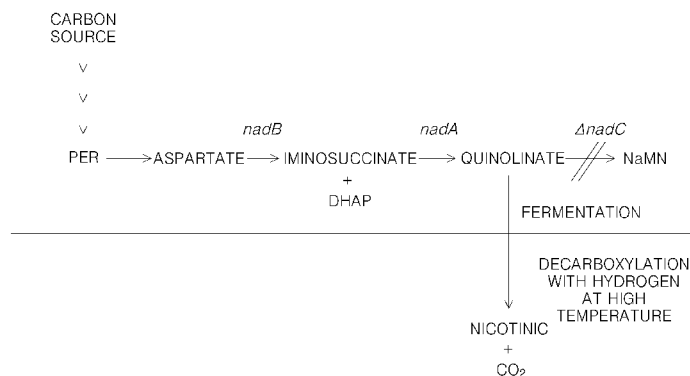




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(54) **Title:** L-ASPARATATE OXIDASE VARIANT AND A METHOD FOR PRODUCING QUINOLINATE OR NICOTINIC ACID USING THE SAME



(57) **Abstract:** To produce quinolinate effectively, a L-aspartate oxidase variant that the feedback regulation by nicotinic acid or NAD is released, and a microorganism including the L-aspartate oxidase variant are provided. Quinolinate may be effectively produced by culturing of the microorganism including the L-aspartate oxidase variant.

Description

Title of Invention: L-ASPARATATE OXIDASE VARIANT AND A METHOD FOR PRODUCING QUINOLINATE OR NICOTINIC ACID USING THE SAME

Technical Field

- [1] The invention relates to L-aspartate oxidase variants, quinolinate-producing microorganisms including genes that encode the L-aspartate oxidase variants, and methods of producing quinolinate or nicotinic acid in high efficiency by using the microorganisms.

Background Art

- [2] Nicotinic acid is an oxide of nicotine and one of vitamin B complex. It is a water soluble vitamin, which is also called niacin, or vitamin B₃ and prevalent in animal and plant. Deficiency of nicotinic acid may cause pellagra disease or neuropathies. In general, nicotinic acid is present in the form of nicotinic acid amide coenzyme, i.e., nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP), in vivo, and is involved in oxidation reduction reactions
- [3] Quinolinate, which is also called quinolinic acid, is produced by oxidation of quinoline. Quinolinic acid is known to have neurotoxicity and cause various neurological disorders. Quinolinate is also known as a precursor of nicotinic acid.
- [4] Nicotinic acid that is widely applicable to the foods and medicinal products may be prepared by a chemical synthetic method or a biological production method. Chemical synthesis of nicotinic acid may result in large quantities of toxic waste including catalysts. Thus, the waste is required for a thorough management and great expenses for disposal. In addition, pyrimidine used as a precursor has various derivatives, and then fluctuations in supply and price of pyrimidine cause an unstable price of nicotinic acid.
- [5] To solve the such problems from the chemical synthesis method, biological methods of producing nicotinic acid by using renewable carbohydrate-derived materials have been studied. Biological production of nicotinic acid is accomplished mainly through two biosynthetic pathways, one of which is a biosynthetic pathway of nicotinic acid from tryptophan as a starting material in eukaryotes, and the other is from aspartic acid as a starting material as in prokaryotes. Both of the pathways use quinolinate as an intermediate, and biosynthesize nicotinic acid from quinolinate by the action of quinolinate phosphoribosyltransferase (*nadC*), nicotinate-mononucleotide adenyl transferase (*nadD*), NAD synthetase (*nadE*), nicotinamide-nucleotide adenyl transferase (NMN *nadR*), and nicotinamidase (*pncA*).

- [6] Biological synthesis methods of nicotinic acid by using recombinant *E. coli* or *Corynebacterium glutamicum* via the pathway of aspartic acid is disclosed (Korean Patent No. 10-1223904). While the inventors of the present invention researched to address the problems from such biological synthesis methods of nicotinic acid and to improve the yield of quinolinate or nicotinic acid, they found enzyme variants involved in the high-yield production of quinolinate and completed the present invention.

Disclosure of Invention

Technical Problem

- [7] The invention provides L-aspartate oxidase variants showing that the feedback regulation by nicotinic acid or nicotinamide adenine dinucleotide (NAD) is released.
- [8] The invention provides quinolinate-producing microorganisms that include the L-aspartate oxidase variants.
- [9] The invention provides methods of producing quinolinate by culturing the microorganisms.
- [10] The invention provides methods of producing nicotinic acid by culturing the microorganisms and decarboxylation of quinolinate.

Solution to Problem

- [11] An aspect of the present invention provides an L-aspartate oxidase variant having an amino acid sequence which a 302nd amino acid in amino acid sequence represented by SEQ ID NO: 1 is substituted with another amino acid.
- [12] L-aspartate oxidase has a catalytic activity of oxidizing L-aspartate to iminosuccinate, as represented in Reaction Scheme 1.
- [13] <Reaction Scheme 1>
- [14] $\text{L-Aspartate} + \text{Fumarate} \rightleftharpoons \alpha\text{-iminosuccinate} + \text{Succinate} + \text{H}^+$
- [15] $\text{L-Aspartate} + \text{Oxygen} \rightleftharpoons \text{Hydrogen peroxide} + \alpha\text{-iminosuccinate} + \text{H}^+$
- [16] L-aspartate oxidase of the present invention may comprise the amino acid sequence represented by SEQ ID NO: 1. However, it is not limited thereto, because there may be the difference in the amino acid sequence of the protein depending on the microbial species or strains. In other words, it can be a mutant protein or artificial variant with an amino acid sequence comprising substitution, deletion, insertion, or addition of one or several amino acids at one or more locations of the amino acid sequence of represented by SEQ ID NO: 1, as long as it can oxidize L-aspartate to iminosuccinate. Herein, "several" may differ depending on the location or type in the three-dimensional structure of amino acid residues of the protein, but specifically means 2 to 20, specifically 2 to 10, and more specifically 2 to 5. In addition, the substitution, deletion, insertion, addition or inversion of the amino acid includes those caused by artificial variants or natural mutation, if based on the difference in the individual or species of

microorganism.

- [17] The polynucleotide encoding the amino acid sequence in of the present invention may comprise the polynucleotide sequence encoding the protein having amino acid sequence represented by SEQ ID NO: 1, or the amino acid sequence of 80% or more, specifically 90% or more, more specifically 95% or more, and particularly specifically 97% or more homology with the same, as long as it has similar activity as L-aspartate oxidase. The most specifically, it may be the polynucleotide sequence represented by SEQ ID NO: 24.
- [18] The term "homology" refers to the identity between two amino acid sequences and may be determined by the well known method well known to those skilled in the art, using BLAST 2.0 to compute the parameter such as score, identity and similarity.
- [19] In addition, the polynucleotide sequence encoding L-aspartate oxidase of the present invention can be hybridized with the polynucleotide of SEQ ID. NO: 24 or the probe prepared from the same under 'stringent conditions', and may be a variant modified polynucleotide sequence encoding L-aspartate oxidase which normally functions. As used herein, "stringent conditions" refer to conditions which allow the specific hybridization between the polynucleotide, and are described specifically, for example, in Molecular Cloning (A Laboratory Manual, J. Sambrook et al., Editors, 2nd Edition, Cold Spring Harbor Laboratory press, Cold Spring Harbor, New York, 1989) or Current Protocols in Molecular Biology (F.M. Ausubel et al., Editors, John Wiley & Sons, Inc., New York). For example, which describes, for example, the hybridization is carried out in the hybridization buffer of 65°C (3.5 SSC, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 2.5 mM NaH₂PO₄ (pH 7), 0.5% SDS, 2 mM EDTA). SSC is 0.15 M sodium chloride/0.15 M sodium citrate of pH 7. After hybridization, the membrane to which DNA is delivered transfered is rinsed with 2 X SSC at room temperature and then rinsed again with 0.1 to 0.5 X SSC/0.1 X SDS at a temperature of 68°C.
- [20] As used herein, the term "an another amino acid" refers to the other amino acid residue except the amino acid originally located in the amino acid sequence prior to the modification. Specifically the another amino acid of the present invention may include one amino acid selected from the group consisting of arginine, glycine, alanine, serine, threonine, cysteine, valine, leucine, isoleucine, methionine, proline, phenylalanine, tyrosine, tryptophan, aspartic acid, glutamic acid, asparagine, glutamine, and histidine except lysine. Specifically, the another amino acid may include one amino acid selected from the group consisting of arginine, valine, leucine, isoleucine, methionine, tryptophan, and histidine. For example, the another amino acid may include arginine.
- [21] As used herein, the term "302nd" refers to the position of amino acid from the methionine of the amino acid sequence represented by SEQ ID NO: 1, since the me-

thionine of the amino acid sequence is counted to the first amino acid residue.

