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(54) Titre : PROCEDE DE GENERATION DE L-LYSINE PAR FERMENTATION DE BACTERIES DONT LE GENE ACONITASE ET/OU L'ELEMENT REGULATEUR A ETE MODIFIE

(54) Title: METHOD FOR PRODUCING L-LYSINE BY MODIFYING ACONITASE GENE AND/OR REGULATORY ELEMENTS THEREOF

(57) Abrégé/Abstract:

Provided is an L-lysine generation method by fermentation, comprising modifying the aconitase gene and/or regulatory element thereof on a bacteria chromosome, such that the enzymatic activity and/or expression quantity of the aconitase are reduced but do not disappear; and generating L-lysine by fermenting the modified bacteria. Also disclosed are methods and uses derived from the present method, and the bacteria that can be used in these methods and uses.

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ABSTRACT

Provided is a method of producing L-lysine by fermentation, which comprises the steps of modifying an aconitase gene and/or regulatory element thereof in a chromosome of a
5 bacterium so that the activity and/or the expression amount of the aconitase of the bacterium are reduced but not eliminated; and producing L-lysine by the fermentation of the modified bacterium. In addition, also provided are methods and uses derived from the method as well as bacteria used in these methods and uses.

Method for Producing L-Lysine by Modifying Aconitase Gene and/or Regulatory Elements thereof

Technical Field

5 The present invention belongs to the field of amino acid fermentation, and specifically relates to a method of producing L-lysine by fermentation and methods and uses derived from the method as well as bacteria used in these methods and uses.

Background Art

10 To produce L-lysine by fermentation with L-lysine-producing bacteria (e.g., *E. coli* of *Escherichia* and bacteria of *Corynebacterium*) has been already widely industrially used. These bacteria may be isolated from the natural environment, and/or may be obtained by mutagenesis or genetically engineered modification. Prior arts focus on the genetically engineered modifications of genes such as *pnt*, *dap*, *ppc* and the like, and pay no attention to a
15 regulatory element of a gene encoding an aconitase (e.g., aconitase A from *Escherichia coli*, an aconitase from *Corynebacterium glutamicum* or *Corynebacterium pekinense*) for the production of L-lysine.

 An aconitase is an enzyme of the tricarboxylic acid cycle, which catalyzes two-step chemical reactions: the transformation of citric acid to aconitic acid and the transformation of
20 aconitic acid of isocitric acid. In the known bacteria of *Escherichia*, the gene *acnA*, the nucleotide sequence of which is shown in SEQ ID No: 1, encodes aconitase A. However, perhaps due to its metabolite far away from the final product L-lysine as well as many and complex intermediary metabolism branches, its role in the L-lysine fermentation has not been suggested. And there are no reports about the effects of an aconitase from other
25 L-lysine-producing bacteria such as *Corynebacterium glutamicum* and the like on the L-lysine fermentation. The aconitases described in documents such as Chinese patents CN1289368A and CN101631871A relate to the fermentation of L-glutamic acid, and are not taught to have any effects on the fermentation of L-lysine.

 After a long research and practice, especially by virtue of some luck, the inventors
30 fortuitously found that a modification of an aconitase gene and regulatory element thereof is

conductive to the increase of an L-lysine yield.

In addition, in prior arts, an expression amount and/or enzyme activity are increased by the increase of copy number and the introduction of site-directed mutation of a useful enzyme gene, or an enzyme activity and/or expression amount are eliminated by the knockout of a harmful enzyme gene. However the inventors found that an aconitase gene and regulatory element thereof are different from those in prior arts and can not be simply increased or knocked out. Especially the elimination of the expression of the gene *acnA* by the knockout results in slow growth of bacteria and difficulty for a practical use, so a novel method of modifying an aconitase gene and/or regulatory element thereof for increasing an L-lysine yield was invented.

Furthermore, the method does not interfere in modification sites in chromosomes of a great amount of L-lysine-producing bacteria modified according to prior arts, so as to further increase the effect and be useful for practically producing L-lysine by using a great variety of bacteria.

Disclosure of the Invention

The problem to be solved by the invention is to provide a novel method of producing L-lysine by fermentation and related methods including a method of increasing a fermentation yield of L-lysine compared to an unmodified bacterium, a use of a modified bacterium for producing L-lysine by fermentation, a use of a modified bacterium for increasing a fermentation yield of L-lysine compared to an unmodified bacterium, and/or a method of modifying a bacterium. Furthermore, the invention also provides a polynucleotide, vector and/or bacterium used in the methods mentioned above.

Specifically, in first aspect, the invention provides a method of producing L-lysine by fermentation, which comprises the steps of:

- (1) modifying an aconitase gene and/or regulatory element thereof in a chromosome of a bacterium so that the activity and/or the expression amount of the aconitase of the bacterium are reduced but not eliminated; and
- (2) producing L-lysine by fermentation with the bacterium obtained by the modification of step (1).

The term "modification" used herein means a change of an object in need of modification resulting in a certain effect. Modification techniques of a gene or a regulatory element in a chromosome include, but are not limited to, mutagenesis, site-directed mutation and/or homologous recombination, preferably the last two. To modify a gene or a regulatory element in a chromosome is an addition, deletion or substitution of one or more nucleotides in the nucleotide sequence of the gene or the regulatory element. These techniques are widely described in documents of molecular biology and microbiology, and many of them have been already commercially available. In the embodiments of the invention, the modification can be carried out according to the principle of homologous recombination by using the pKOV plasmid commercially available by Addgene or using the pK18mobsacB plasmid, so that an unmodified aconitase gene and/or regulatory element thereof in a bacterial chromosome are modified to be a novel aconitase gene and/or regulatory element thereof, and the expression amount and/or the enzyme activity of the aconitase in the modified bacterium are reduced but not eliminated. Therefore preferably the modification used herein is a modification by homologous recombination.

