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(54) Benævnelse: **MIKROORGANISME, DER KAN PRODUCERE L-AMINOSYRE, OG FREMGANGSMÅDE TIL FREMSTILLING AF L-AMINOSYRE VED BRUG AF SAMME**

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Description

Technical Field

[0001] The present invention relates to a microorganism able to produce L-threonine or L-tryptophan and to a method of producing L-threonine or L-tryptophan using the same.

Background Art

[0002] It is known that microorganisms which produce useful products through fermentation require very large amounts of energy such as ATP when the biosynthetic pathway is enhanced.

[0003] As is known in the art, it is very important that the intracellular balance between nicotinamide adenine dinucleotide (NAD(H)) that is produced by catabolic reactions and nicotinamide adenine dinucleotide phosphate NADP(H) that is used in anabolic reactions in microbial metabolic processes. NAD(H) is an intermediate in the catabolic reactions that generate ATP through the oxidation of food and functions as an energy source. And NADP(H) play roles in providing a reducing power in the *in vivo* metabolic process, that is providing the high-energy electrons needed to synthesize molecules by reacting with enzymes which generally catalyze an anabolic reactions. The balance therebetween is regulated either by the phosphorylation of NAD as shown in the following equation 1) or by the dephosphorylation of NADP as shown in the following equation 2).

Equation 1)

$$NAD^+ + ATP \Leftrightarrow NADP^+ + ADP$$

Equation 2)

$$NADP^+ \Leftrightarrow NAD^+ + phosphate$$

[0004] Thus, in order to effectively produce reducing power such as NADPH, a phosphate source such as ATP should be increased together.

[0005] ATP (Adenosine-5'-triphosphate) has a high energy phosphate bond, and generates energy when it is hydrolyzed to ADP and phosphate. ATP is produced mainly by chemiosmotic phosphorylation via the electron transport system in microorganisms or by substrate-level phosphorylation. The produced ATP is degraded to supply the energy required for cells and is reused by regenerating via glycolysis pathway or oxidative phosphorylation.

[0006] Based on this fact, studies have been conducted to apply bacteria's ATP energy regenerating process to the mass production of useful products in order to facilitate energy supply (Biosci Biotechnol Biochem., (1997) 61: 840-845). In studies on the regeneration of ATP in *E. coli,* it was found that the level of ATP in a microorganism is about 150% higher than that in the parent strain when a few genes, including *ysaA* (NCBI Gene ID: 948085), *ydaS* (NCBI Gene ID: 945923) and *ybiX* (NCBI Gene ID: 947502) genes was deficient, respectively, and this finding was applied to the production of glutathione (FEMS Microbiol Lett., (2009) 297:217-224). However, there was no report that directly explains the increase in production of amino acids caused by attenuation in the activities of proteins that are encoded by the genes. WO2005/075626 A1 and WO2008/072891 A1 disclose E. coli that display an enhanced production in L-threonine or L-tryptophan respectively, wherein the tyR gene or the nfR gene respectively have been inactivated.

Disclosure

Technical Problem

[0007] The present inventors have found that increasing the intracellular level of ATP, which is used as the most plentiful energy source in cells producing L-amino acid, is effective to increase the production of L-threonine or L-tryptophan.

[0008] An object of the present invention is to provide a recombinant *E. coli* strain which has an increased L-threonine or L-tryptophan productivity by increasing the productivity of ATP.

[0009] Another object of the present invention is to provide a method of producing L-threonine or L-tryptophan using the recombinant *E. coli* strain.

Technical Solution

[0010] In order to accomplish the above objects, an embodiment of the present invention provides an L-threonine or L-tryptophan producing *E. coli* strain, wherein the strain is a recombinant strain that is modified to attenuate (weaken) activity of at least two proteins selected from the group consisting of a protein YsaA having an amino acid sequence represented by SEQ ID NO: 2, a protein YdaS having an amino acid sequence represented by SEQ ID NO: 4, and a protein YbiX having an amino acid sequence represented by SEQ ID NO: 6, wherein the activity is attenuated by a method selected from the group consisting of: 1) deleting part or all of a polynucleotide encoding the protein; 2) modifying an expression regulatory sequence to reduce the expression of the polynucleotide encoding the protein; 3) modifying the chromosomal polynucleotide sequence encoding the protein to weaken the activity of the protein; and 4) combinations thereof.

[0011] An embodiment of the present invention also provides a method for producing L-threonine or L-tryptophan, which comprises culturing the above-described *E. coli* strain.

Advantageous Effects

[0012] The L-threonine or L-tryptophan productivity of a microorganism having L-threonine or L-tryptophan productivity is improved by increasing the intracellular ATP level in said microorganism. The method enhances production of L-threonine or L-tryptophan by recovering the balance of energy metabolism to increase the cellular activity and reduce the culture time.

Description of Drawings

[0013]

FIG. 1 shows the relative ATP level (%) of an L-threonine-producing strain relative to that of its parent strain.
FIG. 2 shows the relative ATP level (%) of a L-tryptophan-producing strain relative to that of its parent strain.

Best Mode

[0014] Hereinafter, the present invention will be described in detail.