- [22] In general, the activity of L-aspartate oxidase is regulated by nicotinic acid or NAD accumulated in microorganisms, in other words, its feedback regulation is inhibited by nicotinic acid or NAD. The feedback regulation by nicotinic acid of NAD may be released in the L-aspartate oxidase variants of the present invention, unlike common L-aspartate oxidase.
- [23] The other aspect of the present invention provides a polynucleotide having a nucleotide sequence that encodes for the L-aspartate oxidase variants.
- [24] In an embodiment of the present invention, a polynucleotide may have a nucleotide sequence that encodes a L-aspartate oxidase variant having an amino acid sequence which a 302nd amino acid in the amino acid sequence represented by SEQ ID NO:1 is substituted with another amino acid.
- [25] The 302nd amino acid may include one amino acid selected from the group consisting of arginine, glycine, alanine, serine, threonine, cysteine, valine, leucine, isoleucine, methionine, proline, phenylalanine, tyrosine, tryptophan, aspartic acid, glutamic acid, asparagine, glutamine, and histidine. Accordingly, the nucleotide sequence corresponding to the 302nd amino acid may be appropriately substituted. In a specific embodiment, 904th to 906th nucleotides in a nucleotide sequence represented by SEQ ID NO:24 may be appropriately substituted with any combination of nucleotides except for AAG, AAA, TAA, TAG, and TGA.
- [26] Another aspect of the present invention provides a vector including an above-described polynucleotide, which is operably linked to a regulatory sequence.
- [27] The polynucleotide may have a nucleotide sequence that 904th to 906th nucleotides in the nucleotide sequence represented by SEQ ID NO: 24 are appropriately substituted. In a specific embodiment, the polynucleotide may have a nucleotide sequence that 904th to 906th nucleotides in the nucleotide sequence represented by SEQ ID NO: 24 are substituted with any combination of nucleotides except for AAG, AAA, TAA, TAG, and TGA. The polynucleotide may be operably linked to a regulatory sequence. The regulatory sequence may regulate expression of L-aspartate oxidase, and include a promoter, a terminator, or an enhancer.
- [28] The vector of the present invention is not specifically limited, and may be any vector known in the art. For example, the vector may be pCR2.1-TOPO vector (Invitrogen, U.S.A) or pECCG117 (KFCC-10673), but it's not limited thereof.
- [29] The promoter of the present invention may be a lambda PL promoter, a trp promoter, a lac promoter, a T7 promoter, a pPro promoter, a pCJ1 promoter, or a pCJ7 promoter (Korean Patent No. 10-0620092). In a specific embodiment, the promoter may be a pCJ1 promoter, but it's not limited thereof.
- [30] The promoter may be operably linked to a nucleotide sequence encoding a gene. As

used herein, the term "operably linked" refers to a functional linkage between a nucleic acid expression regulatory sequence (for example, a promoter, a signal sequence, an array of transcriptional regulatory factor binding sites, a terminator, or an enhancer) and other nucleotide sequences. Accordingly, the regulatory sequence may regulate transcription and/or translation of the nucleotide sequence encoding the gene.

- [31] Another aspect of the present invention provides a microorganism comprising an above-described polynucleotide, wherein the polynucleotide may comprise a nucleotide sequence encoding an amino acid sequence which 302nd amino acid in the amino acid sequence represented by SEQ ID NO: 1 is substituted with another amino acid.
- [32] In a specific embodiment, according to the substitution of the 302nd amino acid in the amino acid sequence of SEQ ID NO: 1, part of a nucleotide sequence of SEQ ID NO: 24 may be substituted. For example, the polynucleotide may comprise a polynucleotide that 904th to 906th nucleotides in the nucleotide sequence of SEQ ID NO: 24 are substituted with other nucleotides.
- [33] The polynucleotide may be obtained through random mutation or genetic engineering manipulation. A microorganism, in which part of an amino acid sequence of SEQ ID NO: 1 is partially substituted, may be constructed by transformation of the obtained polynucleotide.
- [34] As used herein, the term "transformation" refers to introducing a gene into a host cell to be expressed therein. The transformed gene may be in any gene, for example, that is inserted into a chromosome of the host cell, or that is out of the chromosome of the host cell, as long as the introduced gene is expressible within the host cell. The gene includes a polynucleotide encoding a polypeptide, such as DNA and RNA. For example, the gene may be introduced in the form of an expression cassette, which is a polynucleotide structure including all the elements required for self-expression of the gene, into a host cell. Typically, the expression cassette may include a promoter operably linked to the gene, a transcription termination signal, a ribosome binding site, and a translation termination signal. The expression cassette may be in the form of an expression vector that is self-replicable. The gene may also be introduced into a host cell by itself or in the form of a polynucleotide structure and operably linked to a sequence that is required for expression in the host cell.
- [35] As used herein, the term "microorganism having an ability to produce quinolinate" refers to a microorganism capable of producing quinolinate from a carbon source in a culture medium and of accumulating quinolinate.
- [36] To improve the ability to produce quinolinate, it is required that microorganisms produce large quantities of quinolinate, and the produced quinolinate can be accumulated without being used in other ways. Therefore, in some embodiments of the

present invention, the microorganism having an improved ability to produce quinolinate may be obtained by removing or weakening the activity of quinolinate phosphoribosyltransferase that is involved in a decomposition pathway of quinolinate, by enhancing the expression or activity of quinolinate synthetase that is involved in a synthetic pathway of quinolinate, or by a combination thereof.

- [37] In a specific embodiment, the microorganism having an improved ability to produce quinolinate may be further modified to enhance the activity of quinolinate synthetase. Specifically, the enhanced activity of the quinolinate synthetase may be achieved by additionally introducing the quinolinate synthetase to increase the expression thereof in the microorganism. The enhanced activity of the quinolinate synthetase may also be achieved by replacing a promoter linked to the quinolinate synthetase in the microorganism with a strong promoter. In addition, the enhanced activity of the quinolinate synthetase may be achieved by increasing the activity of the quinolinate synthetase itself.
- [38] In the case where the heterogenous quinolinate synthetase is introduced, a polynucleotide encoding this enzyme may be introduced to increase the expression of the polynucleotide. The polynucleotide encoding the quinolinate synthetase may be expressed in a plasmid of the microorganism or may be inserted into a chromosome of the microorganism and expressed therein.
- [39] The quinolinate synthetase may have an amino acid sequence represented by SEQ ID NO: 29 or may have an amino acid sequence that is homologous thereto. In other words, it is not limited thereto, because there may be the difference in the amino acid sequence of the protein depending on the microbial species or strains. It can be a mutant protein or artificial variant with an amino acid sequence comprising substitution, deletion, insertion, or addition of one or several amino acids at one or more locations of the amino acid sequence of represented by SEQ ID NO: 29, as long as it can synthesize quinolinic acid from iminosuccinic acid. The sequence of gene *nadA* encoding this enzyme can be obtained from the genome sequence (gi: GI:89109380) of *Escherichia coli* (*E. coli*) as disclosed in an article (Mol Syst Biol., 2006;2:2006.0007., Epub 2006 Feb 21) or the database available from the National Center for Biotechnology Information (NCBI) or the DNA Data Bank of Japan (DDBJ). Also, the polynucleotide encoding the amino acid sequence of the present invention may comprise the polynucleotide sequence encoding the protein having amino acid sequence represented by SEQ ID NO: 29, or the amino acid sequence of 80% or more, specifically 90% or more, more specifically 95% or more, and particularly specifically 97% or more homology with the same, as long as it has similar activity as L-aspartate oxidase. The most specifically, it may be the polynucleotide sequence represented by SEQ ID NO: 26.

[40] The quinolinate synthetase has an activity to synthesize quinolinic acid from iminosuccinic acid, as shown in Reaction Scheme 2.

[41] <Reaction Scheme 2>

[42] α -iminosuccinate + Dihydroxyacetone phosphate $\rightleftharpoons$ Quinolate + Phosphate + 2H₂O

[43] Therefore, when the expression of a gene encoding the quinolinate synthetase or the activity of this enzyme is enhanced, the yield of quinolate in cells may be increased.

[44] In some embodiments, in the microorganism having an ability to produce quinolate, the activities of aspartic acid oxidase and quinolinate synthetase may be enhanced by substituting endogenous promoters with strong promoters, by inducing a mutation in the promoters, or by increasing the copy number of the genes. For the substitution with strong promoters, generally known strong promoters, including pTac, pTrc, pPro, pR, pL, pCJ1, pCysK, and the like, may be used.

[45] In a specific embodiment, there is provided a microorganism having an improved ability to produce quinolate, wherein the activity of quinolate phosphoribosyltransferase may be additionally reduced or removed.

[46] The activity of quinolate phosphoribosyltransferase may be reduced or removed by modifying a gene encoding quinolate phosphoribosyltransferase or by using a microRNA that suppresses transcription.