After a long research, the inventors found that in *Escherichia coli*, bacteria of *Corynebacterium* and the like, the elimination of the expression of an aconitase, by using the technique such as the knockout of a gene, results in slow growth of bacteria and even no amino acids produced. Therefore the modification of the invention does reduce and does not eliminate the expression amount of the aconitase of the bacterium obtained by the modification compared to the bacterium without the modification. Preferably the expression amount of aconitase A of the bacterium obtained by the modification is reduced by 20%~95%, more preferably by 50%~90%, e.g. by 65%, 70% or 80%.

Accordingly, the invention also provides other uses or methods. For example, in second aspect, the invention provides a method of increasing a fermentation yield of L-lysine, which comprises the steps of:

- (1) modifying an aconitase gene and/or regulatory element thereof in a chromosome of a bacterium so that the activity and/or the expression amount of the aconitase of the bacterium are reduced but not eliminated; and
- (2) producing L-lysine by fermentation with the bacterium obtained by the modification

of step (1).

L-lysine is an important metabolite of a bacterium, and most of bacteria can generate more or less amount of L-lysine. Although an L-lysine-producing bacterium with low yield is not suitable for cost-efficiently producing L-lysine, yet the methods of the invention can result in the increase of L-lysine and can be used in a cost-insensitive area. Undoubtedly a preferable bacterium used herein is an L-lysine-producing bacterium with high yield, and its yield can be further increased by using the methods of the invention. In addition, in the methods or uses of the invention, except a modification of an aconitase gene and/or regulatory element thereof in a chromosome of a bacterium, no other modifications may be carried out. For example, an aconitase gene in a bacterial chromosome may not be modified while a regulatory element of the aconitase gene is modified, and vice versa. For example, only one of an aconitase gene and regulatory element thereof in a chromosome of a bacterium, especially an L-lysine-producing bacterium with high yield, may be modified.

For example, in third aspect, the invention provides a use of a bacterium obtained by a modification for producing L-lysine by fermentation, wherein the modification is to modify an aconitase gene and/or regulatory element thereof in a chromosome of a bacterium, and the enzyme activity and/or the expression amount of the aconitase of the bacterium obtained by the modification are reduced but not eliminated.

The bacterium obtained by the modification may be used alone, with other L-lysine-producing bacteria, or by other means for producing L-lysine by fermentation. Unless otherwise defined (e.g. without the definition of "obtained by the modification"), the term "bacterium" used herein means an unmodified bacterium or a bacterium without the modification, an aconitase gene and a regulatory element located around the locus of the gene in a chromosome of which are an aconitase gene and a regulatory element without the decrease of the enzyme activity and/or the expression amount of the aconitase, e.g., an aconitase gene and a regulatory element from a wild-type bacterium.

For example, in fourth aspect, the invention provides a use of a bacterium obtained by a modification for increasing a fermentation yield of L-lysine, wherein the modification is to modify an aconitase gene and/or regulatory element thereof in a chromosome of a bacterium, and the enzyme activity and/or the expression amount of the aconitase of the bacterium

obtained by the modification are reduced but not eliminated..

As used herein, a bacterium may be an L-lysine producing bacterium, e.g. a bacterium of *Escherichia* or *Corynebacterium*. In one preferable aspect, a bacterium may be a bacterium of *Escherichia*, more preferably *Escherichia coli*, e.g. filial strains of *Escherichia coli* K-12 including strains derived from the strain W3110; In the other preferable aspect, a bacterium may be a bacterium of *Corynebacterium*, more preferably *Corynebacterium glutamicum* or *Corynebacterium pekinense*. Because few prior arts suggest the role of an aconitase gene and/or regulatory element thereof from a bacterium in the production/fermentation of L-lysine and focus on the loci such as *pnt*, *dap*, *ppc* and the like to modify the genes in chromosomes, there are no reports about a modification of an aconitase gene and/or regulatory element therearound from L-lysine-producing bacteria (especially bacteria of *Escherichia* or *Corynebacterium*, e.g. *E. coli*, *Corynebacterium glutamicum* or *Corynebacterium pekinense*) in prior arts, and thus the L-lysine-producing bacteria substantially have wild-type aconitase genes and regulatory elements therearound. So they may be modified by using the methods of the invention for the increase of a fermentation yield of L-lysine. In the embodiments of the invention, all of L-lysine-producing bacteria with low or high yield can be modified by using the methods of the invention to increase a fermentation yield of L-lysine.

Substantially, in fifth aspect, the invention provides a method of modifying a bacterium, which comprises the step of modifying an aconitase gene and/or regulatory element thereof in a chromosome of a bacterium so that the activity and/or the expression amount of the aconitase of the bacterium obtained by the modification are reduced but not eliminated.

The bacterium obtained by the method of the fifth aspect of the invention can be used for producing or generating L-lysine by fermentation. Therefore in sixth aspect, the invention provides a bacterium obtained by the method of the fifth aspect of the invention.

According to the experiments by the inventors, many of nucleotide sequences of aconitase genes (*acnA*) from bacteria of *Escherichia* (e.g., *E. coli*) are shown in SEQ ID No: 1, while many of nucleotide sequences of aconitase genes from bacteria of *Corynebacterium* (e.g., *Corynebacterium glutamicum* or *Corynebacterium pekinense*) are shown in SEQ ID No: 2. To modify these aconitase genes so as to reduce but not eliminate the enzyme activity

and/or the expression of the aconitases in the modified bacteria, can increase a fermentation yield of L-lysine. Therefore in the embodiments of the invention, the nucleotide sequence of the aconitase gene is shown in SEQ ID No: 1 or 2. For other bacteria, the nucleotide sequences of aconitase genes from the bacteria can be obtained by the techniques such as sequencing and comparing identities of sequences for the modification of the methods of the invention.