[0015] An embodiment of the present invention provides an L-threonine or L-tryptophan producing *E. coli* strain, wherein the strain is a recombinant strain that is modified to attenuate activity of at least two selected from the group consisting of a protein YsaA having an amino acid sequence represented by SEQ ID NO: 2, a protein YdaS having an amino acid sequence represented by SEQ ID NO: 4, and a protein YbiX having an amino acid sequence represented by SEQ ID NO: 6, wherein the activity is attenuated by a method selected from the group consisting of: 1) deleting part or all of a polynucleotide encoding the protein; 2) modifying an expression regulatory sequence to reduce the expression of the polynucleotide encoding the protein; 3) modifying the chromosomal polynucleotide sequence encoding the protein to weaken the activity of the protein; and 4) combinations thereof.

[0016] An L-threonine or L-tryptophan producing microorganism includes any microorganism capable of producing L-threonine or L-tryptophan, such as Escherichia sp. Bacterium, *E. coli,* Coryneform bacterium, *Serratia* sp. bacterium, *Providencia* sp. bacterium, or the like.

[0017] In a specific embodiment of the present invention, recombinant *E. coli* strain CJ600 (KCCM 10812P) (Korean Patent Registration No. 10-0792095) having L-tryptophan productivity is used to produce the *E.coli* strain of the invention. *E. coli* strain CJ600 (KCCM 10812P) is obtained by genetically engineering an recombinant *E. coli* strain (KFCC 10066) having L-phenylalanine productivity so as to desensitize the tryptophan auxotrophy, block L-phenylalanine biosynthesis and enhance tryptophan biosynthesis-related genes.

[0018] In another specific embodiment of the present invention, recombinant *E. coli* strain FTR2533 (KCCM-10541) (Korean Patent Registration No. 10-0576342) having L-threonine productivity is used to produce the *E.coli* strain of the invention. *E. coli* strain FTR2533 (KCCM-10541) is obtained by genetically engineering an *E. coli* mutant strain (KFCC 10718) having L-threonine productivity so as to inactivate the wild-type *galR* gene.

[0019] YsaA, a protein having an amino acid sequence represented by SEQ ID NO: 2, is predicted a hydrogenase of 4Fe-4S ferredoxin-type component, but its exact function has not yet been found.

[0020] YdaS, a protein having an amino acid sequence represented by SEQ ID NO: 4, is predicted a DNA binding transcription regulator, but its exact function has not yet been found.

[0021] YbiX, a protein having an amino acid sequence represented by SEQ ID NO: 6, is one of the Fe (II)-dependent oxygenase superfamily, which functions as an oxidoreductase that oxidizes its substrate using oxygen.

[0022] The polypeptides YsaA, YdaS and YbiX in *E.coli* have the amino acid sequences represented by SEQ ID NOS:

2, 4 and 6, respectively. The amino acid sequences of the proteins may depend on the species or strains of microorganisms.

[0023] Proteins may be mutants or artificial variants that have an amino acid sequence including a substitution, deletion, insertion, addition or inversion of one or several amino acids in one or more positions of the amino acid sequence represented by SEQ ID NO: 2, 4 or 6, as long as the mutants or artificial variants can be helpful in increasing the production of amino acid by attenuating the activities described herein. Herein, the number of "several" amino acids differs depending on the position or type of amino acid residues in the three-dimensional structure of the protein, but is particularly 2-20, specifically 2-10, and more specifically 2-5. In addition, this substitution, deletion, insertion, addition or inversion of amino acids also include those alterations caused by a naturally occurring mutation or artificial variation based on the difference in individuals or species of microorganisms having the activity of the polypeptides.

[0024] As used herein, the term "attenuation" means that the activity of a protein is weakened either by deleting part or all of the gene encoding the protein, or by modifying an expression regulatory sequence to reduce the expression of the gene, or by modifying the chromosomal gene sequence to weaken the activity of the protein, or by combinations thereof.

[0025] The method for deletion of part or all of the polynucleotide encoding the protein may be performed by replacing a polynucleotide which encodes an endogenous target protein in the chromosome, with either a polynucleotide that has a part of the nucleic acid sequence deleted or a marker gene, through a chromosome insertion vector.

[0026] Herein, the term "a part of" a nucleic acid sequence differs depending on the kind of gene, but is regardless of the position thereof, and it is specifically 1-200, more specifically 1-100, and even more specifically 1-50.

[0027] Also, the method of modifying the expression regulatory sequence to reduce the expression of the polynucleotide may be performed either by inducing a mutation in the expression regulatory sequence by the deletion, insertion, non-conservative or conservative substitution, or combinations thereof, of one or more nucleotides to attenuate the activity of the expression regulatory sequence, or by replacing the expression regulatory sequence with weaker activity. The expression regulatory sequence includes a promoter, an operator sequence, a sequence encoding a ribosome-binding site, a sequence regulating the termination of transcription and translation.

[0028] In addition, the method of modifying the chromosomal polynucleotide sequence encoding the protein may be performed either by inducing a mutation in the sequence by the deletion, insertion, non-conservative or conservative substitution, or combinations thereof, of one or more nucleotides to attenuate the activity of the sequence, or by replacing the sequence with an modified nucleotide sequence having weaker activity.

[0029] The polynucleotide encoding the protein can be introduced into a host cell and may be substituted with a codon difficult to express in the host. In addition, the N-terminus or C-terminus thereof may be extended or deleted, and the start codon may be modified to regulate the expression level.

[0030] Each of the polynucleotides disclosed herein may have a polynucleotide sequence encoding a protein having a homology of at least 80%, specifically at least 90%, more specifically at least 95%, and even more specifically at least 97% to the amino acid sequence of each represented by SEQ ID NOS: 2, 4 and 6, as long as the polynucleotide can attenuate the protein activity of the variant. More specifically, the polynucleotides disclosed herein have a polynucleotide sequence represented by SEQ ID NOs: 1, 3 and 5, respectively.

