

[DESCRIPTION]**[Invention Title]**

NOVEL GLUTAMINE-HYDROLYZING GMP SYNTHASE VARIANT AND METHOD FOR PRODUCING PURINE NUCLEOTIDE BY USING SAME

[Technical Field]

The present disclosure relates to a glutamine-hydrolyzing GMP synthase variant and a method for producing a purine nucleotide by using the same.

[Background Art]

Purine nucleotides, which are a type of nucleic acid-based materials, are widely used for food seasoning additives or food and are receiving attention as a flavor-enhancing nucleic acid-based seasonings.

For the production of purine nucleotides, various studies have been conducted to develop microorganisms with high-efficiency production and fermentation process technology. For example, target substance-specific approaches are mainly used, such as increasing the expression of genes encoding enzymes involved in biosynthesis of purine nucleotides or removing unnecessary genes in the biosynthesis (EP 3722430 A1 and US 2020-0347346 A1).

As the demand for purine nucleotides increases, studies for effective purine nucleotide production are still needed.

[Disclosure]**[Technical Problem]**

The present disclosure provides a glutamine-hydrolyzing GMP synthase variant.

[Technical Solution]

An aspect of the present disclosure is to provide a glutamine-hydrolyzing GMP synthase variant.

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

Still another aspect of the present disclosure is to provide a microorganism containing the variant or the polynucleotide encoding the same.

Still another aspect of the disclosure is to provide a method for producing a purine nucleotide, the method including culturing the microorganism.

[Advantageous Effects]

The variants of the present disclosure can be used to produce purine nucleotides with high efficiency.

[Detailed Description of the Invention]

The present disclosure will be specifically described as follows. Each description and embodiment disclosed in this disclosure may also be applied to other descriptions and embodiments. That is, all combinations of various elements disclosed in this disclosure fall within the scope of the present disclosure. Furthermore, the scope of the present disclosure is not limited by the specific description below. Furthermore, throughout the entire specification, many papers and patent documents are referenced, and their citations are provided. The disclosures of cited papers and patent documents are incorporated into the present specification in their entirety by reference, and the level of the technical field within which the present disclosure falls and details of the present disclosure are explained more clearly.

An aspect of the present disclosure provides a glutamine-hydrolyzing GMP synthase variant in which the amino acid corresponding to position 29 in SEQ ID NO: 3 is substituted with another amino acid.

The glutamine-hydrolyzing GMP synthase variant refers to a variant including a substitution of the amino acid corresponding to position 29 from the N-terminus of SEQ ID NO: 3 with another amino acid, in a polypeptide having glutamine-hydrolyzing GMP synthase activity or a glutamine-hydrolyzing GMP synthase.

In the present disclosure, "glutamine-hydrolyzing GMP synthase" is an enzyme involved in the conversion of 5'-xanthosine monophosphate (XMP) into 5'-guanosine monophosphate (hereinafter, GMP).

The glutamine-hydrolyzing GMP synthase of the present disclosure may be glutamine-hydrolyzing GMP synthase or a polypeptide having glutamine-hydrolyzing GMP synthase activity, which is to be modified to prepare a glutamine-hydrolyzing GMP synthase variant provided in the present disclosure.

Specifically, the polypeptide may be a naturally occurring polypeptide or a wild-type polypeptide, or a mature polypeptide thereof, and may include a variant or functional fragment thereof, but any polypeptide is included without limitation as long as it can be a parent of the glutamine-hydrolyzing GMP synthase variant of the present disclosure.

In an embodiment, the glutamine-hydrolyzing GMP synthase of the present disclosure may be derived from the genus *Corynebacterium*, and more specifically *Corynebacterium stationis*, but is not limited thereto.

In an embodiment, the glutamine-hydrolyzing GMP synthase of the present disclosure may be the polypeptide of SEQ ID NO: 3. In an embodiment, the glutamine-hydrolyzing GMP synthase of the present disclosure may be a polypeptide having at least about 60%, 70%, 75%, 80%, 85%, 90%, 93%, 95%, 96%, 97%, 98%, or 99% sequence identity with the polypeptide of SEQ ID NO: 3, and any polypeptide that has activity identical or corresponding to that of a polypeptide consisting of the amino acid sequence of SEQ ID NO: 3 is included, without limitation, within a range of glutamine-hydrolyzing GMP synthase.

Specifically, the glutamine-hydrolyzing GMP synthase may have, contain, or consist essentially of the amino acid sequence set forth in SEQ ID NO: 3, but is not limited thereto.

The glutamine-hydrolyzing GMP synthase to be mutated in the present disclosure may be *Corynebacterium* sp.-derived glutamine-hydrolyzing GMP synthase, and specifically, a polypeptide/protein containing the amino acid sequence set forth in SEQ ID NO: 3 or any polypeptide/protein having the same activity as the synthase may be included without limitation. For example, the polypeptide/protein may contain the amino acid sequence of SEQ ID NO: 3 or an amino acid sequence having at least 80% homology or identity therewith, but is not limited thereto. Specifically, the polypeptide/protein may contain an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% homology or identity with SEQ ID NO: 3. A polypeptide/protein having deletion, modification, substitution, or addition in a part of the sequence thereof is obviously included within a range of the polypeptide/protein to be mutated in the present disclosure as long as it is a polypeptide/protein having such homology or identity and exhibiting glutamine-hydrolyzing GMP synthase activity.

The sequence of the glutamine-hydrolyzing GMP synthase of the present disclosure may be available in the NCBI GenBank, a known database. For example, the glutamine-hydrolyzing GMP synthase of the present disclosure may be the polypeptide encoded by *guaA* gene. For example, the glutamine-hydrolyzing GMP synthase of the present disclosure may be derived from *Corynebacterium stationis*, *Corynebacterium casei*, or *Corynebacterium ammoniagenes*, and examples thereof may be an enzyme expressed by NCBI Reference sequence WP_194285183.1, WP_006823296.1, or WP_040355890.1, but is not limited thereto.

As used herein, the term “homology” or “identity” refers to the degree of similarity between two given amino acid sequences or nucleotide sequences and

may be expressed as a percentage. The terms homology and identity may be often used interchangeably.

The sequence homology or identity of conserved polynucleotides or polypeptides may be determined by a standard alignment algorithm, and default gap penalties established by a program to be used may be used together. Substantially, homologous or identical sequences may generally hybridize with each other as a whole or in part under moderate or highly stringent conditions. It is obvious that the hybridization also includes hybridization with a polynucleotide containing usual codons or codons considering codon degeneracy in the polynucleotide.

The homology, similarity, or identity between any two polynucleotide or polypeptide sequences may be determined using a known computer algorithm, such as the “FASTA” program, by using default parameters as in Pearson *et al.*, (1988) *Proc. Natl. Acad. Sci. USA* 85:2444. Alternatively, the homology, similarity, or identity 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 European Molecular Biology Open Software Suite (EMBOSS) package (including Rice *et al.*, 2000, *Trends Genet.* 16:276–277) (version 5.0.0 or later) (GCG program package (Devereux, J. *et al.*, *Nucleic Acids Research* 12:387 (1984)), BLASTP, BLASTN, FASTA (Atschul, S. F. *et al.*, *J MOLEC BIOL* 215:403 (1990); *Guide to Huge Computers*, Martin J. Bishop, ed., Academic Press, San Diego, 1994, and CARILLO *et al.* (1988) *SIAM J Applied Math* 48:1073). For example, the homology, similarity, or identity may be determined using BLAST or ClustalW from the National Center for Biotechnology Information database.

The homology, similarity, or identity between polynucleotides or polypeptides may be determined by comparing sequence information using the GAP computer program as introduced by, for example, Needleman *et al.* (1970), *J*

Mol Biol. 48:443 as disclosed by Smith and Waterman, *Adv. Appl. Math* (1981) 2:482. Briefly, the GAP program defines the homology, similarity, 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. The default parameters for the GAP program may include: (1) a binary comparison matrix (containing a value 1 for identity and a value 0 for non-identity) 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) (or EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap (or a gap open penalty of 10 and a gap extension penalty of 0.5); and (3) no penalty for end gaps.

As used herein, the term “variant” refers to a polypeptide which has a different sequence but maintain functions or properties compared with the amino acid sequence prior to mutation by conservative substitution and/or modification of at least one amino acid. Such a variant can be usually identified by modifying one or more amino acids in the amino acid sequence of the polypeptide and evaluating the properties of the modified polypeptide. That is, the ability of the variant may be increased, unchanged, or reduced compared with that of a polypeptide prior to mutation. Some variants may include a variant in which at least one part, such as an N-terminal leader sequence or a transmembrane domain, is removed. Other variants may include a variant in which a part of the N-terminus and/or C-terminus of a mature protein is removed. The term “variant” may also be used interchangeably with “modification”, “modified protein”, “modified polypeptide”, “mutant”, “mutein”, “divergent”, or the like, and any term that is used in a sense of being mutated can be used without limitation thereto.