In the invention, the preferable step of modifying an aconitase gene in a chromosome of a bacterium is an addition, deletion or substitution of one or more nucleotides in the nucleotide sequence of the aconitase gene, as long as the enzyme activity and/or the expression amount of the aconitase from the modified bacterium are reduced but not eliminated.

According to the experience of the inventors, although it is usually difficult to increase an activity and/or an expression amount of a bacterium's enzyme since the bacterium has been evolved for a long time and modification sites for the increase need be researched particularly, yet the research for the decrease of the activity and/or the expression amount of the enzyme is much easier. For example, the nucleotide sequence out of domains of an enzyme may be mutated. In the invention, the preferable step of modifying an aconitase gene in a chromosome of a bacterium may be a substitution of one or more nucleotides in the nucleotide sequence of the aconitase gene. For example, the substitution includes a substitution for the initiation codon of the aconitase gene, preferably a substitution of GTG. It can be carried out for *Escherichia coli*, *Corynebacterium glutamicum*, *Corynebacterium* *pekinense* and the like. Also in the invention, the preferable step of modifying an aconitase gene in a chromosome of a bacterium may be a deletion of one or more nucleotides in the nucleotide sequence of the aconitase gene. For example, the deletion includes a deletion in the nucleotide sequence of the aconitase gene, preferably a deletion of 1-120 nucleotides, more preferably a deletion of 1-90 nucleotides, most preferably a deletion of 90 nucleotides, e.g. a deletion of 90 nucleotides before the termination codon in the nucleotide sequence of the aconitase gene. It can be carried out for *Escherichia coli*, *Corynebacterium glutamicum*, *Corynebacterium pekinense* and the like.

As used herein, a regulatory element means a polynucleotide located upstream or downstream of a gene (e.g., an aconitase gene) and regulating the transcription and/or the

expression of the gene in order to have an effect on the expression amount of the gene. It may include non-coding and encoding sequences. A regulatory element may be a promoter, enhancer, repressor or other polynucleotide related to transcription and/or expression control. A preferable regulatory element may be a promoter. In one embodiment of the invention, the nucleotide sequence of the promoter is shown in SEQ ID No: 4 or 6. A preferable regulatory element may also be a repressor, for example a transcription repressor. In one embodiment of the invention, the nucleotide sequence of the transcription repressor is shown in SEQ ID No: 7. By modifying a promoter and/or repressor, an enzyme activity and/or expression amount of an aconitase in a modified bacterium are reduced but not eliminated.

10 In the invention, the preferable step of modifying a regulatory element of an aconitase gene in a chromosome of a bacterium is an addition, deletion or substitution of one or more nucleotides in the nucleotide sequence of the regulatory element of the aconitase gene, as long as the enzyme activity and/or the expression amount of the aconitase from the modified bacterium are reduced but not eliminated.

15 According to the experience of the inventors, although it is usually difficult to increase a transcription activity of a bacterium's promoter or enhancer since the bacterium has been evolved for a long time and modification sites for the increase need be researched particularly, yet the research for the decrease of the transcription activity of the promoter or enhancer is much easier. For example, one or several nucleotide sequence can be added for increasing the distance between a promoter and the initiation codon of a gene, or a promoter with weak transcription activity is substituted for an original promoter. In the invention, the preferable step of modifying a regulatory element of an aconitase gene in a chromosome of a bacterium may be a substitution of one or more nucleotides in a promoter of the aconitase gene, for example a substitution of a promoter with weak transcription activity, more preferably a substitution of the nucleotide sequence shown in SEQ ID No: 3 or 5.

25 Furthermore, in the invention, the preferable step of modifying a regulatory element of an aconitase gene in a chromosome of a bacterium may be an addition of the nucleotide sequence of a transcription repressor for the aconitase gene, for example an addition of the nucleotide sequence of a new transcription repressor or increase of the copy number of the nucleotide sequence of an original transcription repressor. A transcription repressor may be added behind

a promoter (especially a promoter with strong transcription activity) in order to increase its expression amount and inhibit the transcription of an aconitase gene more efficiently. In an embodiment of the invention, the nucleotide sequence shown in SEQ ID No: 8 and 7 in tandem is added.

5 Furthermore, the invention also provides intermediate products such as a polynucleotide and/or vector used in the methods mentioned above, as well as uses of the products. For example, in seventh aspect, the invention provides a polynucleotide, the nucleotide sequence of which is selected from

(a) the nucleotide sequence obtained by a substitution (preferably of GTG) for the
10 initiation codon of the nucleotide sequence shown in SEQ ID No: 1;

(b) the nucleotide sequence obtained by a deletion (preferably of 1-120 nucleotides, more preferably of 1-90 nucleotides, most preferably of 90 nucleotides) in the nucleotide sequence shown in SEQ ID No: 1 or 2, e.g. a deletion of 90 nucleotides before the termination codon in the nucleotide sequence shown in SEQ ID No: 1 or 2; and

15 (c) the nucleotide sequence shown in SEQ ID No: 8 or 7 in tandem.

In eighth aspect, the invention provides a vector, which comprises the polynucleotide of the seventh aspect of the invention.

In ninth aspect, the invention provides a use of the polynucleotide of the seventh aspect of the invention and/or the vector of the eighth aspect of the invention in the method or use of
20 the first, second, third and/or fourth aspect of the invention. That is that in the methods or uses of the first, second, third and/or fourth aspects of the invention, the polynucleotide of the seventh aspect of the invention and/or the vector of the eighth aspect of the invention are used.