[0031] As used herein, the term "homology" refers to the identity between two amino acid sequences. The homology can be determined using well-known methods, for example, the computer program BLAST 2.0 which calculates parameters like as score, identity, and similarity.

[0032] Also, the polynucleotide sequences disclosed herein may be hybridized with the polynucleotide sequences represented by SEQ ID NOS: 1, 3 and 5 and probes produced from the above-described nucleotide sequences under stringent conditions, and may be modified sequences encoding normally functioning proteins.

[0033] As used herein, the term "stringent conditions" refers to conditions that allow specific hybridization between polynucleotides. Alternatively, the term is related to polypeptides or proteins, including derivatives thereof (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).

[0034] Specifically, the "stringent conditions" refer to hybridization at 65 °C in hybridization buffer (3.5×SSC, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 2.5 mM NaH_2PO_4 (pH 7), 0.5% SDS, 2 mM EDTA). SSC is 0.15 M sodium chloride/0.15 M sodium citrate at pH 7. After hybridization, the membrane to which the DNA has been transferred is washed in 2×SSC at room temperature and then in 0.1-0.5×SSC/0.1×SDS at 68 °C.

[0035] As used herein, the term "vector" refers to a DNA construct containing the nucleotide sequence of a target protein-encoding gene operably linked to a suitable regulatory sequence so as to be able to express the target gene in a suitable host cell. The regulatory sequence includes a promoter capable of initiating transcription, any operator for regulating this transcription, a sequence encoding a suitable mRNA ribosome binding site, and a sequence for regulating the termination of transcription and translation. Once transformed into a suitable host, the vector may replicate or function independently of the host genome, or may, in some cases, integrate into the genome itself.

[0036] The vector that is used may be any vector known in the art, as long as it can replicate in a host. Examples of the commonly used vectors may include natural or recombinant plasmids, cosmids, viruses, and bacteriophages. For example, the phage vector or cosmid vectors include pWE15, M13, λBL3, λ BL4, λXII, λSHII, λPII, λ10, λ11, Charon4A, and Charon21A, and the plasmid vectors include pBR, pUC, pBluescriptII, pGEM, pTZ, pCL1920 and pET-type plasmids. Vectors that may be used include any known expression vectors. Specifically, pACYC177, pACYC184, pCL1920, pECCG117, pUC19, pBR322, pMW118 or pCClBAC vectors may be used. Most specifically, pACYC177, pCL1920 and pCClBAC vectors may be used.

[0037] Further, the vector that is used is a vector capable of transforming host cells, to insert the polynucleotide encoding the target protein into the chromosome of the host cell. Specific examples of the vector include the shuttle vector pECCG112 that can self-replicate in both directions in *E. coli* and Coryne-type bacteria (Kap-Soo, Noh, Kor. Jour. Microbiol. July 1991, p149-154).

[0038] Also, the polynucleotide encoding the endogenous target protein in the chromosome can be replaced with a new polynucleotide by a vector for insertion into the bacterial chromosome. Insertion of the polynucleotide into the chromosome can be performed by any method known in the art, for example, homologous recombination. Because the vector can be inserted into the chromosome by homologous recombination, it may further comprise a selection marker for confirming its insertion into the chromosome. The selection marker is used to select a cell transformed with the vector, that is, confirm the insertion of the target polynucleotide. The selection marker that is used may be selected from markers that provide selectable phenotypes, such as drug resistance, auxotrophy, resistance to cytotoxic agents, or surface protein expression. Only cells expressing the selection marker are able to survive or to show different phenotypes under the environment treated with the selective agent, and thus the transformed cells can be selected.

[0039] As used herein, the term "transformation" means introducing a vector comprising the polynucleotide encoding the target protein into a host cell so as to be able to express the protein encoded by the polynucleotide in the host cell. The transformed polynucleotides include all the genes inserted in the chromosome of the host cell or located outside the chromosome, as long as they can be expressed in the host cell. In addition, the polynucleotides include DNA and RNA, which encode the target protein. As long as the polynucleotide can be introduced in the host cell and expressed therein, the gene may be introduced in any form.

[0040] For example, the polynucleotide can be introduced into the host cell in the form of an expression cassette which is a polynucleotide construct including all elements for expressing the gene. The expression cassette includes a promoter which is 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 capable of self-replicating. The polynucleotide may also be introduced into the host cell by itself, and be operably linked to the sequence necessary for expression in the host cell.

[0041] Specifically, attenuation of the activity of the protein that is encoded by the *ysaA, ydaS* or *ybiX* gene may be achieved by deletion of the gene. Specifically, a mutation in the gene can be induced using chemicals or light such as UV light, thereby obtaining a variant having the deleted gene. Alternatively, a variant lacking the activity of the protein can be obtained by substituting the chromosomal gene to the nucleotide lacking the activity by a gene recombination technique, by a method of gene replacement throuugh homologous recombination.