[47] The quinolate phosphoribosyltransferase may have an amino acid sequence represented by SEQ ID NO: 30 or an amino acid sequence that is highly homologous thereto. In other words, it is not limited thereto, because there may be the difference in the amino acid sequence of the protein depending on the microbial species or strains. It can be a mutant protein or artificial variant with an amino acid sequence comprising substitution, deletion, insertion, or addition of one or several amino acids at one or more locations of the amino acid sequence of represented by SEQ ID NO: 30, as long as it can synthesize nicotinic acid (nicotinate) mononucleotide from quinolate.

[48] The quinolate phosphoribosyltransferase may have an activity to synthesize nicotinic acid mononucleotide from quinolate, as shown in Reaction Scheme 3. Therefore, the yield of quinolate in cells may be increased by deleting a gene having the activity to synthesize nicotinic acid mononucleotide or by weakening the activity of the gene.

[49] <Reaction Scheme 3>

[50] 5-Phospho- α -D-ribose 1-diphosphate + Quinolate + 2H⁺ $\rightleftharpoons$ CO₂ + Diphosphate + Nicotinate ribonucleotide

[51] Weakening or removing the activity of quinolate phosphoribosyltransferase may be performed by substituting an endogenous gene encoding quinolate phosphoribosyltransferase with a modified gene to weaken or remove the activity of the enzyme, by

replacing a promoter of the endogenous gene with a weak promoter, or by deleting the endogenous gene encoding the enzyme from chromosome.

- [52] In a specific embodiment, in the microorganism having an improved activity to produce quinolinate, the activity of quinolinate phosphoribosyltransferase converting quinolinate into nicotinic acid mononucleotide may be removed. To this end, the gene *nadC* encoding quinolinate phosphoribosyltransferase may be removed from the genome of the microorganism by homologous recombination. The sequence of the gene *nadC* may be obtained from the genome sequence (GI:89106990) of *E. coli* as disclosed in an article (Mol Syst Biol., 2006;2:2006.0007, Epub 2006 Feb 21), or the database available from the National Center for Biotechnology Information (NCBI) or the DNA Data Bank of Japan (DDBJ). Also, the polynucleotide encoding the amino acid sequence in of the present invention may comprise the polynucleotide sequence encoding the protein having amino acid sequence represented by SEQ ID NO: 30, or the amino acid sequence of 80% or more, specifically 90% or more, more specifically 95% or more, and particularly specifically 97% or more homology with the same, as long as it has similar activity as L-aspartate oxidase. The most specifically, it may be the polynucleotide sequence represented by SEQ ID NO: 25.
- [53] In a specific embodiment, the microorganism having an ability to produce quinolinate may be a prokaryotic microorganism or an eukaryotic microorganism.
- [54] In some embodiments, examples of the microorganism having an ability to produce quinolinate may belong to the genus *Enterbacter*, genus *Escherichia*, genus *Erwinia*, genus *Serratia*, genus *Providencia* genus, genus *Corynebacterium*, and genus *Brevibacterium*, but it's not limited thereto.
- [55] In a specific embodiment, the microorganism having an ability to produce quinolinate may be belong to the genus *Escherichia*.
- [56] Specifically, the microorganism having an ability to produce quinolinate may be *Escherichia coli* (*E. coli*).
- [57] Another aspect of the present invention provides a method of producing quinolinate, comprising: culturing a microorganism that includes a polynucleotide encoding a L-aspartate oxidase which a 302nd amino acid in an amino acid sequence represented by SEQ ID NO:1 is substituted with another amino acid; and recovering quinolinate from a cultured solution.
- [58] The culturing of the microorganism may be performed using a suitable culture medium under suitable culture conditions that are well-known in the art. Such culturing procedures may be used by one of ordinary skill in the art and may be readily adjusted depending on a selected microorganism. The culturing method may include a batch culture type, a continuous culture type, and a fed-batch culture type, but it's not limited thereto. Various examples of culturing methods are disclosed in, for example,

"Biochemical Engineering" (by *James M. Lee*, Prentice-Hall International Editions, pp 138-176").

- [59] The culture medium used in the culturing process is required to satisfy suitable conditions for a selected microorganism. Various culture media for microorganisms are disclosed in, for example, "Manual of Methods for General Bacteriology (by the American Society for Bacteriology, Washington D.C., U.S.A, 1981)". For example, the culture medium may include various carbon sources, nitrogen sources, and trace elements.
- [60] Examples of carbon sources available for the culture medium may include carbohydrates, such as glucose, sucrose, lactose, fructose, maltose, and starch; oils and fats, such as soybean oil, sunflower oil, castor oil, and coconut oil; fatty acids, such as palmitic acid, stearic acid, and linoleic acid; alcohols, such as glycol and ethanol; and organic acids, such as acetic acid, which may be used alone or in combination, but it's not limited thereto.
- [61] Examples of nitrogen sources available for the culture medium may include organic nitrogen sources, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor (CSL), soybean flour, and urea; and inorganic nitrogen sources, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate, and ammonium nitrate, which may be used alone or in combination, but it's not limited thereto.
- [62] Examples of phosphorous sources available for the culture media may include potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and corresponding sodium-containing salts. In some embodiments, the culture medium may also include metal salts such as magnesium sulfate or iron sulfate. In some embodiments, the culture medium may further include amino acids, vitamins, and suitable precursors, in addition to the above-listed components. The culture medium for culturing microorganisms, or individual components may be added to a culture solution in a batch or continuous manner.
- [63] In some embodiments, during the culture, the pH of the culture solution may be adjusted by adding a compound, for example, ammonium hydroxide, potassium hydroxide, ammonia, phosphoric acid, or sulfuric acid in a proper manner. In addition, during the culture, foaming in the culture solution may be suppressed using an anti-foaming agent such as a fatty acid, for example, polyglycol ester. To keep the culture solution in an aerobic condition, oxygen or an oxygen-containing gas (for example, air) may be supplied into the culture solution. The temperature of the culture solution may be maintained in a temperature range of about 20°C to about 45°C, specifically about 25°C to about 40°C. The culturing may be maintained until a target quantity of quinolinate is obtained, specifically, the period may be about 10 hours to 160 hours.

- [64] Another aspect of the present invention provides a method of producing nicotinic acid, comprising: culturing a microorganism that includes a polynucleotide encoding L-aspartate oxidase which 302nd amino acid in an amino acid sequence represented by SEQ ID NO:1 is substituted with another amino acid; and conducting decarboxylation reaction by adding an acid to a cultured product.
- [65] As used herein, the term "decarboxylation reaction" refers to a reaction to produce nicotinic acid by removing a carboxyl group from quinolinate and releasing carbon dioxide.
- [66] In particular, after the culturing of the microorganism, the resulting quinolinate-including culture solution may be subjected to centrifugation or membrane filtration to remove the microorganism. Then, to accelerate the decarboxylation reaction, an acid that provides a hydrogen group may be added into the quinolinate-including culture solution. Any acid may be used without limitation, as long as it can provide hydrogen group to the culture solution.
- [67] In an embodiment, the quinolinate-including culture solution may be used without purification.
- [68] In an embodiment, the acid being added into the culture solution may be hydrochloric acid or sulfuric acid.
- [69] In an embodiment, after the addition of the acid, the culture solution may have a pH of about 5 or less, or may specifically be in a range of about 2 to about 3.
- [70] In an embodiment, the decarboxylation reaction of the culture solution may be performed at a temperature of about 100°C to about 150°C, or may specifically be in a range of about 120°C to about 135°C.
- [71] In an embodiment, the decarboxylation reaction of the culture solution may be performed at a pressure of about 0.1 MPa to about 0.5 Mpa, or may specifically be performed at a pressure of about 0.2 MPa to about 0.4 MPa.
- [72] Upon conducting the decarboxylation under high-temperature and high-pressure conditions for about 1 hour to 3 hours after the addition of an acid into the quinolinate-including culture solution, quinolinate in the culture solution may be converted into nicotinic acid, as shown in Reaction Scheme 4.
- [73] <Reaction Scheme 4>
- [74] $\text{Quinolinate} + 2\text{H}^+ \rightleftharpoons \text{CO}_2 + \text{Nicotinic acid}$
- [75] In an embodiment, the method of producing nicotinic acid may further include recovering and purifying the nicotinic acid.
- [76] In an embodiment, the recovering of nicotinic acid may be conducted by a common method known in the art, including filtration of the culture solution and crystallization processes.

Advantageous Effects of Invention

- [77] According to embodiments of the present invention, quinolinate may be effectively produced by culturing a microorganism including a L-aspartate oxidase variant that the feedback regulation by nicotinic acid or NAD. Nicotinic acid is released. These methods using the microorganism may solve the problems from the conventional chemical synthesis methods, regarding environmental issues due to generation of catalyst byproducts, high-energy consumption and use of non-renewable resources, and the problem of low yield with the conventional biosynthesis methods. Consequently, these methods may efficiently produce quinolinate and nicotinic acid in an environmental-friendly manner.