In tenth aspect, the invention provides a use of the polynucleotide of the seventh aspect of
25 the invention and/or the vector of the eighth aspect of the invention in the preparation of the bacterium of the fifth aspect of the invention. That is that in the preparation process of the bacterium of the fifth aspect of the invention, the polynucleotide of the seventh aspect of the invention and/or the vector of the eighth aspect of the invention are used.

The beneficial effects of the invention include that novel techniques are invented and
30 proved for increasing a fermentation yield of L-lysine when using L-lysine-producing bacteria

with high and low yield; and do not interfere in modification sites in chromosomes of a great amount of L-lysine-producing bacteria with high yield modified according to prior arts, so as to further increase the yield, be useful for practically producing L-lysine by the fermentation of bacteria, and be easy to popularize.

5 For a better understanding of the invention, it will now be described in greater detail by reference to specific examples. It should be noted that the examples only exemplify the invention, and should not be construed as limiting the scope of the invention. According to the description of the invention, various modifications and alterations of the invention are obvious to a person skilled in the art.

10 In addition, the publications cited in the invention are used to illustrate the invention, the contents of which are incorporated herein by reference, as if they have been written down herein.

Examples

15 Embodiments of the invention are further exemplified by the following examples. Unless otherwise specified, the techniques used in the examples are well-known for a person skilled in the art and commercially available devices and reagents (see *Molecular Cloning: A Laboratory Manual* (3rd Edition), Science Press, *Microbiology Experiments* (4th Edition), Higher Education Press, and the manufacturer's instructions of the devices and reagents).

20

Example 1. Substitution of GTG for the initiation codon ATG of aconitase

The genomic chromosome of the wild-type *Escherichia coli* strain, *E. coli* K12 W3110 (commercially available from Biological Resource Center, National Institute of Technology and Evaluation (NITE Biological Resource Center, NBRC)), was extracted as a template for a
25 PCR amplification, by using the primers P1/P2 and P3/P4 respectively. Two DNA fragments of 510bp and 620bp were obtained and named Up1 and Down1 fragments respectively. The process of the PCR amplification was shown as follows: denaturalizing for 30s (seconds) in 94°C, annealing for 30s (seconds) in 52°C, extending for 30s (seconds) in 72°C, and performing for 30 times circularly. And the sequences of the primers were shown as follows:

30 P1: 5'-CGCGGATCCGGAGTCGTCACCATATGCC-3'

P2: 5'-TCTCGTAGGGTTGACGACACAGCTCCTCCTTAATGACAGG-3'

P3: 5'-CCTGTCATTAAGGAGGAGCTGTGTCGTCAACCCTACGAGA-3'

P4: 5'-ATTGCGGCCGCTCCATTCACCGTCCTGCAAT-3'

The two DNA fragments were purified by agarose gel electrophoresis and mixed as a template for overlap PCR amplification by using the primers P1/P4. An approximately 1200bp fragment was obtained and named Up-Down1 fragment. The process of the PCR amplification was shown as follows: denaturalizing for 30s (seconds) in 94° C, annealing for 30s (seconds) in 52°C, extending for 60s (seconds) in 72°C, and performing for 30 times circularly.

The Up-Down1 purified by agarose gel electrophoresis and the pKOV plasmid (commercially available from Addgene) were digested respectively by *Bam* HI/*Not* I. The digestion products were purified by agarose gel electrophoresis and ligated to be a vector pKOV-Up-Down1 for further transform. The vector pKOV-Up-Down1 was determined by sequencing the vector by a sequencing company to have a correct point mutation (A-G) in the gene fragment of *acnA*, and stored for further use.

The constructed plasmid pKOV-Up-Down1 was transformed by electroporation into the L-lysine-producing strain with low yield, *E. coli* NRRL B-12185 (commercially available from Agricultural Research Service Culture Collection (NRRL); see also US4346170A for its construction method), and the L-lysine-producing strain with high yield, *E. coli* K12 W3110 $\Delta 3$ (commercially available from Institute of Microbiology, Chinese Academy of Sciences; the strain is an L-lysine-producing engineering strain mutagenized and mutated from *E. coli* K12 W3110) respectively. The two strains were determined by sequencing to have the wild-type gene of *acnA* (i.e., sites 1333855 to 1336530 of GenBank accession number U00096.2) and upstream and downstream elements thereof in their chromosomes. According to the manufacturer's instruction of pKOV plasmid from Addgene, homologous recombination-positive clones were selected after a recovery culture in LB medium under the conditions of 30° C and 100 rpm for 2h, and were determined by sequencing to have the mutation of the initiation codon ATG to GTG in the wild-type gene of *acnA* in their chromosomes. Finally both of L-lysine-producing *E. coli* strains with low and high yield having the mutation of the initiation codon of *acnA* were obtained and named YP-13633 and

YP-13664 respectively. The expression amount of aconitases in the two modified strains was measured and reduced by approximately 75~85% (different values in different media).