In addition, the variant may include deletions or additions of amino acids

that have a minimal effect on the properties and secondary structures of a polypeptide. For example, a signal (or leader) sequence of the N-terminus of a protein which co-translationally or post-translationally directs transfer of the protein may be conjugated to the N-terminus of the variant. In addition, the variant may also be conjugated to another sequence or a linker so that the variant is identified, purified, or synthesized.

The glutamine-hydrolyzing GMP synthase variant provided in the present disclosure may be a polypeptide containing the amino acid sequence in which the amino acid corresponding to position 29 in SEQ ID NO: 3 is substituted with another amino acid. Specifically, the other amino acid may be selected from glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan, proline, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, and histidine.

The “another amino acid” is not limited as long as the amino acid differs from the amino acid prior to substitution. The expression “substituted with a particular amino acid” in the present disclosure is obvious as being substituted with an amino acid different from the amino acid prior to substitution even though “substituted with another amino acid” is not specifically stated.

In an embodiment, the variant of the present disclosure may be a variant in which the amino acid corresponding to position 29 in the amino acid sequence of SEQ ID NO: 3 as a reference protein is an amino acid except for arginine. Specifically, the amino acid corresponding to position 29 in SEQ ID NO: 3 of the variant may be selected from glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan, proline, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, and histidine.

In an embodiment, the glutamine-hydrolyzing GMP synthase variant of the present disclosure may contain an amino acid sequence in which the amino acid

corresponding to position 29 in the amino acid sequence set forth in SEQ ID NO: 3 is an amino acid except for arginine and which has at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.7%, or 99.8% homology or identity with the amino acid sequence set forth in SEQ ID NO: 3.

For example, the glutamine-hydrolyzing GMP synthase variant of the present disclosure may consist of the amino acid sequence of SEQ ID NO: 1 or may contain an amino acid sequence having at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.7%, or 99.8% identity therewith, but is not limited thereto.

It is obvious that even a variant having an amino acid sequence with deletion, modification, substitution, conservative substitution, or addition in a part thereof may also be included within the scope of the present disclosure as long as the amino acid sequence has such homology or identity and exhibits activity corresponding to the variant of the present disclosure.

For example, there may be sequence addition or deletion, naturally occurring mutation, silent mutation, or conservative substitution, which does not alter functions of the variant of the present disclosure, in the N-terminus, C-terminus, and/or inside of the amino acid sequence.

The “conservative substitution” refers to a substitution of one amino acid with another amino acid having similar structural and/or chemical properties thereto. Such an amino acid substitution may generally occur on the basis of similarities in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or amphipathic nature of residues. Usually, the conservative substitution may have little or no effect on the activity of a protein or a polypeptide.

As used herein, the term “corresponding to” refers to an amino acid residue at a position recited in a polypeptide, or an amino acid residue similar, identical, or homologous to a residue recited in a polypeptide. Identifying an amino acid at a corresponding position may be determining a particular amino acid of a sequence

referring to a particular sequence. As used herein, the term “corresponding region” generally refers to a similar or corresponding site in a related protein or reference protein.

For example, any amino acid sequence is aligned with SEQ ID NO: 3, and based on this, each amino acid residue of the amino acid sequence may be numbered with reference to an amino acid residue numerical position corresponding to an amino acid residue of SEQ ID NO: 3. For example, a sequence alignment algorithm as described in the present disclosure may identify the position of an amino acid or a position of occurrence of modifications, such as substitution, insertion, or deletion, compared with a query sequence (also referred to as a “reference sequence”).

For such alignment, Needleman–Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48:443–453) or Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16:276–277) may be for example used, but without limitation thereto, a sequence alignment program, a pairwise sequence comparison algorithm, or the like, which are known in the art, may be used as appropriate.

Another aspect of the present disclosure provides a polynucleotide encoding the variant of the present disclosure.

As used herein, the term “polynucleotide” refers to a DNA or RNA strand having more than a certain length as a nucleotide polymer which is a long chain of nucleotide monomers linked by covalent bonds.

For example, the polynucleotide encoding the glutamine-hydrolyzing GMP synthase of the present disclosure may have or contain a sequence encoding the nucleotide sequence of SEQ ID NO: 4. For example, the polynucleotide encoding the glutamine-hydrolyzing GMP synthase may consist or consist essentially of the sequence of SEQ ID NO: 4.

The polynucleotide of the present disclosure may be variously modified in a coding region thereof within a range in which the amino acid sequence of the variant of the present disclosure is not changed, considering codon degeneracy, or codons preferred by an organism in which the variant of the present disclosure is to be expressed.

Specifically, the polynucleotide encoding the glutamine-hydrolyzing GMP synthase variant of the present disclosure may have or contain a nucleotide sequence having at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and less than 100% homology or identity with the sequence of SEQ ID NO: 4, or may consist or consist essentially of a nucleotide sequence having at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, and less than 100% homology or identity with the sequence of SEQ ID NO: 4, but is not limited thereto. In the sequences having such homology or identity, a codon encoding the amino acid corresponding to position 29 in SEQ ID NO: 3 may be one of the codons encoding amino acids except for arginine.

In addition, the polynucleotide of the present disclosure may include, without limitation, a sequence that can hybridize, under stringent conditions, with a probe capable of being prepared from a known gene sequence, for example, a sequence complementary to a part or the entirety of the polynucleotide sequence of the present disclosure. The term "stringent condition" refers to a condition that enables specific hybridization between polynucleotides. These conditions are described in detail in the literature (see J. Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory press, Cold Spring Harbor, New York, 1989; and F. M. Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., New York, 9.50–9.51, 11.7–11.8). For example, such conditions may include conditions under which polynucleotides having high homology or identity, such as polynucleotides having at least 70%, at

least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% homology or identity therebetween, hybridize with each other, but polynucleotides having lower homology or identity do not hybridize with each other; or washing conditions for typical Southern hybridization, in which washing is conducted at a salt concentration and a temperature, corresponding to 60°C, 1×SSC, 0.1% SDS; specifically 60°C, 0.1×SSC, 0.1% SDS; and more specifically 68°C, 0.1×SSC, 0.1% SDS, once, specifically twice or three times.

Hybridization requires that two nucleic acids have complementary sequences, although mismatches between bases may be possible depending on hybridization stringency. The term “complementary” is used to describe the relationship between nucleotide bases that can hybridize with each another. For example, as for DNA, adenine is complementary to thymine and cytosine is complementary to guanine. Therefore, the polynucleotide of the present disclosure may also include isolated nucleic acid fragments complementary to the entire sequence as well as to substantially similar nucleic acid sequences.

Specifically, a polynucleotide having homology or identity with the polynucleotide of the present disclosure may be detected using hybridization conditions including a step of hybridization at T_m of 55°C and utilizing the above-described conditions. In addition, the T_m value may be 60°C, 63°C, or 65°C, but is not limited thereto, and may be appropriately controlled by a person skilled in the art according to the purpose.

The appropriate stringency for hybridizing polynucleotides depends on the length of the polynucleotides and the degree of complementarity, and variables thereof are well known in the art (*e.g.*, Sambrook *et al.*, *supra*).

Still another aspect of the present disclosure provides a vector including the polynucleotide encoding the variant of the present disclosure. The vector may be an expression vector for expressing the polynucleotide in a host cell, but

is not limited thereto.

The “vector” of the present disclosure may include a DNA construct containing the nucleotide sequence of a polynucleotide, encoding a target polypeptide, operatively linked to a suitable expression control region (or expression control sequence) so as to express the target polypeptide in an appropriate host. The expression control region may include a promoter capable of initiating transcription, any operator sequence for controlling such transcription, a sequence for encoding a suitable mRNA ribosomal binding site, and a sequence for controlling the termination of transcription and translation. The vector, after transformation into an appropriate host cell, can replicate or function independently of the genome of the host, or may be integrated into the genome itself.

The vector used in the present disclosure is not particularly limited, and any vector known in the art may be used. Examples of the vector that is usually used may include native or recombinant plasmids, cosmids, viruses, and bacteriophages. For example, pWE15, M13, MBL3, MBL4, IXII, ASHII, APII, t10, t11, Charon4A, and Charon21A may be used as phage vectors or cosmid vectors, and pBR-based, pUC-based, pBluescriptII-based, pGEM-based, pTZ-based, pCL-based, and pET-based vectors may be used as plasmid vectors. Specifically, pDZ, pDC, pDCM2, pACYC177, pACYC184, pCL, pECCG117, pUC19, pBR322, pMW118, and pCC1BAC vectors may be used.