Brief Description of Drawings

- [78] FIG. 1 illustrates a pathway to produce nicotinic acid in a method of producing nicotinic acid according to an embodiment of the present invention.

Mode for the Invention

- [79] The present invention will be described in further detail with reference to the following examples. These examples are for illustrative purposes only and are not intended to limit the scope of the invention.

[80]

- [81] **Example 1. Preparation of L-aspartate oxidase variants NAD-feedback regulation is released**

- [82] **<1-1> Construction of plasmid expressing L-aspartate oxidase**

- [83] To prepare L-aspartate oxidase variants, an *E. coli*-derived *nadB* gene encoding the wild L-aspartate oxidase was cloned in an expression vector. To this end, a chromosome of *E. coli* K12 W3110 strain was used as a template. The strain was purchased from the American Type Culture Collection (ATCC No.23257). Based on the nucleotide sequence for the *nadB* gene (NCBI Registration No. "GI:89109380") represented by SEQ ID NO: 24 obtained from the GenBank of the National Institute of Health (NIH GenBank). Primers of SEQ ID NOs: 2 and 3 having the recognition sites of restriction enzymes NdeI and BamHI are constructed to amplify the ORF region of the *nadB* gene, for gene cloning.

- [84] Table 1

[Table 1]

Nucleotide sequence	SEQ ID NO
5' AATTCATATGAATACTCTCCCTGAACATT 3'	2
5' AATTGGATCCCTATAACCACTACGCTTGATCAC 3'	3

- [85] PCR was conducted using chromosomal DNA of *E. coli* K12 W3110 as the template

and oligonucleotides represented by SEQ ID NOs: 2 and 3 as primers. The polymerase used was PfuUltra™ DNA polymerase (Stratagene, U.S.A.), and PCR was conducted by repeating 30 times of a cycle comprising denaturation at 96°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 2 minutes. Through the PCR, an amplified gene of about 1.9 kb including the *nadB* ORF gene and the recognition sites of restriction enzymes NdeI and BamHI was obtained.

- [86] The *nadB* gene obtained through the PCR was recovered by agarose gel elution, and then treated with the restriction enzymes NdeI and BamHI, followed by ligation into a pProLar vector (CloneTech, U.S.A) treated with the restriction enzymes NdeI and BamHI so as to express L-aspartate oxidase from the *nadB* gene linked to a pPro promoter. The resulting vector was named "pPro-*nadB* vector".

[87] **<1-2> Construction of plasmid library of L-aspartate oxidase variant**

- [88] To obtain L-aspartate oxidase variants, a *nadB* gene variant library was constructed using the pPro-*nadB* vector obtained in <1-1> as a template by error-prone PCR in the presence of dGTP and MnSO₄.

- [89] In the error-prone PCR using the pPro-*nadB* recombinant vector as a template, primers 4 and 5 were used, concentrations of dGTP and MnSO₄ in a PCR mix, which were used to control a GC rate and an error rate, were 2 mM and 8 mM, respectively, and the polymerase used was PfuUltra™ DNA polymerase (Stratagene, U.S.A.). PCR was conducted by repeating 30 times of a cycle comprising denaturation at 96°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 2 minutes. Through the PCR, a DNA fragment of about 1.9 kb was obtained. This DNA fragment was purified via agarose gel elution, treated with the restriction enzyme DpnI (NEB, U.S.A) for about 1 hour, and then transformed into *E.coli* DH5α strain (Invitrogen, U.S.A.) via CaCl₂ method. The transformed *E.coli* DH5α strain was smeared on a Luria-Bertani (LB)-kanamycin(Km) (yeast extract 10 g/L, NaCl 5 g/L, tryptone 10 g/L, kanamycin 25 μg/L) plate medium, and cultured overnight at 37°C to obtain kanamycin-resistant colonies. Ten clones were randomly selected from them, followed by sequencing. As a result, the error rate of the *nadB* gene was estimated to be about 4.5 nucleotide/1 kb. The number of strains with a *nadB* gene variant obtained was about 3x 10⁵ or more.

- [90] Table 2

[Table 2]

Nucleotide sequence	SEQ ID NO
5' CTCGAGCATAGCATT TTTTATCC 3'	4
5' CAGTGAGCGAGGAAGCGG 3'	5

[91] **<1-3> Selection of L-aspartate oxidase variants showing released NAD-feedback regulation**

[92] **<1-3-1>Construction of L-aspartate oxidase-deficient strain**

[93] The *nadB* gene negatively affects on the production of quinolinate under the feedback regulation by NAD (J Biol Chem. 1982 Jan 25;257(2):626-32.). For this reason, it is important to discover a *nadB* gene the feedback regulation by NAD is released. To effectively selecting better *naB* gene variants, an endogenous *nadB* gene was removed from a strain. Based on the nucleotide sequence for the *nadB* gene (NCBI Registration No. "GI:89109380") represented by SEQ ID NO: 24 obtained from the GenBank of the National Institute of Health (NIH GenBank), primers of SEQ ID NOs: 6 and 7 able to amplify the downstream region of the *nadB* gene, primers of SEQ ID NOs: 8 and 9 able to amplify the upstream region of the *nadB* gene, and primers of SEQ ID NOs: 10 and 11 able to amplify *loxpCm* were constructed.

[94] Table 3

[Table 3]

Nucleotide sequence	SEQ ID NO
5' CATTATACGAACGGTACCCCAAAGCCTGGGTCAGCGCCGT 3'	6
5' GGCGGATATTCAGCAGTGG 3'	7
5' CCCAAACCAAATTTCCACG 3'	8
5' CGGTAGGTACCGAGCTCGAATTTCTTTGTTTAATTTACTA 3'	9
5' TAGTAAATTAAACAAAGAAATTCGAGCTCGGTACCTACCG 3'	10
5' ACGGCGCTGACCCAGGCTTTGGGGTACCGTTCGTATAATG 3'	11

[95] PCR was performed using a chromosomal DNA of *E. coli* K12 W3110 (ATCC NO. 23257) as a template and oligonucleotides of SEQ ID NOs. 6 and 7 as primers to amplify the upstream and SEQ ID NOs. 8 and 9 as primers to amplify downstream regions of *nadB* gene of 0.4 kb and 0.4 kb, respectively. In addition, PCR was performed using the plasmid vector containing *loxpCm*, pLoxpCat2 vector, as a template and oligonucleotides of SEQ ID NOs. 10 and 11 as primers to amplify *loxpCm* gene having the sequence homologous to *nadB* gene on both ends of 1.0 kb. The polymerase used was PfuUltra™ DNA polymerase (Stratagene, U.S.A), and the PCR was conducted by repeating 30 times of a cycle comprising denaturation at 96°C

for 30 seconds, annealing at 53°C for 30 seconds, and extension at 72°C for 1 minute, followed by agarose gel elution to obtain a *nadB*-upstream fragment, a *nadB* - downstream fragment, and a *loxpCm* fragment. PCR was conducted using these obtained fragments as templates under the PCR conditions by repeating 10 times of the cycle comprising denaturation at 96°C for 60 seconds, denaturation at 50°C for 60 seconds, and extension at 72°C for 1 minute, and 20 repetition of the cycle after addition of primers of SEQ ID NOs. 7 and 8. As a result, a *nadB*-deficient cassette containing the upstream region of *nadB* gene-*loxpCm*-downstream region of *nadB* gene of 1.8 kb was obtained.

[96] *E. coli* K12 W3110 strain containing pKD46 as lambda red recombinase expression vector was transformed with the *nadB*-deficient cassette by electroporation, and then the strain was smeared on a LB plate medium (tryptone 10 g, yeast extract 5 g, NaCl 10 g, chloramphenicol 15 µg/L, and agar 1.5 %) containing chloramphenicol as a selective marker. Then cultured at 37°C overnight to select microorganism strains exhibiting resistance against chloramphenicol.

[97] The selected strains were directly used as templates to perform PCR using primers of SEQ ID NOs. 7 and 8 under the same conditions. The gene sizes of the wild strain and the *nadB*-deficient strain were identified to be 2.4 kb and 0.8 kb, respectively on 1.0% agarose gel, thereby confirming the deletion of *nadB* gene in *E. coli* K12 W3110. The construction of the *nadB*-deficient strain, named *W3110-ΔnadB*, was completed.