Example 2. Mutation of the aconitase gene sequence for the decrease of the activity of aconitase

(1) Construction of *E. coli* strains

90 nucleotides before the termination codon in the gene *acnA* of *E. coli* were deleted for the decrease of the aconitase activity. Specifically, the genomic chromosome of the wild-type *Escherichia coli* strain, *E. coli* K12 W3110, was extracted as a template for PCR amplification, by using the primers P5/P6 and P7/P8 respectively. Two DNA fragments of 752bp and 657bp were obtained and named Up2 and Down2 fragments respectively. The process of the PCR amplification was shown as follows: denaturalizing for 30s (seconds) in 94°C, annealing for 30s (seconds) in 52°C, extending for 30s (seconds) in 72°C, and performing for 30 times circularly. And the sequences of the primers were shown as follows:

P5: 5'-CGCGGATCCCGTCACACGATCCGATACCT-3'

P6: 5'-CGGCAAGCAAATAGTTGTTATACGACTTCCTGGCTACCAT -3'

P7: 5'-ATGGTAGCCAGGAAGTCGTATAACAACCTATTGCTTGCCG-3'

P8: 5'-ATTGCGGCCGC CATGGGGCGATTCCTGATG-3'

The two DNA fragments were purified by agarose gel electrophoresis and mixed as a template for an overlap PCR amplification by using the primers P5/P8. An approximately 1400bp fragment was obtained and named Up-Down2 fragment. The process of the PCR amplification was shown as follows: denaturalizing for 30s (seconds) in 94°C, annealing for 30s (seconds) in 52°C, extending for 60s (seconds) in 72°C, and performing for 30 times circularly.

The Up-Down2 purified by agarose gel electrophoresis and the pKOV plasmid (commercially available from Addgene) were digested respectively by *Bam* HI/*Not* I. The digestion products were purified by agarose gel electrophoresis and ligated to be a vector pKOV-Up-Down2 for further transform. The vector pKOV-Up-Down2 was determined by sequencing the vector by a sequencing company to have a deletion of 90bp bases before the termination codon in the gene fragment of *acnA*, and stored for further use.

According to the manufacturer's instruction of pKOV plasmid from Addgene, the constructed plasmid pKOV-Up-Down2 was transformed by electroporation into the L-lysine-producing strain with low yield, *E. coli* NRRL B-12185 (see also US4346170A for its construction method), and the L-lysine-producing strain with high yield, *E. coli* K12 W3110 $\Delta 3$ respectively. The two strains were determined by sequencing to have the wild-type gene of *acnA* and upstream and downstream elements thereof in their chromosomes. Homologous recombination-positive clones were selected, and were determined by sequencing to have the deletion of 90bp bases before the termination codon in the gene of *acnA* in their chromosomes. Finally both of L-lysine-producing *E. coli* strains with low and high yield, in which the enzyme activities of *acnA* were reduced, were obtained and named YP-13675 and YP-13699 respectively. The aconitase activities in the two modified strains were measured and reduced by approximately 60~80% (different values in different media).

(2) Construction of *Corynebacterium* strains

90 nucleotides before the termination codon in the *acn* gene of *Corynebacterium* were deleted for the decrease of the aconitase activity. The process of construction was substantially the same as that of step (1) mentioned above, except that the genome of *Corynebacterium pekinense* AS1.299 (which is similar to *Corynebacterium glutamicum* and is sometimes mistakenly regarded as *Corynebacterium glutamicum*; commercially available from China General Microbiological Culture Collection Center (CGMCC)) was used as a template for an amplification by using the primers P9~P12 as follows (corresponding to the primers P4~P8 mentioned above respectively), and a 542bp fragment (Up2), a 527bp fragment (Down2), and an approximately 1069bp fragment (Up-Down) were obtained. And the sequences of the primers were shown as follows:

P9: 5'-CGGGATCCTGCAGCTCAGTACTTGGAT-3'
 P10: 5'-AAAGTCTTCTAATTAC TTACTGCGTCGAACTCGACG-3'
 P11: 5'-GTTTCGACGCAGTAAGTAATTAGAAGACTTTTGAT-3'
 P12: 5'-TCCCCCGGGGAATACCGGGTTCGGTGCG-3'

Then the Up-Down2 fragment purified by agarose gel electrophoresis and pK18mobsacB plasmid (commercially available from American Type Culture Collection (ATCC)) were

digested respectively by *Bam* HI/*Sma* I. The digestion products were purified by agarose gel electrophoresis and ligated to be a recombinant vector pK18mobsac*B*-Up-P-Down having a deletion of 90bp bases before the termination codon in the *acn* gene. The constructed plasmid pK18mobsac*B*-Up-Down2 was verified by sequencing and transformed respectively by

5 electroporation into the L-lysine-producing strain with low yield, *Corynebacterium pekinense* AS1.299, the L-lysine-producing strain with low yield, *Corynebacterium glutamicum* ATCC 13032 (commercially available from ATCC), and the L-lysine-producing strain with high yield, *Corynebacterium pekinense* AS1.563 (commercially available from China General Microbiological Culture Collection Center). The last two strains were determined by

10 sequencing to have in their chromosomes the *acn* gene from *Corynebacterium pekinense* AS1.299, the nucleotide sequence of which is shown in SEQ ID NO: 2. Homologous recombination-positive clones were selected after a recovery culture in BHIS medium under the conditions of 30°C and 120 rpm for 2h, and were verified by sequencing. Finally three L-lysine-producing *Corynebacterium* strains with low, low and high yield having the mutation

15 of the *acn* gene were obtained and named YP-14808, YP-14852 and YP-14837.

Example 3. Substitution of a promoter with weak transcription activity for the promoter of aconitase gene

(1) Construction of *E. coli* strains

20 By the analysis of the upstream sequence of *acnA* in *E. coli* K12 W3110, we provided a promoter with weak transcription activity (the sequence of which is shown in SEQ ID No: 3) to replace the region of the wild-type promoter (the sequence of which is shown in SEQ ID No: 4) in the upstream of the ORF of the *acnA* gene for the decrease of the expression of the wild-type *acnA* gene.