For example, a polynucleotide encoding a target polypeptide may be inserted in the chromosome through a vector for chromosomal insertion in a cell. The insertion of the polynucleotide into the chromosome may be performed using any method known in the art, for example, homologous recombination, but is not limited thereto. The vector may further include a selection maker for investigating the insertion or non-insertion of the chromosome. The selection marker is for selecting cells transformed with the vector, that is, identifying the insertion of a target nucleic acid molecule, and markers imparting a selectable

phenotype, such as drug resistance, auxotrophy, cytotoxic drug resistance, or expression of surface protein may be used. Under the circumstances of the treatment with a selective agent, only the cells expressing selection markers can survive or express other phenotypic traits, so that the transformed cells can be selected.

As used herein, the term “transformation” means that a vector containing a polynucleotide encoding a target polypeptide is introduced into a host cell or microorganism to allow the polypeptide encoded by the polynucleotide to be expressed in the host cell. Examples of the transformed polynucleotide may include any polypeptide as long as it can be expressed in a host cell, regardless of whether it is inserted and located into the chromosome of the host cell or located outside of the chromosome. In addition, examples of the polynucleotide include DNA and/or RNA encoding a target protein. The polynucleotide may be introduced in any form as long as the polynucleotide can be introduced and expressed in a host cell. For example, the polynucleotide may be introduced in the form of an expression cassette, which is a gene construct containing all factors required for self-expression. The expression cassette may generally include a promoter operatively linked to the polynucleotide, a transcription termination signal, a ribosome binding site, and a translation terminal signal. The expression cassette may be an expression vector enabling self-replication. In addition, the polynucleotide may be introduced in the form as it is into the host cell to be operatively linked to the sequence required for expression in the host cell, but is not limited thereto.

As above, the term “operatively linked” refers to a functional linkage between a promoter sequence for initiating and mediating the transcription of the polynucleotide encoding the variant of the present disclosure and the polynucleotide sequence.

Still another aspect of the present disclosure provides a microorganism

including the variant of the present disclosure or the polynucleotide encoding the variant.

The microorganism of the present disclosure may include the variant of the present disclosure, a polynucleotide encoding the variant, or a vector including the polynucleotide.

As used herein, the term “microorganism (or strain)” encompasses all of wild-type microorganisms or naturally or artificially genetically modified microorganisms, refers to a microorganism in which a particular mechanism is diminished or enhanced due to the insertion of an exogenous gene or the enhancement or inactivation of activity of an endogenous gene, and may be a microorganism including a genetic modification for production of a target polypeptide, protein, or product.

The microorganism of the present disclosure may be: a microorganism including at least one of the variant of the present disclosure, a polynucleotide encoding the variant of the present disclosure, and a vector including the polynucleotide encoding the variant of the present disclosure; a microorganism modified to express the variant of the present disclosure or the polynucleotide encoding the variant of the present disclosure; a microorganism (e.g., recombinant strain) expressing the variant of the present disclosure or a polynucleotide encoding the variant of the present disclosure; or a microorganism (e.g., recombinant strain) having activity of the variant of the present disclosure, but is not limited thereto.

The microorganisms of the present disclosure may have purine nucleotide-producing ability.

The microorganism of the present disclosure may be a microorganism naturally having glutamine-hydrolyzing GMP synthase or purine nucleotide-producing ability, or a microorganism obtained by introducing the variant of the present disclosure or a polynucleotide encoding the same (or a vector including the polynucleotide) into a parent strain without glutamine-hydrolyzing GMP

synthase or purine nucleotide-producing ability and/or by imparting purine nucleotide-producing ability to the parent strain, but is not limited thereto. For example, the microorganism of the present disclosure may be a microorganism having purine nucleotide-producing ability imparted by the introduction of the glutamine-hydrolyzing GMP synthase variant of the present disclosure. For example, the microorganism of the present disclosure may be a microorganism having purine nucleotide-producing ability enhanced by the introduction of the glutamine-hydrolyzing GMP synthase variant of the present disclosure. However, the microorganism of the present disclosure is not limited thereto.

For example, the strain of the present disclosure is a cell or microorganism transformed by a vector including the polynucleotide of the present disclosure or a polynucleotide encoding the variant of the present disclosure, and thus expresses the variant of the present disclosure, and for the purpose of the present disclosure, the strain of the present disclosure encompasses all the microorganisms that can produce a purine nucleotide by including the variant of the present disclosure. For example, the strain of the present disclosure may be a recombinant strain having enhanced purine nucleotide-producing ability through the expression of the glutamine-hydrolyzing GMP synthase variant by introducing a polynucleotide encoding the variant of the present disclosure into a native wild-type microorganism or a microorganism producing a purine nucleotide. The recombinant strain having enhanced purine nucleotide-producing ability compared with a native wild-type microorganism or a glutamine-hydrolyzing GMP synthase non-modified microorganism (*i.e.*, a microorganism expressing wild-type glutamine-hydrolyzing GMP synthase (SEQ ID NO: 3) or a microorganism not expressing a modified protein), but is not limited thereto.

As used herein, the term “non-modified microorganism” may refer to a wild-type strain or a native strain as it is, or a strain before transformation due to genetic mutation caused by natural or artificial factors, not excluding strains including mutations that may naturally occur in microorganisms. For example,

the non-modified microorganism may refer to a strain to which the glutamine-hydrolyzing GMP synthase variant described herein is not introduced or before the introduction thereof. The “non-modified microorganism” may be used interchangeably with “strain before modification”, “microorganism before modification”, “non-varied strain”, “non-modified strain”, “non-varied microorganism”, or “reference microorganism”.

In an embodiment, the microorganism of the present disclosure may be a microorganism of the genus *Corynebacterium*. For example, the microorganism of the present disclosure may be *Corynebacterium stationis*, *Corynebacterium crudilactis*, *Corynebacterium deserti*, *Corynebacterium efficiens*, *Corynebacterium callunae*, *Corynebacterium glutamicum*, *Corynebacterium singulare*, *Corynebacterium halotolerans*, *Corynebacterium striatum*, *Corynebacterium ammoniagenes*, *Corynebacterium pollutisoli*, *Corynebacterium imitans*, *Corynebacterium testudinoris*, or *Corynebacterium flavescens*. Specifically, the microorganism of the present disclosure may be *Corynebacterium stationis*, but is not limited thereto.

The term “purine nucleotide” herein refers to a nucleotide containing a purine-based structure. The purine nucleotide may be XMP, IMP, GMP, or AMP, and specifically IMP, XMP, or GMP. More specifically, the purine nucleotide may be XMP or GMP, but is not limited thereto.

The “inosine 5'-monophosphate or 5'-inosinic acid (IMP)” and “guanosine-5'-monophosphate or 5'-guanylic acid (GMP)” of the present disclosure, which are intermediates in the nucleic acid biosynthesis metabolic systems, have a physiologically important role in the body and are widely used in foods, pharmaceuticals, and the like. Specifically, IMP itself is known to impart a beef flavor, and GMP, derived from XMP, is known to impart a mushroom flavor. Both of the materials are known to enhance the flavor of monosodium glutamate (MSG), and thus have attracted much attention as a flavor-enhancing nucleic acid-based

seasoning.

In an embodiment, GMP may be produced by conversion from XMP, but is not limited thereto. For example, the amount of GMP production can be increased by the conversion of the increasingly produced XMP to GMP, and thus the GMP is also included within the scope of the present disclosure.

The “XMP” of the present disclosure is also called xanthosine-5'-monophosphate or 5'-xanthylic acid, and may be formed from IMP through the action of IMP dehydrogenase or converted to GMP through the action of GMP synthase.

As used herein, the term “microorganism producing a purine nucleotide” encompasses all of wild-type microorganisms or naturally or artificially genetically modified microorganisms, and refers to a microorganism in which a particular mechanism is diminished or enhanced due to the insertion of an exogenous gene or the enhancement or inactivation of activity of an endogenous gene, wherein the microorganism may have a genetic mutation or enhanced activity for the production of a target purine nucleotide.

The microorganism of the present invention producing a purine nucleotide can retain enhanced ability to produce a target purine nucleotide by containing the glutamine-hydrolyzing GMP synthase variant of the present disclosure.

In an embodiment, the microorganism producing a purine nucleotide or the microorganism having purine nucleotide-producing ability in the present disclosure may be a microorganism in which some of the genes involved in the purine nucleotide biosynthesis are enhanced or diminished or some of the genes involved in the purine nucleotide degradation pathway are enhanced or diminished.

As used herein, the term “diminishment” of the activity of a polypeptide has a concept encompassing all of the reduction of activity or the absence of activity compared with the intrinsic activity. The diminishment may be used

interchangeably with inactivation, deficiency, down-regulation, decrease, reduction, attenuation, or the like.