[98] **<1-3-2> Selection of L-aspartate oxidase variants**

[99] To find a *nadB* gene resistant to the feedback regulation by NAD, 6-aminopyridine-3-carboxylic acid (6-NA, purchased from Aldrich, U.S.A) as a nicotinic acid analogue, and 6-aminonicotinamide (6-Nm, purchased from Aldrich, U.S.A) as a nicotinamide analogue were used. Although a 6-NAD analogue should be used, due to the structural similarity of NAD with nicotinic acid and nicotinamide, resistant *nadB* gene variants were primarily selected using the nicotinic acid analogue and nicotinamide analogue, and then the NAD-feedback resistance of each of the selected *nadB* gene variants was confirmed (ref. Journal of bacteriology, June 1983, p.1126-1136). L-aspartate oxidase variant plasmid library constructed in <1-2> of Example 1 was transformed into *W3110-ΔnadB* strain via CaCl₂ method.

[100] The transformed *W3110-ΔnadB* strain was smeared on a M9-(6,NA)-(6,Nm) plate medium (NaHPO₄-7H₂O 12.8 g, KH₂PO₄ 3 g, NaCl 0.5 g, NH₄Cl 1 g, MgSO₄ 2 mM, CaCl₂ 0.1 mM, casamino acid 1 g, 6-aminopyridine-3-carboxylic acid (6-NA) 0.3 g/L, 6-aminonicotinamide (6-Nm) 0.3 g/L) to select colonies with higher growth rate compared to the wild *nadB*. A total of 1 x 10⁷ colonies were screened and three of them were selected, and then named pPro-*nadB*64, pPro-*nadB*67, and pPro-*nadB*110, respectively. The selected three strains were each inoculated and cultured in a

M9-(6,NA)-(6,Nm) liquid medium (NaHPO₄-7H₂O 12.8 g, KH₂PO₄ 3 g, NaCl 0.5 g, NH₄Cl 1 g, MgSO₄ 2 mM, CaCl₂ 0.1 mM, casamino acid 1 g, 6-aminopyridine-3-carboxylic acid (6-NA) 0.3 g/L, 6-aminonicotinamide (6-Nm) 0.3 g/L), and then optical densities (OD) thereof were compared. As a result, the growth rates of pPro-nadB67 were found to be higher than those of the pPro-nadB64 and pPro-nadB72 in the media containing 6-NA and 6-Nm, as shown in Table 4. Accordingly, the pPro-nadB67 was selected and sequence thereof was identified. As a result, the pPro-nadB67 was found to include arginine as substituent for lysine, the 302nd amino acid from the start amino acid methionine.

[101] *E. coli* K12 W3110 which is deficient of *nadC* gene, and contains a pPro-nadB67(Lys302Arg)-pCJ-nadA by using pProlar vector as an expression vector for nadB gene variant, was named CV01-0518. Then it was deposited under the Budapest Treaty at the Korean Culture Center of Microorganisms (KCCM) with Accession No. KCCM11434P on June 20, 2013.

[102] Table 4

[Table 4]

Growth rate comparison of three *nadB* variants in a liquid media containing 6-NA and 6-Nm

nadB name	OD (4hr)	OD (12hr)
pPro nadB	0.24	1.06
pPro nadB64	0.44	1.12
pPro nadB67	0.62	1.30
pPro nadB72	0.46	1.18

[103]

[104] **Example 2. Quinolinic acid yield comparison between nadB gene variants**

[105] **<2-1> Construction of quinolinate phosphoribosyltransferase-deficient strain**

[106] PCR was conducted using on chromosomal DNA of *E. coli* K12 W3110 as a template to obtain a *nadC* gene that is involved in the decomposition pathway of quinolinate. Based on the nucleotide sequence for the *nadC* gene (NCBI Registration No. "GI:89106990") represented by SEQ ID NO: 25 obtained from the GenBank of the National Institute of Health (NIH GenBank), primers represented by SEQ ID NOs: 12 and 13 able to amplify the downstream region of the *nadC* gene, primers of SEQ ID NOs: 14 and 15 able to amplify the upstream and downstream regions of the *nadC* gene, and *loxP*Cm gene, and primers of SEQ ID NOs: 16 and 17 able to amplify the upstream region of the *nadC* gene were constructed.

[107] Table 5

[Table 5]

Nucleotide sequence	SEQ ID NO
5' CATTATACGAACGGTACCCCCAGTTGAATAAACACCTCTTCA 3'	12
5' TGGCGGCAGGCTAATATT 3'	13
5' GTTCTTCCAGATTCTCTACTTTTCGAGCTCGGTACCTACCG 3'	14
5' TGAAGAGGTGTTTATTCAACTGGGGGTACCGTTCGTATAATG 3'	15
5' ATAACCACCATCAGTTCGATA 3'	16
5' CGGTAGGTACCGAGCTCGAAAAGTAGAGAATCTGGAAGAAC 3'	17

[108] PCR was performed using chromosomal DNA of *E. coli* K12 W3110 as a template and oligonucleotides of SEQ ID NOs: 12 and 13, and 16 and 17 as primers to amplify the upstream and downstream regions of *nadC* gene of 0.5 kb and 0.3 kb, respectively. In addition, PCR was performed using a plasmid vector including *loxpCm*, pLoxpCat2 vector, as a template and oligonucleotides of SEQ ID NOs. 14 and 15 as primers to amplify *loxpCm* gene having the sequence homologous to *nadC* gene on both ends of 1.0 kb. The polymerase used was PfuUltra™ DNA polymerase (Stratagene, U.S.A), and PCR was conducted by repeating 30 times of a cycle comprising denaturation at 96°C for 30 seconds, annealing at 53°C for 30 seconds, and extension at 72°C for 1 minute. As a result, a *nadC*-upstream fragment, a *nadC*-downstream fragment, and a *loxpCm* fragment were obtained. PCR was conducted using these fragments resulting from the above-described PCR as templates under the PCR conditions including 10 repetition of the cycle comprising denaturation at 96°C for 60 seconds, denaturation at 50°C for 60 seconds, and extension at 72°C for 1 minute, and 20 repetition of the cycle after addition of primers of SEQ ID NOs: 12 and 17. As a result, a *nadC*-deficient cassette containing the upstream region of *nadC* gene-*loxpCm*-downstream region of *nadC* gene of 1.8 kb was obtained.

[109] *E. coli* K12 W3110 strain containing pKD46 as expression vector of lambda red re-combinase was transformed with the *nadC*-deficient cassette by electroporation, and then the strain was smeared on a LB plate medium (tryptone 10 g, yeast extract 5 g, NaCl 10 g, and agar 1.5 %) containing chloramphenicol as a selective marker, and cultured at 37°C overnight to select microorganism strains exhibiting resistance against chloramphenicol.

[110] The selected strains were directly used as templates to perform PCR using primers of SEQ ID NOs: 13 and 16 under the same conditions, and the gene sizes of the wild strain and the *nadC*-deficient strain were identified to be 1.6 kb and 1.3 kb, respectively on 1.0% agarose gel, thereby confirming the deletion of *nadC* gene in *E. coli* K12 W3110. The construction of the *nadC*-deficient strain, named *W3110-ΔnadC*, was completed.

[111] **<2-2> Construction of plasmid for expression of aspartate oxidase and quinolinate synthetase**

[112] Two enzymes, aspartate oxidase and quinolinate synthetase, are required to produce quinolinate. Accordingly, a plasmid able to express both *nadB* and *nadA* genes encoding the two enzymes was constructed. First, PCR was performed using chromosomal DNA of *E. coli* W3110 to obtain *nadA* gene encoding quinolinate synthetase. Based on the nucleotide sequence for the *nadA* gene (NCBI Registration No. "GI: 89107601") represented by SEQ ID NO: 26 obtained from the GenBank of the National Institute of Health (NIH GenBank), primers of SEQ ID NOs: 18 and 19 able to amplify the ORF region of the *nadA* gene including ATG and TAA regions and having the recognition sites of restriction enzymes *ApaI* and *NotI* were synthesized.

[113] Table 6

[Table 6]

Nucleotide sequence	SEQ ID NO
5' AATTGGGCCCCATGAGCGTAATGTTTGATCCA 3'	18
5' AATTGCGGCCGCTCGTGCCTACCGCTTCG 3'	19

[114] PCR was performed using chromosomal DNA of *E. coli* K12 W3110 as a template, and oligonucleotides of SEQ ID NOs: 18 and 19 as primers. The polymerase used was PfuUltra™ DNA polymerase (Stratagene, U.S.A), and the PCR was conducted by repeating 30 times of a cycle comprising denaturation at 96°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 1 minute. As a result, an amplified gene of about 1.0 kb including the *nadA* gene and the recognition sites of restriction enzymes *ApaI* and *NotI* was obtained.

[115] A pCJ1 promoter was obtained through PCR using a plasmid including the pCJ1 promoter as a template, based on the disclosure in Korean laid-open Patent No. 10-2006-0068505. Primers represented by SEQ ID NOs: 20 and 21 having the recognition sites of restriction enzymes *BamHI* and *ApaI* were constructed for ligating the pCJ1 promoter with the amplified *nadA* gene.