25 Specifically, the genomic chromosome of the wild-type *Escherichia coli* strain, *E. coli* K12 W3110, was extracted as a template for PCR amplification, by using the primers P13/P14 and P15/P16 respectively. Two DNA fragments of 580bp and 618bp were obtained and named Up3 and Down3 fragments respectively. A plasmid comprising the promoter with weak transcription activity mentioned above was used as a template for PCR amplification by using

30 P17/P18, and a 161bp promoter fragment with weak transcription activity was obtained and

named P fragment. The process of the PCR amplification was shown as follows: denaturalizing for 30s (seconds) in 94°C, annealing for 30s (seconds) in 52°C, extending for 30s (seconds) in 72°C, and performing for 30 times circularly.

The three DNA fragments mentioned above were purified by agarose gel electrophoresis. Then the Up3 and P fragments were mixed as a template for overlap PCR amplification by using the primers P13/P18. An approximately 716bp fragment was obtained and named Up-P fragment. The process of the PCR amplification was shown as follows: denaturalizing for 30s (seconds) in 94°C, annealing for 30s (seconds) in 52°C, extending for 60s (seconds) in 72°C, and performing for 30 times circularly.

The Up-P and Down3 fragments purified by agarose gel electrophoresis were mixed as a template for an overlap PCR amplification by using the primers P13/P16. An approximately 1334bp fragment was obtained and named Up-P-down fragment. The process of the PCR amplification was shown as follows: denaturalizing for 30s (seconds) in 94°C, annealing for 30s (seconds) in 52°C, extending for 60s (seconds) in 72°C, and performing for 30 times circularly.

The sequences of the primers mentioned above were shown as follows:

P13: 5'-CGCGGATCCGAAGAAATTGAGGTCATGTT -3'

P14: 5'-GGTTTCTTAGACGTCGGATTCGTTTCGTTTCTGTTTCATT-3'

P15: 5'-ATCAGCAGGACGCACTGACCCATTAAGGAGGAGCTATGTCTG -3'

P16: 5'-ATTGCGGCCGCTCCATTCACCGTCCTGCAATT -3'

P17: 5'-AATGAAACAGAAACGAAACGCAATCCGACGTCTAAGAAACC -3'

P18: 5'-CGACATAGCTCCTCCTTAATGGGTCAGTGCGTCCTGCTGAT -3'

The Up-P-down purified by agarose gel electrophoresis and the pKOV plasmid (commercially available from Addgene) were digested respectively by *Bam* HI/Not I. The digestion products were purified by agarose gel electrophoresis and ligated to be a vector pKOV-Up-P-Down for further transform. The vector pKOV-Up-P-Down was determined by sequencing the vector by a sequencing company to have a correct sequence of the promoter with weak transcription activity, and stored for further use.

The constructed plasmid pKOV-Up-P-Down was transformed by electroporation into the L-lysine-producing strain with low yield, *E. coli* NRRL B-12185, and the L-lysine-producing

strain with high yield, *E. coli* K12 W3110 $\Delta 3$ respectively. The two strains were determined by sequencing to have the wild-type gene of *acnA* and upstream and downstream elements thereof in their chromosomes, where the upstream promoter comprises sites from 2102518 to 2102713 of GenBank accession number CP004009.1. According to the manufacturer's instruction of pKOV plasmid from Addgene, homologous recombination-positive clones were selected after a recovery culture in LB medium under the conditions of 30°C and 100 rpm for 2h, and were determined by sequencing to have the substitution of the promoter with weak transcription activity for the wild-type promoter in the upstream of the gene *acnA* in their chromosomes. Finally both of L-lysine-producing *E. coli* strains with low and high yield having the mutation of the promoter of *acnA* were obtained and named YP-13627 and YP-13682 respectively. The expression amount of aconitases in the two modified strains was measured and reduced by 65~80% in different media.

(2) Construction of *Corynebacterium* strains

By the analysis of the upstream sequence of *acn* in *Corynebacterium*, we provided a promoter with weak transcription activity (the sequence of which is shown in SEQ ID No: 5) to replace the region of the 166 bp wild-type promoter (the sequence of which is shown in SEQ ID No: 6) in the upstream of the ORF of the *acn* gene from *Corynebacterium*. The process of construction was substantially the same as that of step (1) mentioned above, except that:

The genome of *Corynebacterium pekinense* AS1.299 (commercially available from China General Microbiological Culture Collection Center) was used as a template for an amplification by using the primers P19~P24 as follows (corresponding to the primers P13~P18 mentioned above respectively), and a 573bp fragment (Up3), a 581bp fragment (Down3), a 130bp fragment (P), and an approximately 1284bp fragment (Up-P-down) were obtained. And the sequences of the primers were shown as follows;

P19: 5'-CGGGATCCGCCAAAGCAACCAACCCC -3'

P20: 5'-CTTTTtagTTTTCAACGGTCGGATTTGCTCGAAAT-3'

P21: 5'-GCCGAAAC AAAGTAGCCGAAGCAGACGCCGTCG -3'

P22: 5'-CGGAATTCTGACCTGGTGGACGATAC-3'

P23: 5'-CGAGCAAATCCGACCGTTGAAAATAAAAAGCTGG-3'

P24: 5'-GCGTCTGCTTCGGCTACTTTGTTTCGGCCACCC-3'

Then the Up-P-down fragment purified by agarose gel electrophoresis and the pK18mobsacB plasmid were digested respectively by *Bam* HI/*Eco* RI. The digestion products were purified by agarose gel electrophoresis and ligated to be a recombinant vector pK18mobsacB-Up-P-Down for further substitution of the promoter. The constructed plasmid pK18mobsacB-Up-P-Down was verified by sequencing and transformed respectively by electroporation into the L-lysine-producing strain with low yield, *Corynebacterium* *pekinense* AS1.299, the L-lysine-producing strain with low yield, *Corynebacterium* *glutamicum* ATCC 13032, and the L-lysine-producing strain with high yield, *Corynebacterium* *pekinense* AS1.563. The last two strains were determined by sequencing to have in their chromosomes the *acn* gene and upstream and downstream elements thereof from *Corynebacterium* *pekinense* AS1.299. Homologous recombination-positive clones were selected after a recovery culture in BHIS medium under the conditions of 30°C and 120 rpm for 2h, and were verified by sequencing. Finally three L-lysine-producing *Corynebacterium* strains with low, low and high yield having the mutation of the promoter of *acn* were obtained and named YP-14755, YP-14732 and YP-14780.

