linearized *pDZ* vector (Korean Patent No. 10-0924065 and International Patent Publication No. 2008-033001), which had already been digested with a restriction enzyme (*Xba*I), transformed into *E. coli* DH5 α , and the transformants were plated on a solid LB medium containing kanamycin (25 mg/L).

To prepare vectors with a single mutation for confirming the effects of the single mutations of the *impE2* (S387T, M413T, and N458K), in which three kinds of mutations among the selected variants are integrated, PCR was performed using the ATCC6872 strain as a template along with primer pairs between SEQ ID NO: 56 and each of SEQ ID NOS: 67, 69, and 71, and thereby gene fragments were obtained. Then, PCR was performed using the ATCC6872 strain as a template along with primer pairs between SEQ ID NO: 55 and each of

SEQ ID NOS: 68, 70, and 72, and thereby gene fragments were obtained. Overlapping PCR was performed using the two fragments prepared above, and the thus-obtained gene fragments were digested with a restriction enzyme (*Xba*I). The resulting gene fragments were ligated using T4 ligase to the linearized *pDZ* vector, which had already been digested with a restriction enzyme (*Xba*I), transformed into *E. coli DH5 α* , and the transformants were plated on a solid LB medium containing kanamycin.

[Table 15]

SEQ ID NO	Primer	Sequence (5' to 3')
55	impE mt - R	CATCTAGACCGAGACAAAAACGCCAAACG
56	impEW mt - 1	GCTCTAGACCGCGGATAACGTCGGCATT
57	impEW ttk - 2	CATCCACCACAAAGCAAACGC
58	impEW ttk - 3	CTTTGTGGTGGATGACCCAGATGACCGTTGAGACTT
59	impEW C - 2	AAATGGAGATACCTGAGATGT
60	impEW C - 3	CAGGTATCTCCATTTGCGTTATTGGCTCGACGCTCG
61	impEW v - 2	GGCCGCAAAACCCATCCAGTC
62	impEW v - 3	ATGGGTTTTGCGGCCGTCGCAATCACGACCAGCACC
63	impEW y - 2	ATACAAGGAAGCGAACTCCGA
64	impEW Y - 3	TTCGCTTCCTTGTATACGGTGTCGGCTTGGGCTTTG

After selecting the colonies transformed with the vector into which the target gene was inserted, the plasmids were obtained using a conventionally known plasmid extraction method. The plasmids were named *pDZ-impE2(S387T, M413T, N458K)*, *pDZ-impE2(F123C)*, *pDZ-impE2(I243V)*, *pDZ-impE2(F405Y)*, *pDZ-impE2(S387T)*, *pDZ-impE2(M413T)*, and *pDZ-impE2(N458K)*, according to the mutation inserted into *impE2* of each plasmid.

Example 11: Preparation of strains introduced with *impE2* mutation based on wild-type *impE1*, *impE2* and comparison of their abilities to produce IMP

The four kinds of vectors prepared in Example 10 for introducing novel mutations (*i.e.*, *pDZ-impE2(S387T, M413T, N458K)*, *pDZ-impE2(F123C)*, *pDZ-impE2(I243V)*, and *pDZ-*

impE2(F405Y)) were transformed by a two-step recombination of homologous chromosomes into the *CJI0323_impE1E2(WT)* strain, in which the *impE1E2* of *Corynebacterium stationis* *CJI0323* (an IMP-producing strain prepared in Example 4) was recovered to *WT*. Then, strains in which the *impE2* mutations were introduced on the chromosome were selected by sequence analysis of the polynucleotides, and the strains were named *CJI0323_impE1E2(WT)_impE2(S387T, M413T, N458K)*, *CJI0323_impE1E2(WT)_impE2(F123C)*, *CJI0323_impE1E2(WT)_impE2(I243V)*, and *CJI0323_impE1E2(WT)_impE2(F405Y)*, respectively.

The *Corynebacterium stationis* *CJI032_impE1E2(WT)_impE2(F123C)*, *Corynebacterium stationis* *CJI0323_impE1E2(WT)_impE2(I243V)*, and *Corynebacterium stationis* *CJI0323_impE1E2(WT)_impE2(F405Y)* strains were deposited on November 2, 2018, to the Korean Culture Center of Microorganisms (KCCM), an international depositary authority under the Budapest Treaty, and assigned Accession Numbers KCCM12362P, KCCM12363P, and KCCM12365P, respectively.

The strains were cultured in the same manner as in Example 7 and their IMP concentrations were analyzed. After 48 hours of culture, the concentrations were measured (Table 16).