[116] Table 7

[Table 7]

Nucleotide sequence	SEQ ID NO
5' CCGCGGATCCCACCGCGGGCTTATTCCATTAC 3'	20
5' GATGGGCCCATCTTAATCTCCTAGATTGGGTTTC 3'	21

- [117] PCR was performed using chromosomal DNA of *E. coli* K12 W3110 as a template, and oligonucleotides of SEQ ID NOs: 20 and 21 as primers. The polymerase used was PfuUltra™ DNA polymerase (Stratagene, U.S.A), and the PCR was conducted by repeating 30 times of a cycle comprising denaturation at 96°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 1 minute. As a result, an amplified fragment of about 0.3 kb including the pCJ1 promoter and the recognition sites of the restriction enzymes BamHI and ApaI was obtained.
- [118] The *nadA* gene obtained through the PCR was treated with restriction enzymes ApaI and NotI, and the amplified pCJ1 promoter fragment was treated with restriction enzymes ApaI and BamHI. The *nadA* gene and pCJ1 promoter fragment treated with the restriction enzymes, respectively, were cloned via ligation into the *pPro-nadB* vector obtained in <1-1> that was treated with the restriction enzymes NotI and BamHI. Ultimately a *pPro-nadB-pCJ1-nadA* recombinant vector of 5.9 kb was constructed, in which the *nadB* gene expression is regulated by the pPro promoter as a constitutive promoter, and the *nadA* gene expression is regulated by pCJ1 promoter. The constructed *pPro-nadB-pCJ1-nadA* had the sequence represented by SEQ ID NO: 27. In addition, through the above-described processes, a recombinant vector pPro-nadB67-pCJ1-nadA including the *nadB* variant and the wild *nadA* gene was also constructed. The constructed *pPro-nadB67-pCJ1-nadA* had the sequence represented by SEQ ID NO: 28.
- [119] **<2-2-1> Construction of vector in which 302nd amino acid of nadB gene of pPro-nadB67-pCJ1-nadA is substituted with amino acid except for lysine and arginine**
- [120] To confirm the resistance against NAD-feedback regulation of nadB67 gene variant, including arginine instead of lysine as the 302nd amino acid starting from methionine of L-aspartate oxidase, the 302nd amino acid of the *nadB* gene was substituted with each of 18 different amino acids, except for lysine and arginine. Quick mutagenesis using the recombinant vector pPro-nadB67-pCJ1-nadA as a template was performed. The polymerase used was PfuUltra™ DNA polymerase (Stratagene, U.S.A.). PCR was conducted by repeating 18 times of a cycle comprising denaturation at 96°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 15 minutes. The resulting PCR product was treated with the restriction enzyme DpnI (NEB, U.S.A), and then transformed into *E.coli* DH5α strain (Invitrogen, U.S.A.) via CaCl₂ method.

The transformed *E.coli* DH5 α strain was smeared on a LB-Km plate medium (yeast extract 10 g/L, NaCl 5 g/L, tryptone 10 g/L, kanamycin 25 μ g/L), and cultured overnight at 37°C to select kanamycin-resistant colonies. The resulting colonies were subjected to plasmid extraction and sequencing to identify the substitution of the 302nd amino acid with the 18 different amino acids. The resulting 18 *nadB* variants as plasmids were named as in Table 2.

[121] Table 8

[Table 8]

L-aspartate oxidase variants in which 302nd amino acid was substituted with another amino acid

Name	Original 302 nd amino acid of <i>nadB</i>	Substituted amino acid
pPro-nadB67-pCJ1-nadA	Lysine	Arginine
pPro-nadB67(G)-pCJ1-nadA		Glycine
pPro-nadB67(A)-pCJ1-nadA		Alanine
pPro-nadB67(S)-pCJ1-nadA		Serine
pPro-nadB67(T)-pCJ1-nadA		Threonine
pPro-nadB67(C)-pCJ1-nadA		Cysteine
pPro-nadB67(V)-pCJ1-nadA		Valine
pPro-nadB67(L)-pCJ1-nadA		Leucine
pPro-nadB67(I)-pCJ1-nadA		Isoleucine
pPro-nadB67(M)-pCJ1-nadA		Methionine
pPro-nadB67(P)-pCJ1-nadA		Proline
pPro-nadB67(F)-pCJ1-nadA		Phenylalanine
pPro-nadB67(Y)-pCJ1-nadA		Tyrosine
pPro-nadB67(W)-pCJ1-nadA		Tryptophan
pPro-nadB67(D)-pCJ1-nadA		Aspartic acid
pPro-nadB67(E)-pCJ1-nadA		Glutamic acid
pPro-nadB67(N)-pCJ1-nadA		Asparagine
pPro-nadB67(Q)-pCJ1-nadA		Glutamine
pPro-nadB67(H)-pCJ1-nadA		Histidine

[122] <2-3> Evaluation of quinolinate-producing ability of wild *nadB* and *nadB67*

variants with respect to NAD concentration

- [123] To compare the ability to produce quinolinate in the wild type *nadB* gene and *nadB* gene variants with respect to NAD concentration, a quinolinate titer assay was conducted. After the quinolinate-producing strain (W3110- Δ *nadC*) obtained in <2-1> of Example 2 was transformed with the plasmid *pPro-nadB67-pCJq1-nadA* constructed in <2-2> of Example 2, and each of the 18 types of *nadB67* variants constructed in <2-2-1> of Example 2 via CaCl_2 method. The transformed strains were cultured overnight in a LB-Km plate medium in a 37°C culture batch to obtain a single colony, and then the single colony was inoculated by one platinum pool into 25 ml of a culture medium for the quinolinate potency assay and cultured at 37°C for 24 hours to 72 hours by shaking at 250 rpm. Table 9 shows the composition of the culture medium for producing quinolinate. To identify the NAD feedback resistance of the *nadB67* variants, nicotinic acid (NA) was added to the culture medium at different concentrations, followed by comparison of the produced quantities of quinolinate.

- [124] Table 9

[Table 9]

Composition of the culture medium for quinolinate potency assay

Composition	Concentration (per liter)
Glucose	70 g
Ammonium sulfate	17 g
KH_2PO_4	1.0 g
$\text{MgSO}_4 \cdot \text{H}_2\text{O}$	0.5 g
$\text{FeSO}_4 \cdot \text{H}_2\text{O}$	5 mg
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	5 mg
ZnSO_4	5 mg
Calcium carbonate	30 g
Yeast extract	2 g

- [125] The quantity of quinolinate (quinolinic acid, QA) in each of the culture solutions was analyzed by high performance liquid chromatography (HPLC). The results are shown in Table 10. Table 10 shows the ability to produce quinolinate of the *nadB67* variants with respect to NAD concentration.

- [126] Table 10

[Table 10]

Production quantities of quinolinate with respect to NAD concentrations

Strain	Plasmid	QA (mg/L)		
		NA 0uM	NA 5uM	NA 10uM
W3110- Δ nadC	pPro-nadB-pCJ-nadA	3500	1600	700
	pPro-nadB67-pCJ-nadA	4553	3860	3251
	pPro-nadB67(G)-pCJ-nadA	4560	3830	3058
	pPro-nadB67(A)-pCJ-nadA	4560	3830	3058
	pPro-nadB67(S)-pCJ-nadA	4560	3830	3058
	pPro-nadB67(T)-pCJ-nadA	4560	3830	3058
	pPro-nadB67(C)-pCJ-nadA	4560	3830	3058
	pPro-nadB67(V)-pCJ-nadA	4425	3937	3280
	pPro-nadB67(L)-pCJ-nadA	4425	3937	3280
	pPro-nadB67(I)-pCJ-nadA	4425	3937	3280
	pPro-nadB67(M)-pCJ-nadA	4425	3937	3280
	pPro-nadB67(P)-pCJ-nadA	2223	1879	1975
	pPro-nadB67(F)-pCJ-nadA	4241	3795	3141
	pPro-nadB67(Y)-pCJ-nadA	4241	3795	3141
	pPro-nadB67(W)-pCJ-nadA	4707	3715	3186
	pPro-nadB67(D)-pCJ-nadA	100	100	100
	pPro-nadB67(E)-pCJ-nadA	100	100	100
	pPro-nadB67(N)-pCJ-nadA	4552	3939	3171
	pPro-nadB67(Q)-pCJ-nadA	4552	3939	3171
	pPro-nadB67(H)-pCJ-nadA	4891	3751	3255

[127] Table 11

[Table 11]