[0042] Also, an embodiment of the present invention also provides a method for producing L-threonine or L-tryptophan, the method comprising culturing an L-threonine or L-tryptophan producing *E. coli* strain, wherein the strain is a recombinant strain that is modified to attenuate activity of at least two selected from the group consisting of a protein YsaA having an amino acid sequence represented by SEQ ID NO: 2, a protein YdaS having an amino acid sequence represented by SEQ ID NO: 4, and a protein YbiX having an amino acid sequence represented by SEQ ID NO: 6, wherein the activity is attenuated by a method selected from the group consisting of: 1) deleting part or all of a polynucleotide encoding the protein; 2) modifying an expression regulatory sequence to reduce the expression of the polynucleotide encoding the protein; 3) modifying the chromosomal polynucleotide sequence encoding the protein to weaken the activity of the protein; and 4) combinations thereof.

[0043] The culture process of the present invention can be performed in suitable media and culture conditions known in the art. This culture process can be easily modified by any person skilled in the art depending on the type of strain selected. Examples of the culture process include, but are not limited to, batch culture, continuous culture, and fed-batch culture.

[0044] The media and culture conditions that are used in culture of the microorganism of the present invention may be those as long as that are used in culture of microorganisms belonging to the genus Escherichia, but these should properly satisfy the requirements of the microorganism of the present invention.

[0045] In a specific embodiment of the present invention, the microorganism may be cultured in a conventional medium containing suitable carbon sources, nitrogen sources, amino acids, vitamins and the like under aerobic conditions while adjusting temperature, pH and the like.

[0046] Carbon sources that may be used in the method of the present invention include carbohydrates such as glucose, fructose, sucrose, maltose, mannitol, sorbitol; alcohols such as sugar alcohol, glycerol, pyruvic acid, lactic acid and citric

acid; and amino acids such as organic acid, glutamic acid, methionine and lysine. In addition, natural organic nutrient sources such as starch hydrolysates, molasses, blackstrap molasses, rice bran, cassava, bagasse and corn steep liquor may be used. Specifically, the organic nutrient sources include glucose and sterile pretreated molasses (i.e., molasses converted to reduced sugars), and suitable amounts of carbon sources may be used without limitation.

[0047] Nitrogen sources that may be used in the method of the present invention include inorganic nitrogen sources such as ammonia, ammonium sulfate, ammonium chloride, ammonium acetate, ammonium phosphate, ammonium carbonate, and ammonium nitrate; amino acids such as glutamic acid, methionine and glutamine; and organic nitrogen sources such as peptone, NZ-amine, meat extract, yeast extract, malt extract, corn steep liquor, casein hydrolysate, fish meal or its digested product, defatted soybean cake or its digested product, etc. These nitrogen sources may be used alone or in combination. The medium may contain potassium phosphate monobasic, potassium phosphate dibasic and corresponding sodium-containing salts, as phosphorus sources.

[0048] Inorganic compounds that may be used in the method of the present invention include sodium chloride, calcium chloride, iron chloride, magnesium sulfate, iron sulfate, manganese sulfate and calcium carbonate. In addition, the medium may contain amino acids, vitamins and suitable precursors. These media or precursors may be added to the medium in a batch or continuous manner.

[0049] Compounds such as ammonium hydroxide, potassium hydroxide, ammonia, phosphoric acid and sulfuric acid may be added to the medium in a suitable manner during culture to adjust the pH of the culture medium.

[0050] In addition, during culture, a defoaming agent such as fatty acid polyglycol ester may be used to suppress the formation of bubbles. Further, in order to maintain the culture medium in an aerobic state, oxygen or oxygen-containing gas may be injected into the culture medium. In addition, in order to maintain the culture medium in an anaerobic or non-aerobic state, no gas is injected, or nitrogen, hydrogen or carbon dioxide gas may be injected into the culture medium. The culture medium is typically maintained at a temperature ranging from 27 °C to 37 °C, and specifically from 30 °C to 35 °C. Culture of the microorganism can be continued until the desired level of the useful substance will be obtained. Specifically, the culture period is from 10 to 100 hours.

[0051] The method of the present invention may further comprise purifying or recovering the L-amino acid produced in the culture step. The purification or recovery process can be performed by purifying or recovering the desired L-amino acid from the culture medium using a suitable method selected depending on the method used for culture of the microorganism, for example, a batch, continuous or fed-batch culture method.

[0052] Hereinafter, the present invention will be described in further detail with reference to examples. It is to be understood, however, that these examples are for illustrative purposes.

Examples

Example 1: Construction of L-threonine and L-tryptophan producing strains having attenuated activity of protein that is encoded by *ysaA, ydaS* or *ybiX* gene

[0053] In this Example, each of the *ysaA, ydaS* and *ybiX* gene in the L-tryptophan producing strain KCCM10812P (Korean Patent Registration No. 10-0792095) and the L-threonine producing strain KCCM10541 (Korean Patent Registration No. 10-0576342) was deleted by homologous recombination.

[0054] The L-tryptophan producing parent strain KCCM10812P is a strain derived from an *E. coli* variant KFCC 10066 having L-phenylalanine productivity. It is a recombinant *E. coli* strain having L-tryptophan productivity, characterized in that chromosomal tryptophan auxotrophy was desensitized, the *pheA, trpR, mtr* and *tnaAB* genes were attenuated and the *aroG* and *trpE* genes were modified.

[0055] Also, the L-threonine producing parent strain KCCM10541 is a strain derived from *E. coli* KFCC10718 (Korean Patent Laid-Open Publication No. 1992-0008365). It has resistance to L-methionine analogue, a methionine auxotroph phenotype, resistance to L-threonine analogue, a leaky isoleucine auxotroph phenotype, resistance to L-lysine analogue, and resistance to α-aminobutyric acid, and is capable of producing L-threonine.