[Table 16]

Concentration of IMP produced by strains introduced with <i>impE2</i> mutations (g/L)	
Strain	Average IMP
<i>CJI0323_impE1E2(WT)</i>	2.32
<i>CJI0323_impE1E2(WT)_impE2(S387T, M413T, N458K)</i>	3.35
<i>CJI0323_impE1E2(WT)_impE2(F123C)</i>	2.62
<i>CJI0323_impE1E2(WT)_impE2(I243V)</i>	2.74
<i>CJI0323_impE1E2(WT)_impE2(F405Y)</i>	2.90

With respect to IMP concentration, it was confirmed that the four novel modified strains showed a maximum increase of 44% compared to that of the *CJI0323_impE1E2(WT)* strain. The increase in the amount of IMP production due to mutations of the ImpE protein of the present disclosure can be interpreted to be very meaningful.

Example 12: Preparation of strains introduced with *impE2* mutation based on

CJI0323::*impE2*(G64D) and comparison of abilities to produce IMP

The four kinds of vectors prepared in Example 10 for introducing novel mutations (*i.e.*, *pDZ-impE2*(S387T, M413T, N458K), *pDZ-impE2*(F123C), *pDZ-impE*(I243V), and *pDZ-impE2*(F405Y)) were transformed into the *CJI0323_impE2*(G64D) strain (*i.e.*, an IMP-producing strain) by a two-step recombination of homologous chromosomes. Then, strains in which the *impE2* mutations were introduced on the chromosome were selected by sequence analysis of the polynucleotides, and the strains were named *CJI0323::*impE2*(G64D)_impE2*(S387T, M413T, N458K), *CJI0323::*impE2*(G64D)_impE2*(F123C), *CJI0323::*impE2*(G64D)_impEp*(I243V), and *CJI0323::*impE2*(G64D)_impE2p*(F405Y), respectively, according to the inserted *impE2* mutation.

The strains were cultured in the same manner as in Example 7 and their IMP concentrations were analyzed (Table 17).

[Table 17]

Concentration of IMP produced by strains introduced with <i>impE2</i> mutations (g/L)	
Strain	Average IMP
<i>CJI0323::<i>impE2</i>(G64D)</i>	11.53
<i>CJI0323::<i>impE2</i>(G64D)_impE2</i> (S387T, M413T, N458K)	13.47
<i>CJI0323::<i>impE2</i>(G64D)_impE2</i> (F123C)	12.90
<i>CJI0323::<i>impE2</i>(G64D)_impE2</i> (I243V)	13.17
<i>CJI0323::<i>impE2</i>(G64D)_impE2</i> (F405Y)	12.70

With respect to IMP concentration, it was confirmed that the four novel modified strains showed a maximum increase of 17% compared to that of the *CJI0323::*impE2*(G64D)* strain. The increase in the amount of IMP production due to mutations of the ImpE protein of the present disclosure can be interpreted to be very meaningful.

Then, the seven kinds of vectors prepared above (*i.e.*, *pDZ-impE2*(S387T, M413T, N458K), *pDZ-impE2*(F123C), *pDZ-impE*(I243V), *pDZ-impE2*(F405Y), *pDZ-impE2*(S387T), *pDZ-impE2*(M413T), and *pDZ-impE2*(N458K)), alone or in combination, were transformed into the *CJI0323_impE1E2*(WT) strain or *CJI0323::*impE2*(G64D)* strain. The prepared

strains were named *CJI0323_impE1E2(WT)_impE2(S387T)*, *CJI0323_impE1E2(WT)_impE2(M413T)*, *CJI0323_impE1E2(WT)_impE2(N458K)*, *CJI0323_impE1E2(WT)_impE2(F123C, I243V, S387T, F405Y, M413T, N458K)*, *CJI0323::impE2(G64D)_impE2(S387T)*, *CJI0323::impE2(G64D)_impE2(M413T)*, *CJI0323::impE2(G64D)_impE2(N458K)*, *CJI0323::impE2(G64D)_impE2(I243V, S387T, M413T, N458K)*, *CJI0323::impE2(G64D)_impE2(S387T, F405Y, M413T, N458K)*, *CJI0323::impE2(G64D)_impE2(I243V, S387T, F405Y, M413T, N458K)*, *CJI0323::impE2(G64D)_impE2(F123C, S387T, M413T, N458K)*, and *CJI0323::impE2(G64D)_impE2(F123C, I243V, S387T, F405Y, M413T, N458K)*, and their abilities to produce IMP were measured in the same manner as described above (Table 18).