Relative yield of quinolinate with respect to NAD concentration

Strain	Plasmid	Relative Percentage (%)		
		NA 0 uM	NA 5 uM	NA 10 uM
W3110- Δ nadC	pPro-nadB-pCJ-nadA	100	46	20
	pPro-nadB67-pCJ-nadA	100	85	71
	pPro-nadB67(G)-pCJ-nadA	100	84	67
	pPro-nadB67(A)-pCJ-nadA	100	84	67
	pPro-nadB67(S)-pCJ-nadA	100	84	67
	pPro-nadB67(T)-pCJ-nadA	100	84	67
	pPro-nadB67(C)-pCJ-nadA	100	84	67
	pPro-nadB67(V)-pCJ-nadA	100	89	74
	pPro-nadB67(L)-pCJ-nadA	100	89	74
	pPro-nadB67(I)-pCJ-nadA	100	89	74
	pPro-nadB67(M)-pCJ-nadA	100	89	74
	pPro-nadB67(P)-pCJ-nadA	100	85	89
	pPro-nadB67(F)-pCJ-nadA	100	89	74
	pPro-nadB67(Y)-pCJ-nadA	100	89	74
	pPro-nadB67(W)-pCJ-nadA	100	79	68
	pPro-nadB67(D)-pCJ-nadA	100	100	100
	pPro-nadB67(E)-pCJ-nadA	100	100	100
	pPro-nadB67(N)-pCJ-nadA	100	87	70
	pPro-nadB67(Q)-pCJ-nadA	100	87	70
	pPro-nadB67(H)-pCJ-nadA	100	77	67

[128] For the two quinolinate-producing variants including the plasmids pPro-nadB67(D)-pCJ-nadA and pPro-nadB67(E)-pCJ-nadA, the nadB gene was destroyed and lost activity. As a result, the quinolinate-producing variants mostly had very low quinolinate (QA) levels, the level was converted to a residual relative yield in percentage (%). It was based on 100% of the produced QA level when a nicotinic acid (NA) is 0 uM.

[129] Referring to Table 11, the yield of quinolinate was less than 50% when the pPro-

nadB-pCJ-nadA plasmid including the wild type nadB gene was used, and the culture medium contains nicotinic acid, over 5 μ M or greater. Whereas the yield of quinolinate was 70% or greater when the pPro-nadB67-pCJ-nadA plasmid and 302nd variants recombinant plasmids including the *nadB* gene variant were used. W3110- Δ nadC strain is an NAD auxotroph that requires supply of external NAD for growth. W3110- Δ nadC strain is unable to directly accept NAD, and uses heterogenous nicotinic acid (NA) or nicotinamide (Nm) to produce NAD. When an amount of intracellular NAD is 1 mM or greater, the *nadB* gene is feedback-regulated to maintain the amount of intracellular NAD constant. Accordingly, the production of QA may be limited by the *nadB* gene that is feedback-regulated. Adding NA to a culture medium means an increase of NAD in a strain. Accordingly, the production quantities of QA were compared between the strains with the addition of NA to the culture medium at different concentrations to identify a degree of feedback regulation in the nadB gene variants with respect to NAD concentration. As a result, the nadB gene variants with substituted 302nd amino acids were found that the feedback regulation by NAD is released.

[130] **<2-4> Production of nicotinic acid through decarboxylation reaction**

[131] To identify the ability to produce nicotinic acid of the quinolinate-producing strains into which nadB variants were introduced, the yields of quinolinate in Table 10 were used. The production method of nicotinic acid used herein was decarboxylation of the quinolinate-including culture solution under high-temperature and high-pressure conditions based on the disclosure of Korean Patent No. 10-1223904, to convert quinolinate to nicotinic acid. To remove cells from the quinolinate-containing culture solution, centrifugation was performed at about 3000 rpm to about 4000 rpm for about 10 minutes to about 30 minutes. The quinolinate-containing supernatant resulting from the centrifugation was separated and used as a sample for decarboxylation reaction.

[132] The decarboxylation reaction was conducted at about 135°C at about 0.2 MPa for about 3 hours. The samples used are shown in Table 12. An aqueous solution of quinolinate (a standard product of Sigma-Aldrich Co.) in deionized water was used as a control group. Each samples of the aqueous solutions of quinolinate was titrated with sodium hydroxide, ammonia water, hydrochloric acid, or sulfuric acid, specifically the conversion of quinolinate into nicotinic acid took place at pH of 2. Table 12 shows the production quantities of nicotinic acid converted from quinolinate of Table 10 through high-temperature and high-pressure reaction.

[133] Table 12

[Table 12]

Strain	Plasmid	Quinolinate (mg/L)	Nicotinic acid (mg/L)
W3110- Δ nadC	pPro-nadB-pCJ-nadA	3500	2555
	pPro-nadB67-pCJ-nadA	4553	3324
	pPro-nadB67(G)-pCJ-nadA	4560	3329
	pPro-nadB67(A)-pCJ-nadA	4560	3329
	pPro-nadB67(S)-pCJ-nadA	4560	3329
	pPro-nadB67(T)-pCJ-nadA	4560	3329
	pPro-nadB67(C)-pCJ-nadA	4560	3329
	pPro-nadB67(V)-pCJ-nadA	4425	3230
	pPro-nadB67(L)-pCJ-nadA	4425	3230
	pPro-nadB67(I)-pCJ-nadA	4425	3230
	pPro-nadB67(M)-pCJ-nadA	4425	3230
	pPro-nadB67(P)-pCJ-nadA	2223	1623
	pPro-nadB67(F)-pCJ-nadA	4241	3096
	pPro-nadB67(Y)-pCJ-nadA	4241	3096
	pPro-nadB67(W)-pCJ-nadA	4707	3436
	pPro-nadB67(D)-pCJ-nadA	100	73
	pPro-nadB67(E)-pCJ-nadA	100	73
	pPro-nadB67(N)-pCJ-nadA	4552	3323
	pPro-nadB67(Q)-pCJ-nadA	4552	3323
	pPro-nadB67(H)-pCJ-nadA	4891	3570

[134] The experiment of converting quinolinate in aqueous solution (deionized water) into nicotinic acid, as disclosed in the prior reference Chinese Patent No. 101353322, was performed at 135°C and at 0.2 MPa for about 3 hours. The temperature and the pressure levels were lower than 150°C to 250°C in the prior reference, respectively. The results are shown in Table 12.

[135]

[136] **Example 3: Confirmation of NAD feedback resistance activity of L-aspartate oxidase variants**

[137] **<3-1> Construction of L-aspartate oxidase over-expression plasmid**

[138] To more clarify the NAD-feedback resistance activities of L-aspartate oxidase variants, the NAD-feedback resistance of a L-aspartate oxidase variant encoded by the nadB67 variant selected in <Example 1-3-2> was evaluated. To clone the wild type nadB and the nadB variants into a pCDF-duet vector containing a histidine (His)-tag, PCR was performed using the wild type pPro-nadB plasmid and the recombinant pPro-nadB67 as templates, respectively. For use in PCR, primers of SEQ ID NOs: 22 and 23 able to amplify the ORF region of the wild type nadB and the variant nadB67 gene that includes ATG and TAA regions and having the recognition sites of the restriction enzymes BamHI and NdeI were constructed.

[139] Table 13

[Table 13]

Nucleotide sequence	SEQ ID NO.
5' AATTGGATCCGATGAATACTCTCCCTGAACATT 3'	22
5' AATTCATATGTTATCTGTTTATGTAATGATTGC 3'	23

[140] The polymerase used was PfuUltra™ DNA polymerase (Stratagene, U.S.A), and the PCR was conducted by repeating 30 times of a cycle comprising denaturation at 96°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 2 minutes. As a result, amplified genes of about 1.9 kb of the wild type nadB and the nadB67 variant including the recognition sites of the restriction enzymes BamHI and NdeI were obtained.

[141] The genes of the wild type nadB and nadB67 variant obtained through the PCR were treated with the restriction enzymes BamHI and NdeI, and then cloned via ligation into a pCDF-duet vector treated with the restriction enzymes BamHI and NdeI. Ultimately recombinant vectors including the genes of the wild type nadB and the nadB67 variant, respectively, was constructed wherein the expression of each of the genes is controllable by a T7 promoter as a constitutive promoter and the gene contains the His-tag capable of protein purification. The constructed recombinant vectors were named pT7-nadB and pT7-nadB67, respectively. Other recombinant vectors in which valine, leucine, isoleucine, and histidine were substituted for the 302nd amino acid of the wild type nadB, respectively, were constructed using the same method, and named pT7-nadB67(V), pT7-nadB67(L), pT7-nadB67(I), pT7-nadB67(H), respectively.