Example 4. Increase of the copy number of the gene *acnR* encoding the transcription repressor of aconitase gene

The copy number of the gene *acnR* encoding the transcription repressor of *acn* was increased for the decrease of the transcription level of the *acn* gene. Specifically, the genome of *Corynebacterium* *pekinense* AS1.299 was used as a template for PCR amplification by using the primers P25/P26 and P27/P28 respectively. Two DNA fragments of 715bp and 797bp were obtained and named Up4 and Down4 fragments. The template was used or a PCR amplification by using the primers P29/P30. A 567 bp *acnR* fragment was obtained and named R fragment, the nucleotide sequence of which is shown in SEQ ID No: 7. In addition, the expression plasmid pXMJ19 (commercially available from Biovector Science Lab, Inc) was used as a template for a PCR amplification by using the primers P31 and P32. An 164bp promoter P_{tac} with strong transcription activity was obtained and named P_{tac} fragment, the

nucleotide sequence of which is shown in SEQ ID No: 8. The process of the PCR amplification was shown as follows: denaturalizing for 30s (seconds) in 94°C, annealing for 30s (seconds) in 52°C, extending for 30s (seconds) in 72°C, and performing for 30 times circularly.

5 The sequences of the primers mentioned above were shown as follows:

P25: 5'-CGGGATCCTTCGCAACCGATAGAGCA-3'

P26: 5'-CACGAATTATGCAGAATAAGCCTTTAAGTAACAA-3'

P27: 5'-TAAACGCGACTAAGCGTGACCATTAAAAGGCT-3'

P28: 5'-CGGAATTCAAAAGCCTATTAAGTGTC-3'

10 P29: 5'-TTCACACAGGAAAGTGTCCGTAGCGGCAGGCGA-3'

P30: 5'-TTTAATGGTCACGC TTAGTCGCGTTTACGGACAG-3'

P31: 5'-TTAAAGGCTTATTCTGCATAATTCGTGTCGCTC-3'

P32: 5'-GCCGCTACGGACACTTTCCTGTGTGAAATTGTTA-3'

The R and P_{tac} fragments were purified by agarose gel electrophoresis and mixed as a
 15 template for an overlap PCR amplification by using the primers P31/P30. An approximately 731bp fragment was obtained and named P_{tac} -R fragment. The Up4 and P_{tac} -R fragments were purified by agarose gel electrophoresis and mixed as a template for overlap PCR amplification by using the primers P25/P30. An approximately 1446bp fragment was obtained and named Up4- P_{tac} -R fragment. The Up4- P_{tac} -R and Down4 fragments were purified by agarose gel
 20 electrophoresis and mixed as a template for an overlap PCR amplification by using the primers P25/P28. An approximately 2243bp fragment was obtained and named Up- P_{tac} -R-Down fragment. The process of the PCR amplification was shown as follows: denaturalizing for 30s (seconds) in 94°C, annealing for 30s (seconds) in 52°C, extending for 60s (seconds) in 72°C, and performing for 30 times circularly.

25 Then the Up- P_{tac} -R-Down purified by agarose gel electrophoresis and the pK18mobsacB plasmid were digested respectively by *Bam* HI/*Eco* RI. The digestion products were purified by agarose gel electrophoresis and ligated to be a recombinant vector pK18mobsacB-Up- P_{tac} -R-Down for further insertion of additional copies of *acnR* into a non-coding region of a chromosome. The vector pK18mobsacB-Up- P_{tac} -R-Down was
 30 determined by sequencing the vector by a sequencing company to have the P_{tac} -*acnR* gene

fragments, and stored for further use.

The constructed plasmid pK18mobsacB-Up-P_{tac}-R-Down was transformed respectively by electroporation into the L-lysine-producing strain with low yield, *Corynebacterium pekinense* AS1.299, the L-lysine-producing strain with low yield, *Corynebacterium glutamicum* ATCC 13032, and the L-lysine-producing strain with high yield, *Corynebacterium pekinense* AS1.563. Homologous recombination-positive clones were selected after a recovery culture in BHIS medium under the conditions of 30°C and 100 rpm for 2h, and were verified by sequencing. Finally three L-lysine-producing *Corynebacterium* strains with low, low and high yield, into non-coding regions of the chromosomes of which additional copies of the gene *acnR* were inserted, were obtained and named YP-14857, YP-14896 and YP-14860.

Example 5. L-lysine fermentation experiments

For the fermentation of *E. coli*, 25mL of the seed medium shown in table 1 was inoculated with each of the strains *E. coli* K12 W3110 $\Delta 3$, *E. coli* NRRL B-12185 and *E. coli* strains constructed by examples 1~3 respectively, and cultured under the conditions of 37°C and 220 rpm for 9 h. Then 25mL of the fermentation medium shown in table 1 was inoculated with 1mL culture product of the seed medium, and cultured under the conditions of 37°C and 220 rpm for 48 h. The yield of L-lysine was measured by HPLC after the culture process.