The *Corynebacterium stationis* *CJI0323_impE1E2(WT)_impE2(S387T)*, *Corynebacterium stationis* *CJI0323_impE1E2(WT)_impE2(M413T)*, and *CJI0323_impE1E2(WT)_impE2(N458K)* strains were deposited on November 2, 2018, to the Korean Culture Center of Microorganisms (KCCM), an international depositary authority under the Budapest Treaty, and assigned Accession Numbers KCCM12364P, KCCM12366P, and KCCM12367P, respectively.

[Table 18]

Concentration of IMP produced by strains with a single <i>impE2</i> mutation and strains introduced with combined <i>impE2</i> mutations (g/L)	
Strain	Average IMP
<i>CJI0323_impE1E2(WT)</i>	2.32
<i>CJI0323::impE2(G64D)</i>	11.52
<i>CJI0323_impE1E2(WT)_impE2(S387T)</i>	2.7
<i>CJI0323_impE1E2(WT)_impE2(M413T)</i>	3.1
<i>CJI0323_impE1E2(WT)_impE2(N458K)</i>	3.0
<i>CJI0323_impE1E2(WT)_impE2(F123C, I243V, S387T, F405Y, M413T, N458K)</i>	4.7
<i>CJI0323::impE2(G64D)_impE2(S387T)</i>	12.94
<i>CJI0323::impE2(G64D)_impE2(M413T)</i>	13.0
<i>CJI0323::impE2(G64D)_impE2(N458K)</i>	13.1
<i>CJI0323::impE2(G64D)_impE2(I243V, S387T, M413T, N458K)</i>	13.6
<i>CJI0323::impE2(G64D)_impE2(S387T, F405Y, M413T, N458K)</i>	13.7

<i>CJI0323::impE2(G64D)_impE2(F123C, S387T, M413T, N458K)</i>	13.82
<i>CJI0323::impE2(G64D)_impE2(I243V, S387T, F405Y, M413T, N458K),</i>	14.0
<i>CJI0323::impE2(G64D)_impE2(F123C, I243V, S387T, F405Y, M413T, N458K)</i>	14.27

As shown in the above tables, it was confirmed that single mutations (*i.e.*, *impE2(S387T)*, *impE2(M413T)*, and *impE2(N458K)*) showed a maximum increase of 33.6% compared to that of the wild-type strain, and all of the strains with a combination of novel mutations showed a maximum increase of 102.5%. Additionally, when a novel mutation alone was introduced into the *CJI0323::impE2(G64D)* strain, the ability to produce IMP was increased as shown in Tables 17 and 18, whereas when a combination of mutations was introduced into the strain, the strain was shown to have a more improved ability to produce IMP. In particular, when both a mutation of the *CJI0323::impE2(G64D)* strain and a novel mutation(s) are integrated into the strain, the ability to produce IMP was increased about 515% compared to that of the wild-type strain, while showing an about 24% increase compared to that of the *CJI0323::impE2(G64D)* strain. It was also confirmed that the novel mutations discovered in the present disclosure were shown to increase the ability to produce IMP even by a single mutation, and when these mutations were introduced in combination, the ability to produce IMP was increased even further.

Example 13: Enhancement of *impE2* based on IMP-producing strains

Example 13-1: Preparation of strains introduced with *impE2* mutation based on IMP-producing strain

To confirm the effects of introducing an *impE2* mutation into strain, An IMP-producing strain was prepared in which the activities of adenylosuccinate synthetase and IMP dehydrogenase corresponding to the degradation pathway of IMP in the *ATCC6872* strain were attenuated. The initiation codon was changed by changing the first base from ‘a’ to ‘t’ in each nucleotide sequence of the two genes *purA* and *guaB*, which encode the two enzymes. The strain in which the expression of the two genes was attenuated in the *ATCC6872* strain was named *CJI9088*. The *pDZ-impE2(S387T, M413T, N458K)*, *pDZ-impE2(F123C)*, *pDZ-impE2(I243V)*, and *pDZ-impE2(F405Y)* vectors prepared in Example 10 were transformed into

the *CJI9088* strain, either alone or in combination, by electroporation. Then, the strains in which the vectors were inserted into the chromosome by recombination of the homologous sequences were selected on a medium containing kanamycin (25 mg/L). The selected primary strains were subjected to a second cross-over, and the strains in which the modification of a target gene was introduced were selected. The introduction of the modification in the finally transformed strains was confirmed by performing PCR using the primer pair of SEQ ID NOS: 13 and 14, followed by nucleotide sequencing analysis.