[142] **<3-2> Purification of L-aspartate oxidase**

[143] After the recombinant vector pT7-nadB, pT7-nadB67, pT7-nadB67(V), pT7-nadB67(L), pT7-nadB67(I), or pT7-nadB67(H) was transformed into a tuner strain via CaCl₂ method, the transformed strain was smeared on a LB-SP (yeast extract 10 g/L, NaCl 5 g/L, tryptone 10 g/L, and spectinomycin 50 µg/L) plate medium, and

cultured overnight at 37°C to select spectinomycin-resistant colonies. One colony was selected from them and cultured in a LB-SP (yeast extract 10 g/L, NaCl 5 g/L, tryptone 10 g/L, spectinomycin 50 µg/L) liquid medium, and then a isopropyl-1-thio-β-D-galactopyranoside (IPTG) derivative was added thereto when the growth optical density (OD) value reached to 0.4, and cultured at about 18°C overnight. Cells including the overexpressed wild type L-aspartate oxidase and L-aspartate oxidase variants were recovered from the culture medium, followed by purification of the wild type L-aspartate oxidase and L-aspartate oxidase variants from the recovered cells by Ni-NTA spin kit (Quiagen, U.S.A). The recovered protein was 50% of a total protein, about 2% of the recovered protein was recovered as L-aspartate oxidase.

[144] **<3-3> Activity test of L-aspartate oxidase variants**

[145] The L-aspartate oxidase converts aspartate as a substrate to iminoaspartate in the presence of an FAD as cofactor, and the iminoaspartate is converted to oxaloacetate. Hydrogen peroxide (H₂O₂), that is generated through the above-described reaction, was used to identify the activity of the nadB. Absorbance at 560 nm of a reaction product of H₂O₂ with a product from the reaction was measured using an Amplex Red (Invitrogen, Korea) to determine the activity of the nadB. Here, NAD was added at different concentrations to create a condition for competitive inhibition to FAD so as to evaluate the resistance of L-aspartate oxidase against the feedback regulation by NAD. The Relative activity of the wild type L-aspartate oxidase at a NAD concentration of 1 mM was less than 50 %, while the relative activities of the L-aspartate oxidase variants at the same NAD concentration remained about 70% or higher (Table 14), thereby indicating that the feedback regulation by NAD was released in L-aspartate oxidase variants.

[146] Table 14

[Table 14]

Activity comparison between L-aspartate oxidases with respect to NAD concentration

Relativeactivity						
NAD con- centration(mM)	Relative (%)					
	Wild type-nad B	Variant- nadB67	Variant - nadB67(V)	Variant - nadB67(L)	Variant nadB67(I)	Variant - nadB67(H)
0	100	100	100	100	100	100
0.05	95	102	100	100	100	100
0.3	82	96	95	95	95	95
0.5	65	75	77	76	76	77
1	22	74	75	74	75	74

[147]

[148] Depository Institution: Korean Culture Center of Microorganisms (KCCM)
(International Depository Authority)

[149] Accession No : KCCM11434

[150] Date of deposit : June 20, 2013

[151]

[152] While the invention has been particularly shown and described with reference to exemplary embodiments thereof, it will be understood that various changes in form and details may be made therein without departing from the spirit and scope of the following claims.

Industrial Applicability

[153] The present invention is related to L-aspartate oxidase variants that are resistant against the feedback regulation by nicotinic acid or NAD, and thus may effectively produce quinolinate. Quinolinate may be effectively produced by culturing of a microorganism including such a L-aspartate oxidase variant according to the embodiments of the present invention. Quinolinate may also be effectively produced using the microorganism in which the activity of quinolinate synthetase is additionally enhanced or the activity of quinolinate phosphoribosyltransferase is additionally weakened. Through such transformation of the microorganism, the yield of quinolinate or nicotinic acid may be improved, which is industrially and highly useful.

[154]

[155]



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

INTERNATIONAL FORM

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

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

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : <i>Escherichia coli</i> CV01-0518	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM11434P
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 June. 20. 2013. (date of the original deposit) ¹	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : 351-221, Yurim B/D Hongje-1-dong Seodaemun-gu SEOUL 120-091 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: June. 20. 2013.

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

Form BP/4

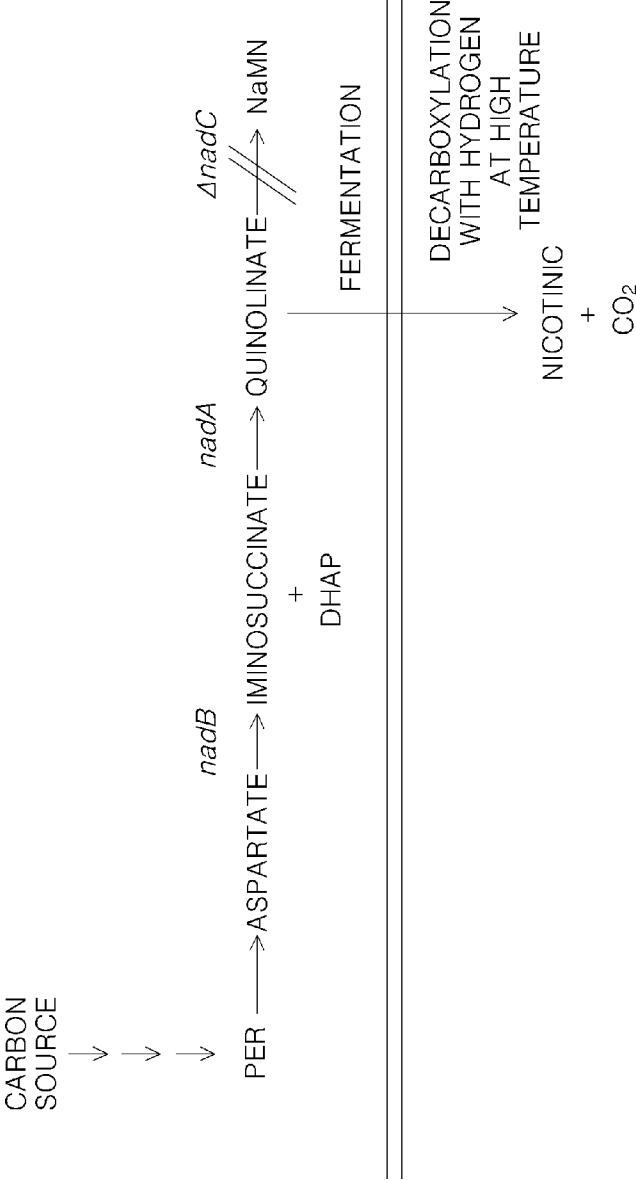
Sole page

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Claims

- [Claim 1] An L-aspartate oxidase variant having an amino acid sequence of which a 302nd amino acid in an amino acid sequence represented by SEQ ID NO: 1 is substituted with another amino acid.
- [Claim 2] The L-aspartate oxidase variant according to claim 1, wherein another amino acid is selected from the group consisting of arginine, valine, leucine, isoleucine, methionine, tryptophan, and histidine.
- [Claim 3] The L-aspartate oxidase variant according to claim 1, wherein the feedback regulation by nicotinic acid or NAD is released.
- [Claim 4] A polynucleotide comprising a nucleotide sequence that encoding an amino acid sequence which a 302nd amino acid in an amino acid sequence represented by SEQ ID NO: 1 is substituted with another amino acid.
- [Claim 5] A vector comprising the polynucleotide of claim 4 which is operably linked to a regulatory sequence.
- [Claim 6] A microorganism comprising the polynucleotide of claim 4.
- [Claim 7] The microorganism according to claim 6, wherein the microorganism belongs to the genus *Escherichia*.
- [Claim 8] The microorganism according to claim 6, wherein the activity of quinolinate synthetase is enhanced.
- [Claim 9] The microorganism according to claim 6, wherein the activity of endogenous quinolinate phosphoribosyltransferase is reduced.
- [Claim 10] A method of producing quinolinate, the method comprising: culturing the microorganism of claim 6 in a medium; and recovering quinolinate from the cultured medium.
- [Claim 11] A method of producing nicotinic acid, the method comprising: culturing the microorganism of claim 6 in a medium; and conducting a decarboxylation reaction by adding an acid to the cultured medium.

[Fig. 1]



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2014/006817**A. CLASSIFICATION OF SUBJECT MATTER****C12N 9/02(2006.01)i, C12N 15/53(2006.01)i, C12N 1/21(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N 9/02; C07C 51/235; C12P 17/10; C12P 17/12; C12N 15/77; C12N 1/20; C12N 15/31; C12N 15/53; C12N 1/21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal), NCBI, google scholar & Keywords: L-aspartate oxidase, quinolinate, nicotinic acid, NAD, E. coli, nadB, 302nd amino acid, variant

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KR 10-1223904 B1 (CJ CHEILJEDANG CORPORATION) 21 January 2013 - See claims 1-5; and paragraphs 19,24	1-11
A	US 6271031 B1 (FALCO; SAVERO CARL et al.) 07 August 2001 - See claims 1,4,5; and columns 1-4	1-11
A	KR 10-0620092 B1 (CJ CORPORATION) 08 September 2006 - See claims 1-3; and pages 3,4	1-11



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search

04 November 2014 (04.11.2014)

Date of mailing of the international search report

04 November 2014 (04.11.2014)

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2014/006817

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