The diminishment may also include: a case where the activity of the polypeptide itself is reduced or eliminated compared with the activity of the polypeptide possessed by the original microorganism due to the mutation or the like of the polynucleotide encoding the polypeptide; a case where the activity and/or concentration (expression level) of the overall polypeptides in the cell is lower compared with the native strain due to the inhibition of the expression of a gene of a polynucleotide encoding the polypeptide or the inhibition of the translation into the polypeptide encoding the polynucleotide or the inhibition of translation into the polypeptide; a case where the expression of the polynucleotide is not made; and/or a case where the polypeptide has no activity in spite of the expression of the polynucleotide. The “intrinsic activity” refers to the activity of a specific polypeptide originally possessed by a parental strain before modification or a wild-type or non-modified microorganism, when the strain or microorganism is transformed by genetic mutation caused by natural or artificial factors. This term may be used interchangeably with the “activity before modification”. The “inactivation, deficiency, decrease, down-regulation, reduction, or attenuation” of the activity of a polypeptide compared with the intrinsic activity thereof means that the activity of the polypeptide is lowered compared with the activity of a specific polypeptide originally possessed by a parent strain before transformation or a non-modified microorganism.

The diminishment of the activity of the polypeptide may be performing by any method known in the art, but is not limited thereto, and may be attained by applying various methods well known in the art (e.g., Nakashima N. *et al.*, Bacterial cellular engineering by genome editing and gene silencing. *Int J Mol Sci.* 2014;15(2):2773–2793, and Sambrook *et al. Molecular Cloning* 2012, etc.).

Specifically, the diminishment of the polypeptide of the present disclosure

may be attained by:

- 1) deletion of a part or the entirety of the gene encoding the polypeptide;
- 2) modification of an expression control region (or expression control sequence) to reduce the expression of a gene encoding the polypeptide;
- 3) modification of the amino acid sequence constituting the polypeptide to eliminate or diminish the activity of the polypeptide (e.g., deletion/substitution/addition of at least one amino acid in the amino acid sequence);
- 4) modification of the gene sequence encoding the polynucleotide to eliminate or diminish the activity of the polypeptide (e.g., deletion/substitution/addition of at least one nucleotide in the nucleotide sequence of the polypeptide gene to encode the polypeptide modified to eliminate or diminish the activity of the polypeptide);
- 5) modification of a nucleotide sequence encoding an initiation codon or 5'-UTR region of the gene transcript encoding the polypeptide;
- 6) introduction of an antisense oligonucleotide (e.g., antisense RNA) complementarily binding to the gene transcript encoding the polypeptide;
- 7) addition of a sequence complementary to the Shine–Dalgarno sequence of the gene encoding the polypeptide to the upstream of the Shine–Dalgarno sequence to form a secondary structure that makes the attachment of ribosomes impossible;
- 8) addition of a promoter, which is to be reverse-transcribed, to the 3' end of the open reading frame (ORF) of the gene sequence encoding the polypeptide (reverse transcription engineering, RTE); or
- 9) combination of two or more selected from items 1) to 8), but is not particularly limited thereto.

For example, these are described as follows.

The deletion of a part or the entirety of the gene encoding the polypeptide in item 1) may be the elimination of the entirety of the polynucleotide encoding an

endogenous target protein in the chromosome, the replacement with a polynucleotide with deletion of some nucleotides, or the replacement with a marker gene.

The modification of an expression control region (or expression control sequence) in item 2) may be the mutation on the expression control region (or expression control sequence) through deletion, insertion, non-conservative or conservative substitution, or a combination thereof, or the replacement with a sequence having more diminished activity. The expression control region includes a promoter, an operator sequence, a sequence for encoding a ribosomal binding site, and a sequence for controlling the termination of transcription and translation, but is not limited thereto.

The modification of the amino acid sequence or the polynucleotide sequence in items 3) and 4) may be the modification on the sequence through deletion, insertion, non-conservative or conservative substitution, or a combination thereof in the amino acid sequence of the polypeptide or the polynucleotide sequence encoding the polypeptide, or a replacement with an amino acid sequence or polynucleotide sequence modified to have more diminished activity or an amino acid sequence or polynucleotide sequence improved to have no activity, so as to diminish activity of the polypeptide. For example, the expression of the gene may be inhibited or attenuated by introducing mutation into the polynucleotide sequence to form a termination codon.

The modification of a nucleotide sequence encoding an initiation codon or 5'-UTR region of the gene transcript encoding the polypeptide in item 5) may be, for example, the substitution with a nucleotide sequence encoding, rather than an endogenous initiation codon, another initiation codon having a lower expression rate of the polypeptide, but is not limited thereto.

The introduction of an antisense oligonucleotide (e.g., antisense RNA) complementarily binding to the gene transcript encoding the polypeptide in item 6) may be referred to, for example, the literature (Weintraub, H. *et al.*, Antisense-

RNA as a molecular tool for genetic analysis, *Reviews - Trends in Genetics*, Vol. 1(1) 1986].

The addition of a sequence complementary to the Shine–Dalgarno sequence of the gene encoding the polypeptide to the upstream of the Shine–Dalgarno sequence so as to form a secondary structure that makes the attachment of ribosomes impossible in item 7) may be making mRNA translation impossible or reducing the rate thereof.

The addition of a promoter, which is to be reverse-transcribed, to the 3' end of the open reading frame (ORF) of the gene sequence encoding the polypeptide (reverse transcription engineering, RTE) in item 8) may be making an antisense nucleotide complementary to the gene transcript encoding the polypeptide to thereby diminish the activity of the polypeptide.

As used herein, the term “enhancement” in activity of a polypeptide refers to an increase in activity of the polypeptide compared with the intrinsic activity thereof. The enhancement may be used interchangeably with activation, up-regulation, overexpression, increase, or the like. Herein, the activation, enhancement, up-regulation, overexpression, and increase may include all of exhibiting the activity that has not been originally possessed or exhibiting the activity enhanced compared with the intrinsic activity or activity before modification. The “intrinsic activity” refers to the activity of a specific polypeptide originally possessed by a parental strain before transformation thereof or a non-modified microorganism when the strain or microorganism is transformed by genetic mutation caused by natural or artificial factors. This term may be used interchangeably with the “activity before modification”. The “enhancement”, “up-regulation”, “overexpression”, or “increase” in activity of a polypeptide compared with the intrinsic activity thereof means an improvement in activity and/or concentration (expression level) of a specific polypeptide originally possessed by a parent strain before transformation or a non-modified microorganism.

The enhancement can be attained through the introduction of an

exogenous polypeptide or the activity enhancement and/or concentration (expression level) of an endogenous polypeptide. The enhancement of the activity of the polypeptide can be identified by an increase in activity degree or expression level of the corresponding polypeptide or amount of a product produced from the corresponding polypeptide.

The enhancement of the activity of the polypeptide may be attained by applying various methods well known in the art, and is not limited as long as the method can enhance the activity of the target polypeptide compared with the pre-transformation microorganism. Specifically, genetic engineering and/or protein engineering well known to a person skilled in the art, which is a routine method of molecular biology, may be used, but is not limited thereto (e.g., Sitnicka *et al.* Functional Analysis of Genes. *Advances in Cell Biology*. 2010, Vol. 2. 1–16, Sambrook *et al.* *Molecular Cloning* 2012, or the like).

Specifically, the enhancement of the polypeptide of the present disclosure may be attained by:

- 1) increase in intracellular copy number of the polynucleotide encoding the polypeptide;
- 2) replacement of a gene expression control region on the chromosome encoding the polypeptide with a sequence having higher activity;
- 3) modification of a nucleotide sequence encoding an initiation codon or 5'-UTR region of the gene transcript encoding the polypeptide;
- 4) modification of the amino acid sequence of the polypeptide to enhance activity of the polypeptide;
- 5) modification of the polynucleotide sequence encoding the polypeptide to enhance the activity of the polypeptide (e.g., modification of the polynucleotide sequence of the polypeptide gene to encode a polypeptide modified to enhance activity of the polypeptide);
- 6) introduction of an exogenous polypeptide exhibiting activity of the polypeptide or an exogenous polypeptide encoding the same;

- 7) codon optimization of the polynucleotide encoding the polypeptide;
- 8) modification or chemical modification of an exposed site selected by analysis of a tertiary structure of the polypeptide; or
- 9) combination of two or more selected from items 1) to 8), but is not particularly limited thereto.

More specifically, the enhancement of the polypeptide is described as follows.

The increase in intracellular copy number of the polynucleotide encoding the polypeptide in item 1) may be attained by the introduction, into a host cell, of a vector to which the polynucleotide encoding the corresponding polypeptide is operatively linked and which can replicate and function independently of the host. Alternatively, the increase in item 1) may be attained by the introduction of one or more copies of the polynucleotide encoding the corresponding polypeptide into the chromosome in a host cell. The introduction into the chromosome may be performed by the introduction, into a host cell, of a vector capable of inserting the polynucleotide into the chromosome in the host cell. The polynucleotide is as described above.