The ability of the prepared strains (*i.e.*, *CJI9088*, *CJI9088_impE2(S387T, M413T, N458K)*, *CJI9088_impE2(F123C)*, *CJI9088_impE2(I243V)*, *CJI9088_impE2(F405Y)*, and *CJI9088_impE2(F123C, I243V, S387T, F405Y, M413T, N458K)* to produce IMP was evaluated. Upon completion of the culture, the amount of IMP production was measured by HPLC and the results are shown in Table 19 below.

[Table 19]

Strain	IMP (g/L)
<i>CJI9088</i>	0.52
<i>CJI9088_impE2(S387T, M413T, N458K)</i>	3.75
<i>CJI9088_impE2(F123C)</i>	0.94
<i>CJI9088_impE2(I243V)</i>	1.07
<i>CJI9088_impE2(F405Y)</i>	1.21
<i>CJI9088_impE2(F123C, I243V, S387T, F405Y, M413T, N458K)</i>	4.32

Upon confirming the amount of IMP accumulated in the culture medium, it was confirmed that these strains showed an increase of IMP production by at least 80%, and a maximum increase of 730%, compared to the parent strain, *CJI9088*. Accordingly, the increase in the amount of IMP production due to mutations of the ImpE protein of the present disclosure can be interpreted to be very meaningful.

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, mutations, 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
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330, DONGHO-RO,
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I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: <i>Corynebacterium stationis</i> CN01-0323	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM12151P
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
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Name : Korean Culture Center of Microorganisms Address : Yurim B/D 45, Hongjenae-2ga-gil Seodaemun-gu SEOUL 03641 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: November. 02. 2018

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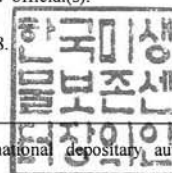
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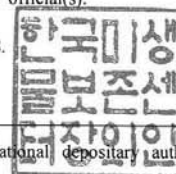
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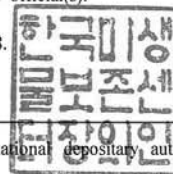
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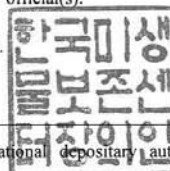
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— KOREAN CULTURE CENTER OF MICROORGANISMS KOREAN FEDERATION OF CULTURE COLLECTIONS

Yoolim Bldg., 45, Hongjenae 2ga-gil, Seodaemun-gu, Seoul, 03641, Korea Tel: 82-2-391-0950, 396-0950 Fax: 82-2-392-2859



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

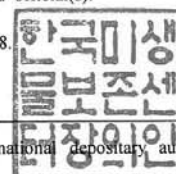
INTERNATIONAL FORM

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 DEPOSIT
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 stationis</i> CJI0323_impE1E2(WT)_impE2(N458K)	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM12367P
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. 02. 2018 (date of the original deposit). ¹	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : Yurim B/D 45, Hongjeonae-2ga-gil Seodaemun-gu SEOUL 03641 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: November. 02. 2018.

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.



Form BP/4 (sole page)

— 한국미생물보존센터 사단법인 한국문화재단
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[CLAIMS]

[Claim 1]

A protein variant exporting 5'-inosine monophosphate, wherein the protein variant comprises a substitution of the 123rd amino acid with cysteine, a substitution of the 243rd amino acid with valine, a substitution of the 387th amino acid with threonine, a substitution of the 405th amino acid with tyrosine, a substitution of the 413th amino acid with threonine, a substitution of the 458th amino acid with lysine, or a combination thereof, in the amino acid sequence of SEQ ID NO: 2.

[Claim 2]

The protein variant according to claim 1, wherein the protein variant further comprises a substitution of the 2nd amino acid with isoleucine, a substitution of the 64th amino acid with glutamic acid or aspartate, or a combination thereof.

[Claim 3]

A polynucleotide encoding the protein variant according to claim 1 or 2.

[Claim 4]

A vector comprising the polynucleotide according to claim 3.

[Claim 5]

A microorganism of the genus *Corynebacterium* producing 5'-inosine monophosphate, which comprises the protein variant of claim 1 or 2; or a vector comprising the polynucleotide encoding the protein variant of claim 1 or 2.

[Claim 6]

The microorganism of the genus *Corynebacterium* according to claim 5, wherein the microorganism of the genus *Corynebacterium* is *Corynebacterium stationis*.

[Claim 7]

A method for preparing 5'-inosine monophosphate, comprising culturing the microorganism of the genus *Corynebacterium* of claim 5 in a medium; and recovering 5'-inosine monophosphate from the microorganism or medium.

[Claim 8]

The method according to claim 7, wherein the microorganism of the genus *Corynebacterium* is *Corynebacterium stationis*.