[0056] The *ysaA, ydaS* and *ybiX* genes to be deleted have the polynucleotide sequences represented by SEQ ID NOS: 1, 2 and 3, respectively.

[0057] For this purpose, the one-step inactivation method (developed by Datsenko KA et al.) that is a mutagenesis technique using lambda red recombinase was used (Proc Natl Acad Sci USA., (2000) 97: 6640-6645). As a marker for confirming insertion into the genes, the chloramphenicol-resistant gene of pUCprmfmloxC was used (Korean Patent Laid-Open Publication No. 2009-0075549) .

[0058] About 1200-bp gene fragments were amplified by polymerase chain reaction (hereinafter referred to as PCR) using pUCprmfmloxC as a template and a pair of primers 1 and 2, a pair of primers 7 and 8 and a pair of primers 13 and 14, which have a portion of each of the three genes and a portion of the chloramphenicol-resistant gene of pUCprmfmloxC. The PCR was performed for 30 cycles, each consisting of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 1 min.

Table 1

Primer No.	Sequence	SEQ ID NO
1	5'-GTAGGGACGCGCTCTCTGGCACTCTGCTGTTTTAGTGCAAAGGAGTGATCAGGTGACACTATAGAACGCG-3'	7
2	5'-GGCATAAACAAAGCGCACTGTTCCGGCGTTGAGAAACGCCGGAAAACGTTTAGTGGATCTGATGGGTACC-3'	8
3	5'-GCTTTGGACAAGTGCCAAAACTTTAACATTTCCTTCGTTGGATCAAAGCAGTAGGGACGCGCTCTCTGGC-3'	9
4	5'-ATTGAATTTGGAAGAATTTGTAGGCCGGATAAGGCGTTTACGCCGCATCTGGCATAAACAAAGCGCACTG-3'	10
5	5'-GAGAGAAAAATCTCCTGAAA-3'	11
6	5'-CCTACATGATTTCTGCAATA-3'	12
7	5'-ATTGCGTTAGGCGTCGCCTAATATTTCTGTGTGTTTTTGGAGTTCATTCGAGGTGACACTATAGAACGCG-3'	13
8	5'-ATTCGATGTGCTCATGCTTGATTTTCATGAATCATTTGCCTCTTGATGTTTAGTGGATCTGATGGGTACC-3'	14
9	5'-TTACATTAGGCAATCCCTACCCTTACTGCATTAGGCACAGCCTATTGACAATTGCGTTAGGCGTCGCCTA-3'	15
10	5'-ATTGGCTACCCATGCCTGCCCTTTTCGGCTGCTAGGGCAAACAACACTGATTCGATGTGCTCATGCTTG-3'	16
11	5'-TATAGAGCCTTTCTTAATCC-3'	17
12	5'- CGCAGATATTCTTCAGTAAT-3'	18
13	5'-CATTTCTGATTCAGATGTGGGGCGCAGGCCCCCACTTTTTGGAGAAATTGTAGGTGACACTATAGAACGCG-3'	19
14	5'-TGTACAGTTAAGTGTAGCTAATCCAGGGACGAACTCGGGCAGTTCAAGCATAGTGGATCTGATGGGTACC-3'	20

(continued)

Primer No.	Sequence	SEQ ID NO
15	5'-ACCGTTATCACCCGGGCGAGCCAAGAACCTTCTTGCTCACAGCCAATATGCATTTCTGATTCAGATGTGG-3'	21
16	5'-GTCATCGTTAGCCCAACCGGATGCCATATCGACCTCCCCATATCAATACTTGTACAGTTAAGTGTAGCTA-3	22
17	5'-AAAGGTTCAGACGGCGCGGT -3'	23
18	5'-TAAGCGCACGCCAGGAATGG-3'	24

[0059] Also, the DNA fragments obtained by the PCR amplification were electrophoresed on 0.8% agarose gel, and then eluted and used as templates in secondary PCR. Secondary PCR was performed so that the 5' and 3' terminal regions of the primary DNA fragments had 20 pairs of complementary nucleotide bases. About 1300-bp gene fragments were amplified by PCR using the eluted primary PCR products as templates and a pair of primers 3 and 4, a pair of primers 9 and 10 and a pair of primers 15 and 16, which have include the 5' and 3' regions of the genes. The PCR was performed for 30 cycles, each consisting of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 1 min. The DNA fragments obtained by the PCR amplification were electrophoresed on 0.8% agarose gel, and then eluted and used in recombination.

[0060] According to the method developed by Datsenko KA et al. (Proc Natl Acad Sci USA., (2000) 97:6640 6645), an *E. coli* strain transformed with a pKD46 were made competent, and then transformed with the 1300-bp gene fragments obtained by PCR. The resulting strains were selected on LB medium having resistance to chloramphenicol. PCR was performed using a pair of primers 5 and 6, a pair of primers 11 and 12 and a pair of primers 17 and 18, and the amplification products had sizes of 1450, 1530 and 1640 bp, respectively, and were confirmed that the genes were deleted.

[0061] pKD46 was removed from the primary recombinant strains having chloramphenicol resistance, and then a pJW168 vector was introduced into the strains, and the chloramphenicol marker gene was removed from the bacterial cells (Gene, (2000) 247,255-264). The resulting bacterial cells were about 400-bp, 500-bp and 600-bp amplification products obtained by a pair of primers 5 and 6, a pair of primers 11 and 12 and a pair of primers 17 and 18, and were confirmed that the desired gene deletion was achieved.