The replacement of a gene expression control region on the chromosome encoding the polypeptide with a sequence with strong activity in item 2) may be, for example, the modification on the sequence through deletion, insertion, non-conservative or conservative substitution, or a combination thereof, or the replacement with a sequence having stronger activity, to further enhance the activity of the expression control region. The expression control region may include, but not particularly limited to, a promoter, an operator sequence, a sequence encoding a ribosome binding site, a sequence controlling the termination of transcription and translation, and the like. For example, it may be the replacement of the original promoter with a stronger promoter, but is not limited thereto.

Examples of the known stronger promoter may be CJ1 to CJ7 promoters

(US 7662943 B2), lac promoter, trp promoter, trc promoter, tac promoter, lamda phage PR promoter, PL promoter, tet promoter, gapA promoter, SPL7 promoter, SPL13 (sm3) promoter (US 10584338 B2), O2 promoter (US 10273491 B2), tkt promoter, yccA promoter, and the like, but are not limited thereto.

The modification of a nucleotide sequence encoding an initiation codon or 5'-UTR region of the gene transcript encoding the polypeptide in item 3) may be, for example, the substitution with a nucleotide sequence encoding, rather than an endogenous initiation codon, another initiation codon having a higher expression rate of a polypeptide, but is not limited thereto.

The modification of the amino acid sequence or the polynucleotide sequence in items 4) and 5) may be the modification on the sequence through deletion, insertion, non-conservative or conservative substitution, or a combination thereof in the amino acid sequence of the polypeptide or the polynucleotide sequence encoding the polypeptide, or the replacement with an amino acid sequence or polynucleotide sequence modified to have stronger activity or an amino acid sequence or polynucleotide sequence modified to have increased activity, to enhance activity of the polypeptide. Specifically, the replacement may be performed by inserting the polynucleotide into the chromosome by homologous recombination, but is not limited thereto. The vector used herein may further include a selection maker for checking the insertion of the chromosome. The selection marker is as described above.

The introduction of an exogenous polypeptide exhibiting activity of the polypeptide in item 6) may be the introduction, into a host cell, of an exogenous polynucleotide encoding a polypeptide exhibiting the same/similar activity with regard to the polypeptide. The exogenous polynucleotide is not limited to the origin or sequence thereof as long as the exogenous polynucleotide exhibits the same/similar activity with regard to the polynucleotide. The introduction may be performed by any known transformation method appropriately selected by a person skilled in the art, and through the expression of the introduced

polynucleotide in the host cell, the polypeptide is produced and the activity thereof can be enhanced.

The codon optimization of the polynucleotide encoding the polypeptide in item 7) may be the codon optimization of an endogenous polynucleotide to increase transcription or translation thereof in a host cell, or the codon optimization of an exogenous polynucleotide to allow optimized transcription or translation thereof in a host cell.

The modification or chemical modification of an exposed site selected by analysis of a tertiary structure of the polypeptide in item 8) may be, for example, the modification or chemical modification of an exposed site to be modified or chemically modified by comparing sequence information of a polypeptide to be analyzed with a database that stores sequence information of existing proteins, determining a template protein candidate according to similarity of the sequences, and identifying the structure based thereon.

Such enhancement of the activity of the polypeptide may mean that the activity, concentration, or expression level of the corresponding polypeptide is increased relative to the activity or concentration of the polypeptide expressed in a wild type or a microbial strain before modification, or that the amount of a product produced from the corresponding polypeptide is increased, but is not limited thereto.

The modification of a part or the entirety of the polynucleotide in the microorganism of the present disclosure may be induced by: (a) genome editing adopting homologous recombination or engineered nuclease (*e.g.*, CRISPR-Cas9) using a vector for chromosomal insertion in a microorganism or and/or (b) the treatment with light, such as ultraviolet light and radiation, and/or chemicals, but is not limited thereto. The method of modifying a part or the entirety of the gene may include a method by DNA recombinant technology. For example, a nucleotide sequence or vector containing a nucleotide sequence homologous to a target gene may be introduced into the microorganism to bring about homologous

recombination, resulting in deletion in a part or the entirety of the gene. The nucleotide sequence or vector to be introduced may include a dominant selection marker, but is not limited thereto.

Still another aspect of the present disclosure provides a method for producing a purine nucleotide, the method including culturing a microorganism including the variant of the present disclosure or a polynucleotide encoding the same.

The purine nucleotide production method of the present disclosure may include culturing, in a medium, a microorganism including the variant of the present disclosure, a polynucleotide encoding the same, or a vector including the polynucleotide.

As used herein the term “culture” refers to growing the microorganism of the present disclosure in appropriately adjusted environment conditions. The culture procedure of the present disclosure may be performed according to appropriate media or culture conditions known in the art. Such culture may be easily adjusted according to the selected strain by a person skilled in the art. Specifically, the culture may be in a batch type, a continuous type, and/or a fed-batch type, but is not limited thereto.

As used herein, the “medium” refers to a mixture containing, as main ingredients, nutrient materials required for culturing the microorganism, wherein the medium supplies nutrient materials, growth factors, and the like, including water that is essential for survival and growth. Specifically, as for the media and other culture conditions used for culturing the microorganism of the present disclosure, any medium that is used for usual culture of microorganisms may be used without particular limitation. However, the microorganisms of the present disclosure may be cultured under aerobic conditions in a conventional medium containing appropriate carbon sources, nitrogen sources, phosphorus sources, inorganic compounds, amino acids, and/or vitamins, while the temperature, pH,

and the like are adjusted.

Specifically, a culture medium for the genus *Corynebacterium* strain may be found in the literature ("Manual of Methods for General Bacteriology" by the American Society for Bacteriology (Washington D.C., USA, 1981)).

In the present disclosure, the carbon source may include carbohydrates, such as glucose, saccharose, lactose, fructose, sucrose, and maltose; sugar alcohols, such as mannitol and sorbitol; organic acids, such as pyruvic acid, lactic acid, and citric acid; and amino acids, such as glutamic acid, methionine, and lysine. In addition, natural organic nutrient sources may be used, such as starch hydrolysates, molasses, blackstrap molasses, rice bran, cassava, bagasse, and corn steep liquor, and specifically, carbohydrates, such as glucose and sterile pretreated molasses (*i.e.*, molasses converted to reduced sugars) may be used, and appropriate amounts of other carbon sources may be used without limitation. These carbon sources may be used alone or in a combination of two or more, but is not limited thereto.

As for the nitrogen sources, inorganic nitrogen sources, such as ammonia, ammonium sulfate, ammonium chloride, ammonium acetate, ammonium phosphate, ammonium carbonate, and ammonium nitrate; amino acids, such as glutamic acid, methionine, and glutamine; and organic nitrogen sources, such as peptone, NZ-amine, meat extracts, yeast extracts, malt extracts, corn steep liquor, casein hydrolysates, fishes or their decomposition products, defatted soybean cake or its degradation products, and the like may be used. These nitrogen sources may be used alone or in a combination of two or more thereof, but are not limited thereto.

Examples of the phosphate sources may include potassium phosphate monobasic, potassium phosphate dibasic, and sodium-containing salts corresponding thereto. As for inorganic compounds, sodium chloride, calcium chloride, iron chloride, magnesium sulfate, iron sulfate, manganese sulfate, calcium carbonate, and the like may be used, and besides, amino acids, vitamins,

and/or suitable precursors may be included. These constituent ingredients or precursors may be added to the medium in a batch or continuous manner. However, the present disclosure is not limited thereto.

The pH of the medium may be adjusted by adding compounds, such as ammonium hydroxide, potassium hydroxide, ammonia, phosphoric acid, and sulfuric acid, to the medium in an appropriate manner during the culture of the microorganism of the present disclosure. In addition, an anti-foaming agent, such as a fatty acid polyglycol ester, may be added to suppress foam formation during the culture. In addition, oxygen or oxygen-containing gas may be injected into the medium to maintain the aerobic state of the medium, or nitrogen, hydrogen or carbon dioxide gas may be injected, or no gas may be injected, to maintain the anaerobic or non-aerobic state of the medium, but is not limited thereto.

In the culture of the present disclosure, the culture temperature may be maintained at 20°C to 45°C, specifically 25°C to 40°C, and the culture may be performed for about 10 hours to 160 hours, but is not limited thereto.

The purine nucleotide produced by the culture of the present disclosure may be released into the medium or may remain in cells without being released.

The purine nucleotide production method of the present disclosure may further include preparing the microorganism of the present disclosure, preparing a medium for culturing the microorganism, or a combination of these steps (regardless of the order, in any order), for example, before or after the culturing step.

The purine nucleotide production method of the present disclosure may further include recovering the purine nucleotide from a medium resulting from the culture (a medium in which culture has been performed) or the microorganism. The recovering step may be further included after the culturing step.