Table 1 Culture medium for *E. coli*

	Seed medium	Fermentation medium
(Ingredient)	(g/L)	(g/L)
glucose	15	40
ammonium sulphate	4	10
potassium dihydrogen phosphate	3	1.6
magnesium sulphate heptahydrate	0.4	1
ferrous sulphate heptahydrate	0.01	0.03
manganese sulfate monohydrate	0.01	0.03
yeast extract	2.0	4.0

calcium carbonate		25
KOH	pH 7.0	pH 7.0
L-tyrosine		0.1
L-methionine		0.5
L-threonine		0.1
L-isoleucine		0.05

For the fermentation of *Corynebacterium*, 30mL of the seed medium shown in table 2 was inoculated with each of the strains *Corynebacterium* AS1.299, ATCC13032, AS1.563 and *Corynebacterium* strains constructed by examples 2~4 respectively, and cultured under the conditions of 30°C and 220 rpm for 8h. Then 30mL of the fermentation medium shown in table 2 was inoculated with 1mL culture product of the seed medium, and cultured under the conditions of 30°C and 220 rpm for 48 h. The yield of L-lysine was measured by HPLC after the culture process.

Table 2 Culture medium for *Corynebacterium*

	Seed medium	Fermentation medium
(Ingredient)	(g/L)	(g/L)
glucose	20	40
ammonium sulphate	5	20
potassium dihydrogen phosphate	1	1.6
magnesium sulphate heptahydrate	0.7	0.8
ferrous sulphate heptahydrate	0.01	0.01
manganese sulfate monohydrate	0.01	0.01
yeast extract	5.0	4.0
calcium carbonate		20
KOH	pH 7.0	pH 7.0

10

The results are show in table 3. To mutate (e.g., delete or replace) the aconitase structure of the strains of *Escherichia coli* and *Corynebacterium* for the decrease of the activity of the

enzyme, or to modify (e.g., replace and insert) the regulatory elements of the aconitase genes of the strains of *Escherichia coli* and *Corynebacterium* for the decrease of the expression amount of the enzyme, is conducive to the increase of L-lysine yield.

Table 3 Yield of L-Lysine by Strains

Strain	Yield of L-lysine (g/L)	Yield increase ratio (%)
<i>E. coli</i> NRRL B-12185	1.5	-
YP-13633	2.1	40
YP-13675	1.8	20
YP-13627	2.0	33
<i>E. coli</i> K12 W3110 $\Delta 3$	10.2	-
YP-13664	16.1	57.8
YP-13699	12.5	22.5
YP-13682	14.1	38.2
<i>Corynebacterium</i> AS1.299	1.2	-
YP-14808	1.6	33
YP-14755	1.9	58
YP-14857	1.4	17
<i>Corynebacterium</i> ATCC 13032	1.1	-
YP-14852	1.5	36
YP-14732	2.0	82
YP-14896	1.3	18
<i>Corynebacterium</i> AS1.563	23.5	-
YP-14837	27.4	16.6
YP-14780	31.2	32.8
YP-14860	25.6	8.9

CLAIMS

1. A method of producing L-lysine by fermentation or of increasing a fermentation yield of
5 L-lysine, which comprises the steps of:

(1) modifying an aconitase gene or regulatory element thereof in a chromosome of an L-lysine producing bacterium so that the activity or the expression amount of the aconitase of the bacterium is reduced but not eliminated; and

(2) producing L-lysine by fermentation with the bacterium obtained by the modification
10 of step (1),

wherein modifying the aconitase gene or regulatory element thereof comprises:

- (i) a substitution of the initiation codon of the nucleotide sequence of SEQ ID NO: 1 of the aconitase gene in the chromosome of an L-lysine producing *Escherichia coli* to GTG;
- 15 (ii) a substitution of the nucleotide sequence of SEQ ID NO: 4 of a promoter of the aconitase gene in the chromosome of an L-lysine producing *Escherichia coli* to the nucleotide sequence of SEQ ID NO: 3;
- (iii) a substitution of the nucleotide sequence of SEQ ID NO: 6 of a promoter of the aconitase gene in the chromosome of an L-lysine-producing *Corynebacterium glutamicum* or *Corynebacterium pekinese* to the nucleotide sequence of SEQ ID
20 NO: 5;
- (iv) a deletion of 90 nucleotides before the termination codon in the nucleotide sequence of the aconitase gene of SEQ ID NO: 1 in the chromosome of an L-lysine producing *Escherichia coli*; or
- 25 (v) an addition in the nucleotide sequence of a transcription repressor of the aconitase gene of SEQ ID NO: 7.

2. The method according to claim 1, wherein the step of modifying an aconitase gene or regulatory element element thereof in a chromosome of an L-lysine producing bacterium so
30 that the activity or the expression amount of the aconitase of the bacterium is reduced but not

eliminated is replaced by the step of modifying an aconitase gene and a regulatory element thereof in a chromosome of an L-lysine producing bacterium so that the activity or the expression amount of the aconitase of the bacterium is reduced but not eliminated.

5 3. The method according to claim 1, wherein the step of modifying an aconitase gene or regulatory element thereof in a chromosome of an L-lysine producing bacterium so that the activity or the expression amount of the aconitase of the bacterium is reduced but not eliminated is replaced by the step of modifying an aconitase gene or regulatory element thereof in a chromosome of an L-lysine producing bacterium so that the activity and the
10 expression amount of the aconitase of the bacterium are reduced but not eliminated.

4. The method according to claim 2, wherein the activity and the expression amount of the aconitase of the bacterium are reduced but not eliminated.

15 5. The method according to any one of claims 1 to 4, wherein the step of an addition in the nucleotide sequence of a transcription repressor of the aconitase gene of SEQ ID NO: 7 is replaced by the step of an addition in the nucleotide sequence of a transcription repressor of the aconitase gene of SEQ ID NO: 8 and SEQ ID NO: 7 in tandem.