[0062] According to the above-described method, the L-threonine producing strains KCCM10541 Δ*ysaA,* KCCM10541 Δ*ydaS* and KCCM10541 Δ*ybiX* were constructed. Also, the L-tryptophan producing strains KCCM10812P Δ*ysaA,* KCCM10812P Δ*ydaS* and KCCM10812P Δ*ybiX* were constructed.

Example 2: Construction of recombinant L-threonine and L-tryptophan producing strains having deletion of two or more of *ysaA* , *ydaS* and *ybiX* genes

[0063] According to the method described in Example, recombinant strains having a deletion of two or more of the genes were constructed.

[0064] A pKD46 vector for using lambda red recombinase was introduced into the strains having a deletion of any one of the genes, and then the strains were made competent. Also, gene fragments amplified by PCR to include a portion of the three genes and the chloramphenicol-resistant gene of pUCprmfmloxC were transformed into different strains having a deletion of one of the genes. The resulting strains were screened on LB medium having chloramphenicol resistance, and deletion of a combination of the genes was confirmed by the use of the primer pairs described in Example 1.

[0065] According to the above-described method, the L-threonine producing strains KCCM10541 Δ*ysaA* Δ*ydaS*, KCCM10541 Δ*ydaS* Δ*ybiX*, KCCM10541 Δ*ybiX* Δ*ysaA* and KCCM10541 Δ*ysaA* Δ*ydaS* Δ*ybiX* were constructed. Also, the L-tryptophan producing strains KCCM10812P Δ*ysaA* Δ*ydaS*, KCCM10812P Δ*ydaS* Δ*ybiX*, KCCM10812P Δ*ybiX* Δ*ysaA* and KCCM10812P Δ*ysaA* Δ*ydaS* Δ*ybiX* were constructed.

[0066] Among the recombinant strains obtained as described above, KCCM10541 Δ*ysaA* Δ*ydaS* Δ*ybiX* and KCCM10812P Δ*ysaA* Δ*ydaS* Δ*yMX* were named "*E. coli* CA03-4257P" and "*E. coli* CA04-2002", respectively, and deposited at the Korean Culture Center of Microorganisms (361-221, Hongje 1-dong, Seodaemun-gu, Seoul, Korea), an international depository authority, on December 29, 2011 under the accession numbers KCCM11243P and KCCM11245P, respectively.

Example 3: Measurement of levels of ATP in constructed L-threonine producing strains and L-tryptophan producing strains

[0067] In this Example, the levels of ATP in the strains constructed in Examples 1 and 2 were quantitatively measured.

[0068] For this purpose, the method developed by Kiyotaka Y. Hara et al., which uses luciferase, was used ("An Efficient Method for Quantitative determination of Cellular ATP Synthetic Activity", J Biom Scre, (2006) V11:No.3:PP310-17).

[0069] Specifically, the strains having different genetic characters were cultured overnight in LB liquid medium containing glucose. The supernatant was removed by centrifugation, the bacterial cells were washed with 100 mM Tris-Cl (pH 7.5), and then treated with PB buffer (permeable buffer: 40%[v/v] glucose, 0.8%[v/v] Triton X-100) for 30 minutes to release intracellular ATP. Next, the supernatant was removed by centrifugation, and luciferin as a substrate for luciferase was added to the cells. The cells were allowed to stand for 10 minutes, and then luciferase activity in the cells was measured with a luminometer to quantitatively determine the ATP level. The results of the measurement are shown in FIGS. 1 and 2. All the results were recorded as the average of three repeated experiments.

[0070] As can be seen in FIGS. 1 and 2, the levels of ATP in the strains constructed from the L-threonine producing strain and the L-tryptophan producing strain in Examples 1 and 2 all increased. In addition, the ATP level was higher in

the strains having a deletion of a combination of the genes than in the strains having a deletion of one of the gene.

Example 4: Examination of titer of L-threonine producing strain, which has attenuated activity of alone or a combination of enzymes that are encoded by *ysaA*, *ydaS* and *ybiX* gene, in glucose-containing medium

[0071] According to the methods described in Examples 1 and 2, alone or a combination of the *ysaA*, *ydaS* and *ybiX* genes was deleted from the L-threonine producing strain KCCM10541 (Korean Patent Registration No. 10-0576342) to increase the intracellular ATP level. The titers of the resulting strains were evaluated using glucose as a carbon source.

[0072] Specifically, the strains having different genetic characters were cultured overnight on LB solid medium in an incubator at 33 °C and inoculated by a platinum loop into 25 mL of glucose-containing medium having the composition shown in Table 2 below. Then, the strains were incubated in an incubator at 33 °C and at 200 rpm for 50 hours. The results are shown in Table 3 below. All the results were recorded as the average of three flask results.