The recovering may be collecting a desired purine nucleotide by using an appropriate method known in the art according to the method for culturing a microorganism of the present disclosure, for example, a batch, continuous, or fed-batch type culture. For example, centrifugation, filtration, treatment with a crystallized protein precipitating agent (salting-out), extraction, ultrasonic disruption, ultrafiltration, dialysis, various types of chromatography, such as molecular sieve chromatography (gel filtration), adsorption chromatography, ion exchange chromatography, and affinity chromatography, HPLC, and a combination of these methods may be used, and a desired purine nucleotide may be recovered from the medium or microorganism by using an appropriate method known in the art.

The purine nucleotide production method of the present disclosure may further include a purification step. The purification may be performed by using an appropriate method known in the art. In an exemplary embodiment, when the purine nucleotide production method of the present disclosure includes both the recovering step and the purification step, the recovering step and the purification step may be performed continuously or discontinuously regardless of the order, or may be performed simultaneously or integrated into one step, but is not limited thereto.

In an exemplary embodiment, the GMP production method of the present disclosure may further include converting XMP into GMP. In the GMP production method of the present disclosure, the converting step may be further included after the culturing step or the covering step. The converting step may be performed by using an appropriate method known in the art. For example, the conversion may be performed using Coryneform bacteria, *E. coli*, or 5'-xanthylic acid aminase (KR 10-0655902 B1, US 2013-0095529 A1, or the like), but is not limited thereto.

In the method of the present disclosure, the variant, polynucleotide, vector, strain, purine nucleotide, and the like are as described in the other aspects above.

Still another aspect of the present disclosure is to provide a composition for producing a purine nucleotide, the composition containing the variant of the present disclosure, the polynucleotide encoding the variant, a microorganism including a vector including the polynucleotide or the polynucleotide of the present disclosure; a medium obtained by culturing the microorganism; or a composition thereof.

The composition of the present disclosure may further contain any appropriate excipient that is usually used in the composition for producing a purine nucleotide, and examples of the excipient may include a preserving agent, a wetting agent, a dispersing agent, a suspending agent, a buffer, a stabilizing agent, or an isotonic agent, but are not limited thereto.

In the composition of the present disclosure, the variant, polynucleotide, vector, microorganism, medium, purine nucleotide, and the like are as described in the other aspects above.

Still another aspect of the present disclosure is to provide a method for increasing purine nucleotide-producing ability of a microorganism, the method including modifying the microorganism to express the variant of the present disclosure.

In the method of the present application, the variant, polynucleotide, vector, microorganism, medium, purine nucleotide, and the like are as described in the other aspects above.

Still another aspect of the present disclosure provides use of the variant of the present disclosure for producing a purine nucleotide.

Still another aspect of the present disclosure provides use of the variant of the present disclosure, the polynucleotide encoding the same, or the microorganism including the vector including the same.

The variant, polynucleotide, vector, microorganism, and the like are as described in the other aspects above.

[Mode for Carrying Out the Invention]

Hereinafter, the present disclosure will be described in detail with reference with examples and test examples. However, these examples and test examples are given for specifically illustrating the present disclosure, and the scope of the present disclosure is not limited thereto.

Example 1: Preparation of *guaA* Synthase-Diminished Variant

To discover a *guaA* variant for XMP production, a *guaA* mutant library, a gene encoding the variant, was constructed.

Example 1-1: Preparation of Vector Containing *guaA*

To prepare a *guaA* mutant library, a recombinant vector containing *guaA* was first constructed. PCR was performed using the chromosome of *Corynebacterium stationis* ATCC6872 as a template along with the primers of SEQ ID NO: 5 and SEQ ID NO: 6, and the amplification product was cloned into pCR2.1 vector by using a TOPO Cloning Kit (Invitrogen), and the resultant vector was named pCR-*guaA*.

TABLE 1

SEQ ID NO	Primer name	Sequence (5'–3')
5	pCR- <i>guaA</i> -F	GCGATGTGATTGCCGGTG
6	pCR- <i>guaA</i> -R	GCTCTAGGAAGTCAGCAG

Example 1-2: Construction of *guaA* Mutant Library

A *guaA* mutant library was constructed using as a template the pCR-*guaA* vector constructed in Example 1-1. The library was prepared by using an error-

prone PCR kit (Clontech Diversify® PCR Random Mutagenesis Kit), and *guaA* mutant PCR products with different sequences were obtained by random mutagenesis using the primers of SEQ ID NOS: 7 and 8 according to the manual of a manufacturer. The amplification products were cloned using TOPO Cloning Kit (Invitrogen), and then transformed into *E. coli* DH5α and spread on an LB solid medium containing kanamycin (25 mg/L). The transformed *E. coli* colonies were collected to extract plasmids, which were named pCR-guaA-library.

TABLE 2

SEQ ID NO	Primer name	Sequence (5'–3')
7	pCR-guaA-library-F	TGCCCAATACGCACAGCTG
8	pCR-guaA-library-R	GGCTGGGGCTTTGTCC

Example 1-3: Evaluation of Constructed Library and Selection of Strain

The pCR-guaA-library prepared in Example 1-2 was transformed into the *Corynebacterium stationis* ATCC6872 wild-type strain by electroporation, and then the strain was spread on a nutrient medium containing 25 mg/L kanamycin to obtain 5,000 strain colonies into which the mutant gene was inserted. The respective colonies were named C.st ATCC6872_pCR_guaA(mt)1 to C.st ATCC6872_pCR_guaA(mt)5000.

- Nutrient medium: 1% peptone, 1% beef extract, 0.25% sodium chloride, 1% yeast extract, 150 mg/L adenine, 150 mg/L guanine, 2% agar, pH 7.2 (based on 1 L of distilled water).

Each of the obtained 5,000 colonies was autoclaved under pressure, inoculated in 100 µL of a seed medium containing kanamycin (25 mg/L), cultured in a 96-deep-well plate with shaking at 30°C, 1200 rpm for 24 hours using a

microplate shaker (TAITEC), and then used as a seed culture. The autoclaved fermentation medium of 320 μ L (240 μ L of a main medium + 80 μ L of a separate sterilized medium) was dispensed into a 96-deep well plate, and then each 15 μ L of the seed culture was inoculated thereto, followed by culture with shaking under the same conditions as above for 72 hours.

To analyze the amount of XMP produced in the culture, 640 μ L of sterile water was dispensed into the culture after the completion of culture, followed by centrifugation at 4000 rpm for 15 minutes, and then 3 μ L of the supernatant was transferred to a 96-well UV plate into which 297 μ L of distilled water was dispensed. Next, a microplate reader was used to perform shaking for 30 seconds, and a spectrophotometer was used to measure the absorbance at 25°C and a wavelength of 260 nm. Then, ten colonies of mutant strains showing an increase in absorbance compared with the wild-type strain were selected. Other colonies showed similar or decreased degrees of absorbance compared with the control.

The ten selected strains were repeatedly measured for absorbance by way of the above method and investigated for the amount of XMP production, and one species of strain (C.st ATCC6872_pCR_guaA(mt)726) having significantly enhanced XMP-producing ability compared with the wild-type strain was selected.

To verify the validity of the finally selected strain, a fermentation titer test was performed.

The *Corynebacterium stationis* ATCC6872 wild-type strain and the selected C.st ATCC6872_pCR_guaA(mt)726 strain were inoculated in 14 mL tubes containing 2.5 mL of the seed medium below and cultured with shaking at 170 rpm for 24 hours at 30°C. In a 250 mL corner-baffle flask containing 32 mL of the following production medium (24 mL of a main medium + 8 mL of a separate sterile medium), 0.7 mL of the seed culture was inoculated and cultured

with shaking at 170 rpm for 75 hours at 30°C.

The compositions of the seed medium, main medium, and separate sterile medium are as follows.

<XMP Flask seed medium>

30 g/L glucose, 15 g/L peptone, 15 g/L of yeast extract, 2.5 g/L sodium chloride, 3 g/L urea, 150 mg/L adenine, 150 mg/L guanine, pH 7.0 (on the basis of 1 L of distilled water).

<XMP Flask Production Medium (Main Medium)>

50 g/L glucose, 10 g/L magnesium sulfate, 3 g/L yeast extract, 100 mg/L calcium chloride, 20 mg/L iron sulfate, 10 mg/L manganese sulfate, 10 mg/L zinc sulfate, 0.8 mg/L copper sulfate, 20 mg/L histidine, 15 mg/L cystine, 15 mg/L beta-alanine, 100 µg/L biotin, 5 mg/L thiamine, 50mg/L adenine, 25 mg/L guanine, 15 mg/L niacin, pH 7.0 (on the basis of 1 L of distilled water).

<XMP Flask Production Medium (Separate Sterile Medium)>

18 g/L potassium phosphate monobasic, 42 g/L potassium phosphate dibasic, 7 g/L urea, 5 g/L ammonium sulfate (on the based on 1 L of distilled water)

The results of measuring the amount of XMP production by a method using HPLC after completion of culture are shown in Table 3.

TABLE 3

Strain	XMP concentration (g/L)
C.st ATCC6872	0
C.st ATCC6872_pCR_guaA(mt)726	4.83

As shown in Table 3, the concentration of XMP produced was 4.83 g/L in

C.st ATCC6872_pCR_guaA(mt)726 compared with the wild-type strain.

Example 1-4: Identification of Mutation Through Gene Sequencing

To identify the sequence of the *guaA* variant of the C.st ATCC6872_pCR_guaA(mt)726 strain, the C.st ATCC6872_pCR_guaA(mt)726 strain was subjected to PCR using the primers of SEQ ID NOS: 8 and 9 and sequencing. As a result of comparison with the sequence of the *guaA* gene of the wild-type strain, the C.st ATCC6872_pCR_guaA(mt)726 strain was identified to include a mutation in which the amino acid at position 29 in the *guaA* gene is substituted from arginine to cysteine (the nucleotide at position 85 being substituted from c to t: SEQ ID NO: 2).

The identified variant sequence is shown in Table 4.

TABLE 4

SEQ ID NO	Variant Sequence
1	MTQPATTPRPVLVVDGFAQYAQLIARRVCEASIYSEVVPHSATV KEIKAKNPAALILSGGPSSVYADGAPQLKPELLELGVPVFGICYG FQAMNHALGGNVAQTGDREYGRTEITHTGGVLHDGLEENHKV WMSHGDAVDKAPEGFTVTASSAGAPVAAMECVAKQMAGVQY HPEVMHSPHGQEVLVRFLTEVAGLEQWTWSANIAQQLIDDVRA QIGPEGRAICGLSGGVDSAVAAALVQRAIGDRLTCVFVDHGLLR AGEREQVEKDFVASTGAKLITAHEADAFLSKLAGVTDPEAKRK AIGAEFIRSFERAQALESPEDESTVDFLVQGTLYPDVVESGG GDGTANIKSHHNVGGLPDDVEFELVEPLRLLFKDEVRAVGREL GLPEEIVARQPFPGPGLGIRIIGEVTEERLEILRQADLIARTELTN AGLDGDIWQCPVLLADVRSVGVQGDGRITYGHPIVLRPVSSE DAMTADWTRVPYDVLEKISTRITNEVNDVNRVVVDITSKPPGTI EWE

In the following examples, it was investigated whether the *guaA* mutation had an effect on XMP production.

Example 2: Identification of Effect of *guaA* Variant

Example 2-1: Construction of Vector for *guaA* Variant Expression

To investigate the effect, on XMP production, of the variant (R29C; SEQ ID NO: 1) in which arginine at position 29 in the *guaA* enzyme amino acid sequence was substituted with cysteine, a vector for preparing an expression strain thereof was constructed as follows by using the plasmid pDCM2 (Korea Publication No. 10-2020-0136813) for gene insertion and replacement in the *Corynebacterium* chromosome.

PCR was performed by using the chromosome of *Corynebacterium stationis* ATCC6872 as a template along with the primer pairs of SEQ ID NOS: 10 and 11 and SEQ ID NOS: 12 and 13. Solg™ Pfu-X DNA polymerase was used as the polymerase, and PCR was performed under PCR amplification conditions: denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and polymerization at 72°C for 60 seconds, and then a polymerization reaction at 72°C for 5 minutes to thereby obtain each PCR product. The amplification product was mixed with the pDCM2 vector previously prepared by digestion with SmaI restriction enzyme, and cloned by the Gibson assembly (DG Gibson *et al.*, *NATURE METHODS*, Vol. 6 No. 5, MAY 2009, NEBuilder HiFi DNA Assembly Master Mix) method to obtain a recombinant plasmid, which was named pDCM2-*guaA*(R29C). The cloning was performed by mixing the Gibson assembly reagent and each of the gene fragments in a calculated number of moles, followed by preservation at 50°C for 1 hour.

Example 2-2: Preparation of *guaA* Variant-Expressing Strain

The pDCM2-guaA(R29C) vector constructed in the above example was transformed into the *Corynebacterium stationis* ATCC6872 strain by electroporation, and strains having the vector inserted into the chromosome by recombination of homologous sequences were selected on a medium containing 25 mg/L kanamycin. The selected primary strains were subjected to secondary cross-over to select a strain having mutation introduced into the target gene. The introduction of gene mutation in the finally transformed strain was investigated by PCR using the primer pair of SEQ ID NO: 14 and SEQ ID NO: 15 and sequencing, and the confirmed strain was named C.st ATCC6872::guaA(R29C).

Example 2-3: Comparison of XMP-Producing Ability of *guaA* Variant-Expressing Strain

The XMP-producing abilities of the C.st ATCC6872::guaA(R29C) strain prepared in Example 2-2 and the wild-type strain were investigated through the fermentation titer evaluation method in Example 1-3. After completion of culture, the amount of XMP production was measured by HPLC, and the culture results are shown in Table 5.

The test was repeated three times, and the mean values of the analysis results are shown in Table 5.

TABLE 5

Strain	XMP concentration (g/L)
C.st ATCC6872	0
C.st ATCC6872::guaA(R29C)	5.21

As shown in Table 5, the concentration of XMP produced was 5.21 g/L in the C.st ATCC6872::guaA(R29C) strain.

The C.st ATCC6872::guaA(R29C) strain was named CN02-2299, which

was deposited with the Korea Microorganism Conservation Center, a depository institution under the Budapest Treaty, on 9 August 2021, and given accession number KCCM13029P.

Example 3: Substitution of Amino Acid with Other Amino Acids in *guaA* Mutation

Example 3-1: Construction of Vector for Amino Acid Substitution and Insertion for *guaA* Mutation

It was identified through the above example that XMP could be produced by *guaA*(R29C) mutation. To investigate the positional importance of *guaA* mutation, vectors for substitution of the amino acid at position 29 with other amino acids were constructed, and the effect on XMP-producing ability was investigated. Site-directed mutagenesis was performed by using the pDCM2-*guaA*(R29C) vector constructed in Example 2-1 as a template.

Specifically, PCR was performed using the primer pairs of SEQ ID NOS: 10 and 16 and SEQ ID NOS: 17 and 13, primer pairs of SEQ ID NOS: 10 and 18 and SEQ ID NOS: 19 and 13, primer pairs of SEQ ID NOS: 10 and 20 and SEQ ID NOS: 21 and 13, primer pairs of SEQ ID NOS: 10 and 22 and SEQ ID NOS: 23 and 13, primer pairs of SEQ ID NOS: 10 and 24 and SEQ ID NOS: 25 and 13, primer pairs of SEQ ID NOS: 10 and 26 and SEQ ID NOS: 27 and 13, primer pairs of SEQ ID NOS: 10 and 28 and SEQ ID NOS: 29 and 13, primer pairs of SEQ ID NOS: 10 and 30 and SEQ ID NOS: 31 and 13, primer pairs of SEQ ID NOS: 10 and 32 and SEQ ID NOS: 33 and 13, primer pairs of SEQ ID NOS: 10 and 34 and SEQ ID NOS: 35 and 13, primer pairs of SEQ ID NOS: 10 and 36 and SEQ ID NOS: 37 and 13, primer pairs of SEQ ID NOS: 10 and 38 and SEQ ID NOS: 39 and 13, primer pairs of SEQ ID NOS: 10 and 40 and SEQ ID NOS: 41 and 13, primer pairs of SEQ ID NOS: 10 and 42 and SEQ ID NOS: 43 and 13, primer pairs of SEQ ID NOS: 10 and 44 and SEQ ID NOS: 45 and 13, primer

pairs of SEQ ID NOS: 10 and 46 and SEQ ID NOS: 47 and 13, primer pairs of SEQ ID NOS: 10 and 48 and SEQ ID NOS: 49 and 13, and primer pairs of SEQ ID NOS: 10 and 50 and SEQ ID NOS: 51 and 13. Solg™ Pfu-X DNA polymerase was used as the polymerase, and PCR was performed under PCR amplification conditions: with denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and polymerization at 72°C for 60 seconds, and then a polymerization reaction at 72°C for 5 minutes to thereby obtain each PCR product. The respective amplification products were mixed with the pDCM2 vector previously prepared by digestion with SmaI restriction enzyme, and cloned by the Gibson assembly method to obtain recombinant plasmids, and the plasmid information thus obtained is shown in Table 6. The cloning was performed by mixing the Gibson assembly reagent and the respective fragments in a calculated number of moles, followed by preservation at 50°C for 1 hour.