Table 2

Composition	Concentration (per liter)
Glucose	70 g
KH_2PO_4	2 g
$(NH_4)_2SO_4$	25 g
$MgSO_4 \cdot H_2O$	1 g
$FeSO_4 \cdot H_2O$	5 mg
$MnSO_4 \cdot H_2O$	5 mg
Yeast extract	2 g
Calcium carbonate	30 g
pH	6.8

Table 3

Strain	OD	Glucose consumption (g/L)*	L-threonine (g/L)**
KCCM10541	23.7	30.3	31.8
KCCM10541 Δ*ysaA*	24.6	33.4	32.2
KCCM10541 Δ*ydaS*	23.5	34.7	33.0
KCCM10541 Δ*ybiX*	22.7	33.9	32.7
KCCM10541 Δ*ysaA* Δ*ydaS*	24.9	35.1	32.9
KCCM10541 Δ*ydsS* Δ*ybiX*	24.5	36	33.1
KCCM10541 Δ*ybiX* Δ*ysaA*	25.0	32.1	33.0
KCCM10541 Δ*ysaA* Δ*ydaS* Δ *ybiX*	26.1	36.9	33.9
* measured at 30 hours ** measured at 50 hours			

[0073] As can be seen in Table 3 above, we have demonstrated that the glucose utilization of the recombinant L-threonine producing *E.coli* strains increased by up to about 22% compared to that of the parent strain, and the production of threonine of the recombinant strains increased by up to about 7% compared to that in the parent strain. In view of the ATP level shown in FIG. 1, these results indicate that the glucose consumption rate or amino acid productivity of the recombinant strains was increased by the increased ATP level.

Example 5: Examination of titer of L-threonine producing strain, which has attenuated activity of alone or a combination of enzymes that are encoded by *ysaA*, *ydaS* and *ybiX* gene, in sucrose-containing medium

[0074] According to the methods described in Examples 1 and 2, alone or a combination of the *ysaA*, *ydaS* and *ybiX* genes was deleted from the L-threonine producing strain KCCM10541 (Korean Patent Registration No. 10-0576342) to increase the intracellular ATP level. The titers of the resulting strains were evaluated using sucrose as a carbon source.

[0075] Specifically, the strains having different genetic characters were cultured overnight on LB solid medium in an incubator at 33 °C and inoculated by a platinum loop into 25 mL of sucrose-containing medium having the composition shown in Table 4 below. Then, the strains were incubated in an incubator at 33 °C and at 200 rpm for 48 hours. The results are shown in Table 5 below. All the results were recorded as the average of three flask results.

Table 4

Composition	Concentration (per liter)
Sucrose	70 g
KH_2PO_4	2 g
$(NH_4)_2SO_4$	25 g
$MgSO_4 \cdot H_2O$	1 g
$FeSO_4 \cdot H_2O$	5 mg
$MnSO_4 \cdot H_2O$	5 mg
Yeast extract	2 g
Calcium carbonate	30 g
pH	6.8

Table 5

Strain	OD	sucrose consumption (g/L)*	L-threonine (g/L)**
KCCM10541	26.2	40.0	37.0
KCCM10541 Δ*ysaA*	27.1	40.9	37.5
KCCM10541 Δ*daS*	26.7	41.7	37.8
KCCM10541 Δ*ybiX*	25	41.1	38.1
KCCM10541 Δ*ysaA* Δ*ydaS*	27.5	42.1	38.0
KCCM10541 Δ*dsS* Δ*ybiX*	27.2	43.0	38.1
KCCM10541 Δ*ybiX* Δ*ysaA*	28.3	42.8	38.7
KCCM10541 Δ*ysaA* Δ*ydaS* Δ*ybiX*	27.9	43.9	38.9
* measured at 24 hours ** measured at 48 hours			

[0076] As can be seen in Table 5 above, we have demonstrated that the sucrose utilization of the recombinant L-threonine producing *E.coli* strains increased by up to about 10% compared to that of the parent strain, and the production of threonine of the recombinant strains increased by up to about 5% compared to that in the parent strain. In view of the ATP level shown in FIG. 1, these results indicate that the activity and sucrose consumption rate or amino acid productivity of the recombinant strains were increased by the increased ATP level.

Example 6: Examination of titer of L-tryptophan producing strain, which has attenuated activity of alone or a combination of enzymes that are encoded by *ysaA*, *ydaS* and *ybiX* gene, in glucose-containing medium

[0077] According to the methods described in Examples 1 and 2, alone or a combination of the *ysaA*, *ydaS* and *ybiX* genes was deleted from the L-tryptophan producing strain KCCM10812P (Korean Patent Registration No. 10-0792095)

to increase the intracellular ATP level. The titers of the resulting strains were evaluated using glucose as a carbon source.

[0078] In order to examine the titer, the strains were inoculated by a platinum loop on LB solid medium and then cultured overnight in an incubator. And, it was inoculated by a platinum loop into 25 mL of flask titer medium having the composition shown in Table 6 below. Then, the strains were incubated in an incubator at 37 °C and at 200 rpm for 48 hours. The results are shown in Table 7 below. All the results were recorded as the average of three flask results.

Table 6

Composition	Concentration (per liter)
Glucose	60 g
K_2HPO_4	1 g
$(NH_4)_2SO_4$	10 g
NaCl	1 g
$MgSO_4 \cdot H_2O$	1 g
Sodium citrate	5 g
Yeast extract	2 g
Calcium carbonate	40 g
Sodium citrate	5 g
Phenylalanine	0.15 g
Tyrosine	0.1 g
PH	6.8

Table 7

Strain	OD	Glucose consumption (g/L)*	L-tryptophan (g/L)**
KCCM10812P	18.2	47.2	5.7
KCCM10812P Δ*ysaA*	18.3	48.3	6.9
KCCM10812P Δ*ydaS*	18	49.1	6.6
KCCM10812P Δ*ybiX*	17.7	50	6.0
KCCM10812P Δ*ysaA*Δ*ydaS*	17.9	48.4	7.5
KCCM10812P Δ*ydaS*Δ*ybiX*	18.7	49.3	7.6
KCCM10812P Δ*ybiX*Δ*ysaA*	19.9	49	7.3
KCCM10812P Δ*ysaA*Δ*ydaS*Δ*ybiX*	18.9	51.9	7.9
* measured at 33 hours ** measured at 48 hours			

[0079] As can be seen in Table 7 above, we have demonstrated that the glucose consumption of the recombinant L-threonine producing *E. coli* strains increased by up to about 10% compared to that of the parent strain, and the production of tryptophan of the recombinant strains increased by up to about 38% compared to that in the parent strain. In view of the ATP level shown in FIG. 2, these results indicate that the activity and glucose consumption rate or amino acid productivity of the recombinant strains were increased by the increased ATP level.