TABLE 6

NO	Plasmid Name
1	pDCM2-guaA(R29G)
2	pDCM2-guaA(R29F)
3	pDCM2-guaA(R29S)
4	pDCM2-guaA(R29Y)
5	pDCM2-guaA(R29W)
6	pDCM2-guaA(R29I)
7	pDCM2-guaA(R29V)
8	pDCM2-guaA(R29P)
9	pDCM2-guaA(R29T)
10	pDCM2-guaA(R29A)
11	pDCM2-guaA(R29D)

12	pDCM2-guaA(R29N)
13	pDCM2-guaA(R29H)
14	pDCM2-guaA(R29L)
15	pDCM2-guaA(R29M)
16	pDCM2-guaA(R29Q)
17	pDCM2-guaA(R29K)
18	pDCM2-guaA(R29E)

Example 3-2: Preparation of Strains Having Substitutions of Mutation in *guaA* Variant with Other Amino Acids and Comparison of XMP-Producing Ability

The 18 vectors for mutation introduction obtained in Example 3-1 were transformed into the *Corynebacterium stationis* ATCC6872 wild-type strain, and strains having the vectors inserted into the chromosome by recombination of homologous sequences were selected on a medium containing 25 mg/L kanamycin. The selected primary strains were subjected to secondary cross-over to select strains having mutation introduced into the target gene. The introduction of gene mutation in the finally transformed strains was investigated by PCR using the primer pair of SEQ ID NO: 14 and SEQ ID NO: 15 and sequencing, and the strain names according to the inserted mutations are shown in Table 7 below.

TABLE 7

NO	Strain name
1	C.st ATCC6872:: <i>guaA</i> (R29G)
2	C.st ATCC6872:: <i>guaA</i> (R29F)
3	C.st ATCC6872:: <i>guaA</i> (R29S)
4	C.st ATCC6872:: <i>guaA</i> (R29Y)

5	C.st ATCC6872::guaA(R29W)
6	C.st ATCC6872::guaA(R29I)
7	C.st ATCC6872::guaA(R29V)
8	C.st ATCC6872::guaA(R29P)
9	C.st ATCC6872::guaA(R29T)
10	C.st ATCC6872::guaA(R29A)
11	C.st ATCC6872::guaA(R29D)
12	C.st ATCC6872::guaA(R29N)
13	C.st ATCC6872::guaA(R29H)
14	C.st ATCC6872::guaA(R29L)
15	C.st ATCC6872::guaA(R29M)
16	C.st ATCC6872::guaA(R29Q)
17	C.st ATCC6872::guaA(R29K)
18	C.st ATCC6872::guaA(R29E)

The prepared strains, the C.st ATCC6872::guaA(R29C) strain, and the wild-type strain were cultured and analyzed for the XMP concentration by way of the fermentation titer evaluation method in Example 1-3. The test was repeated three times, and the mean values of the analysis results are shown in Table 8.

TABLE 8

Strain	XMP concentration (g/L)
C.st ATCC6872	0
C.st ATCC6872::guaA(R29C)	5.01
C.st ATCC6872::guaA(R29G)	4.95
C.st ATCC6872::guaA(R29F)	4.33
C.st ATCC6872::guaA(R29S)	4.01
C.st ATCC6872::guaA(R29Y)	4.88

C.st ATCC6872:: <i>guaA</i> (R29W)	2.24
C.st ATCC6872:: <i>guaA</i> (R29I)	4.77
C.st ATCC6872:: <i>guaA</i> (R29V)	4.89
C.st ATCC6872:: <i>guaA</i> (R29P)	4.18
C.st ATCC6872:: <i>guaA</i> (R29T)	4.77
C.st ATCC6872:: <i>guaA</i> (R29A)	4.22
C.st ATCC6872:: <i>guaA</i> (R29D)	4.16
C.st ATCC6872:: <i>guaA</i> (R29N)	4.93
C.st ATCC6872:: <i>guaA</i> (R29H)	0.25
C.st ATCC6872:: <i>guaA</i> (R29L)	4.76
C.st ATCC6872:: <i>guaA</i> (R29M)	4.24
C.st ATCC6872:: <i>guaA</i> (R29Q)	4.88
C.st ATCC6872:: <i>guaA</i> (R29K)	4.93
C.st ATCC6872:: <i>guaA</i> (R29E)	4.99

Referring to Table 8, the strains including *guaA* in which the amino acid at position 29 in the amino acid sequence encoded by the *guaA* gene is substituted with other amino acids except for arginine were identified to produce XMP compared with the wild-type strain. That is, the amino acid at position 29 in the amino acid sequence encoded by *guaA* gene corresponded to a major mutation position in XMP production, and more specifically, when the amino acid at position 29 in the amino acid sequence encoded by the *guaA* gene was substituted with other amino acids except for arginine (cysteine, glycine, phenylalanine, serine, tyrosine, tryptophan, isoleucine, valine, proline, threonine, alanine, aspartic acid, asparagine, histidine, leucine, methionine, glutamine, lysine, and glutamic acid), the amounts of XMP produced by the microorganisms containing such mutation can be greatly increased.

It can be therefore seen that the variants having substitutions of the amino acid at position 29 in *guaA* with other amino acids can be advantageously used in

the production of a purine nucleotide.

Example 4: Identification of GMP Producing Ability of *guaA* Variant-Expressing Strain

The production of GMP was investigated using an XMP culture of *C.st* ATCC6872::*guaA*(R29C) as an XMP-producing strain by the following method (see Korean Patent No. KR 10-0655902 B1 and U.S. Patent Publication No. US 2013-0095529 A1).

The *C.st* ATCC6872::*guaA*(R29C) strain was cultured by the fermentation titer evaluation method in Example 1-3. After completion of culture, the amount of XMP production was measured by a method using HPLC. For a conversion reaction of the produced XMP to GMP, the following conversion reaction additives and *E. coli* XMP aminase were added to the fermentation liquid in the corner-baffle flask to perform a conversion reaction at 40°C for 2.5 hours.

<Conversion Reaction Additives>

1.8 g/L phytic acid, 4.8 g/L magnesium sulfate, 3 mL/L nymeen, 2% xylene, 100 mg/L adenine, 7.7 g/L sodium phosphate dibasic (Na₂HPO₄), 2 g/L glutamine, 46 g/L glucose

As a result of the test, the conversion rate indicating the amount of GMP production compared with the amount of XMP consumption is shown in Table 9.

TABLE 9

Strain name	Concentration (g/L)		Conversion rate (%) (GMP production/XMP consumption)
	XMP	GMP	
<i>C.st</i> ATCC6872:: <i>guaA</i> (R29C)	4.91	3.54	72.1

As shown in Table 9, 3.54 g/L GMP was produced through the conversion reaction from XMP produced by the *C.st* ATCC6872::*guaA*(R29C) strain.

It was therefore identified that the variant having a substitution of the amino acid at position 29 in *guaA* with another amino acid increased the GMP production, and thus it can be seen that the variant provided in the present disclosure can be advantageously used in the production of a purine nucleotide.

As set forth above, a person skilled 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 departing from the technical spirit or essential characteristics thereof. Therefore, the embodiments described above should be construed as being exemplified and not limiting the present disclosure. The scope of the present disclosure should be understood that all changes or modifications derived from the definitions and scopes of the claims and their equivalents fall within the scope of the disclosure.



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> CN02-2299	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM13029P
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 August. 09. 2021 (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: August. 09. 2021.

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

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[CLAIMS]

[Claim 1]

A glutamine-hydrolyzing GMP synthase variant in which the amino acid corresponding to position 29 in SEQ ID NO: 3 is substituted with another amino acid.

[Claim 2]

The glutamine-hydrolyzing GMP synthase variant of claim 1, wherein the other amino acid is selected from glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan, proline, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, and histidine.

[Claim 3]

A polypeptide encoding the variant of claim 1.

[Claim 4]

A microorganism comprising a glutamine-hydrolyzing GMP synthase variant in which the amino acid corresponding to position 29 in SEQ ID NO: 3 is substituted with another amino acid, or a polynucleotide encoding the variant.

[Claim 5]

The glutamine-hydrolyzing GMP synthase variant of claim 4, wherein the microorganism is *Corynebacterium stationis*.

[Claim 6]

A method for producing a purine nucleotide, the method comprising culturing the microorganism of claim 4 or 5.

[Claim 7]

The method of claim 6, further comprising recovering the purine nucleotide from a culture medium or the microorganism.

[Claim 8]

A composition for producing a purine nucleotide, the composition comprising at least one of: a glutamine-hydrolyzing GMP synthase variant in which the amino acid corresponding to position 29 in SEQ ID NO: 3 is substituted with another amino acid; a microorganism comprising the variant; and a medium in which the microorganism has been cultured.

[Claim 9]

A method for increasing purine nucleotide-producing ability of a microorganism, the method comprising modifying the microorganism to express the variant of claim 1.