Accession Number

[0080]

Depository authority: Korean Culture Center of Microorganisms (International)

Accession Number: KCCM11243P
Deposition date: December 29, 2011
Depository authority: Korean Culture Center of Microorganisms (International)
Accession Number: KCCM11245P
Deposition date: December 29, 2011

<110> CJ Cheiljedang Corporation

<120> MICROORGANISMS PRODUCING L-AMINO ACIDS AND PROCESS FOR PRODUCING L-AMINO ACIDS USING THEREOF

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Patentkrav

1. En L-threonin eller L-tryptofan, som producerer *E. coli* -stamme, hvori stammen er en rekombinant stamme, der er ændret til at dæmpe aktivitet af mindst to af de proteiner, der er valgt fra den gruppe, der består af et protein YsaA, der har en aminosyresekvens, der er repræsenteret af SEQ ID NO: 2, et protein YdaS, der har en aminosyresekvens, der er repræsenteret af SEQ ID NO: 4, og et protein YbiX, der har en aminosyresekvens, der er repræsenteret af SEQ ID NO: 6, hvori aktiviteten dæmpes ved at slette del af eller det hele af en polynukleotid, der koder proteinet.

2. L-threonin eller L-tryptofan, som producerer *E. coli* -stamme i henhold til krav 1, hvori proteinet YsaA er kodet af en polynukleotidsekvens, der er repræsenteret af SEQ ID NO: 1, proteinet YdaS, der er kodet af en polynukleotidsekvens, der er repræsenteret af SEQ ID NO: 3, og proteinet YbiX, der er kodet af en polynukleotidsekvens, der er repræsenteret af SEQ ID NO: 5.

3. L-threonin eller L-tryptofan, som producerer *E. coli* stamme i henhold til krav 1, hvori *E. coli*-stammen er L-threonin, der producerer *Escherichia coli* CA03-4257P (KCCM11243P).

4. L-threonin eller L-tryptofan, som producerer *E. coli* stamme i henhold til krav 1, hvori *E. coli*-stammen er L-tryptofan, der producerer *Escherichia coli* CA04-2002 (KCCM11245P).

5. En fremgangsmåde til fremstilling af L-threonin eller L-tryptofan, hvor fremgangsmåden omfatter dyrkning af en L-threonin eller L-tryptofan, som producerer *E. coli*-stamme, hvori stammen er en rekombinant stamme, der er ændret til at dæmpe aktivitet af mindst to af de proteiner,

der er valgt fra den gruppe, der består af et protein (YsaA), der har en aminosyresekvens, der er repræsenteret af SEQ ID NO: 2, et protein (YdaS), der har en aminosyresekvens, der er repræsenteret af SEQ ID NO: 4, og et protein (YbiX), der har en aminosyresekvens, der er repræsenteret af SEQ ID NO: 6, hvori aktiviteten dæmpes ved at slette del af eller det hele af en polynukleotid, der koder proteinet.

6. Fremgangsmåden i henhold til krav 5, hvori *E. coli* stammen er L-threonin, der producerer *Escherichia coli* CA03-4257P (KCCM11243P).

7. Fremgangsmåden i henhold til krav 5, hvori *E. coli*-stammen er L-tryptofan, der producerer *Escherichia coli* CA04-2002 (KCCM11245P).

DRAWINGS

Figure 1.

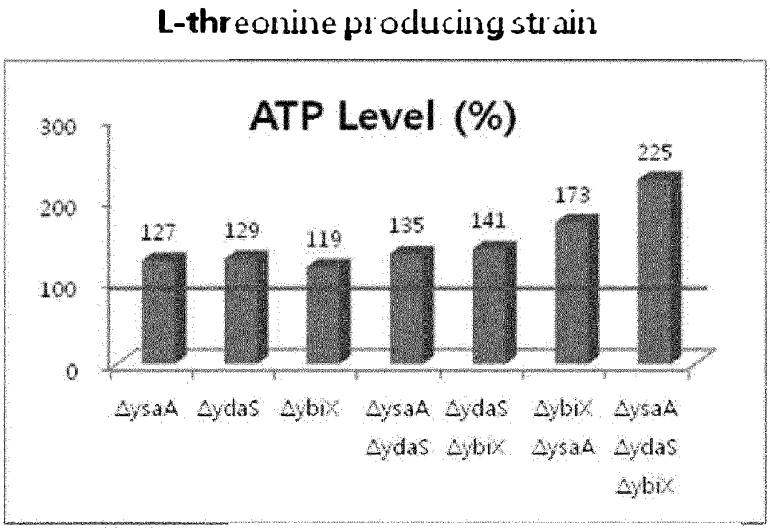

Figure 2.