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(54) PROCESS FOR THE PREPARATION OF L-THREONINE USING STRAINS OF THE ENTEROBACTERIACEAE FAMILY

VERFAHREN ZUR HERSTELLUNG VON L-THREONIN UNTER VERWENDUNG VON STÄMMEN DER FAMILIE ENTEROBACTERIACEAE

PROCEDE DE PREPARATION DE L-THREONINE A L'AIDE DE SOUCHES DE LA FAMILLE DES ENTEROBACTERIACEAE

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(56) References cited:
DE-A- 19 539 952 DE-C- 19 949 579

• OKAMOTO K ET AL: "HYPERPRODUCTION OF L-THREONINE BY AN ESCHERICHIA COLI MUTANT WITH IMPAIRED L-THREONINE UPTAKE" BIOSCIENCE BIOTECHNOLOGY BIOCHEMISTRY, JAPAN SOC. FOR BIOSCIENCE, BIOTECHNOLOGY AND AGROCHEM. TOKYO, JP, vol. 61, no. 11, November 1997 (1997-11), pages 1877-1882, XP001018682 ISSN: 0916-8451

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Description

Field of the Invention

[0001] This invention relates to a process for the preparation of L-threonine, using strains of the Enterobacteriaceae family in which at least the cysB gene and optionally one or more of the genes of the cysteine biosynthesis pathway (cysteine biosynthetic pathway) chosen from the group consisting of cysG, cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysI, cysH, cysE and sbp is (are) over-expressed.

Prior Art

[0002] L-Amino acids, in particular L-threonine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal nutrition.

[0003] It is known to prepare L-amino acids by fermentation of strains of Enterobacteriaceae, in particular Escherichia coli (E. coli) and Serratia marcescens. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as e.g. stirring and supply of oxygen, or the composition of the nutrient media, such as e.g. the sugar concentration during the fermentation, or the working up to the product form, by e.g. ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

[0004] Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites, such as e.g. the threonine analogue α-amino-β-hydroxyvaleric acid (AHV), or are auxotrophic for metabolites of regulatory importance and produce L-amino acids, such as e.g. L-threonine, are obtained in this manner.

[0005] Methods of the recombinant DNA technique have also been employed for some years for improving the strain of strains of the Enterobacteriaceae family which produce L-amino acids, by amplifying individual amino acid biosynthesis genes and investigating the effect on the production.

Object of the Invention

[0006] The object of the invention is to provide new measures for improved fermentative preparation of L-threonine.

Summary of the Invention

[0007] The invention provides a process for the fermentative preparation of L-threonine, using microorganisms of the Enterobacteriaceae family which in particular already produce L-amino acids and in which at least the cysB gene and optionally one or more of the nucleotide sequence(s) which code(s) for the genes of the cysteine biosynthesis pathway (cysteine biosynthetic pathway) chosen from the group consisting of cysG, cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysI, cysH, cysE and sbp, or nucleotide sequences which code for the gene product of said gene is/are over-expressed.

[0008] The process according to the invention for the preparation of L-threonine comprises the following steps:

- a) fermentation of the microorganisms of the Enterobacteriaceae family which produce L-threonine and in which the cysB gene and optionally one or more of the genes of the cysteine biosynthesis pathway chosen from the group consisting of cysG, cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysI, cysH, cysE and sbp, or nucleotide sequences which code for the gene product of said gene is/are over-expressed,

- b) concentration of said L-amino acid in the medium or in the cells of the microorganisms, and

- c) isolation of said L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100%) thereof optionally remaining in the product.

Detailed Description of the Invention

[0009] The use of endogenous genes is preferred. "Endogenous genes" or "endogenous nucleotide sequences" are understood as meaning the genes or nucleotide sequences present in the population of a species.

[0010] Where L-amino acids or amino acids are mentioned in the following, this means, including its salts, L-threonine, L-lysine.

[0011] The term "enhancement" in this connection describes the increase in the intracellular activity or concentration of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by
increasing the number of copies of the gene or genes, using a potent promoter or a gene or allele which codes for a corresponding enzyme or protein with a high activity, and optionally combining these measures.

[0012] By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on that of the wild-type protein or the activity or concentration of the protein in the starting microorganism.

[0013] The process comprises carrying out the following steps:

a) fermentation of microorganisms of the Enterobacteriaceae family in which the cysB gene and optionally one or more of the genes of the cysteine biosynthesis pathway chosen from the group consisting of cysG, cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysI, cysH, cysE and sbp is (are) overexpressed,

b) concentration of the L-threonine in the medium or in the cells of the microorganisms of the Enterobacteriaceae family, and

c) isolation of said L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100 %) thereof optionally remaining in the product.

[0014] The microorganisms which the present invention provides can produce L-threonine from glucose, sucrose, lactose, fructose, maltose, molasses, optionally starch, optionally cellulose or from glycerol and ethanol. They are representatives of the Enterobacteriaceae family chosen from the genera Escherichia, Erwinia, Providencia and Serratia. The genera Escherichia and Serratia are preferred. Of the genus Escherichia the species Escherichia coli and of the genus Serratia the species Serratia marcescens are to be mentioned in particular.

[0015] Suitable strains, which produce L-threonine of the genus Escherichia, in particular of the species Escherichia coli, are, for example

Escherichia coli TF427
Escherichia coli H4578
Escherichia coli KY10935
Escherichia coli VNIgenetika MG442
Escherichia coli VNIgenetika M1
Escherichia coli VNIgenetika 472T23
Escherichia coli BKIIM B-3996
Escherichia coli kat 13
Escherichia coli KCCM-10132

[0016] Suitable L-threonine-producing strains of the genus Serratia, in particular of the species Serratia marcescens, are, for example

Serratia marcescens HNr21
Serratia marcescens TLr156
Serratia marcescens T2000

[0017] Strains from the Enterobacteriaceae family which produce L-threonine have, inter alia, one or more genetic or phenotypic features chosen from the group consisting of: resistance to α-amino-β-hydroxyvaleric acid, resistance to thialysine, resistance to ethionine, resistance to α-methylserine, resistance to diaminosuccinic acid, resistance to α-aminobutyric acid, resistance to borrelidin, resistance to rifampicin, resistance to valine analogues, such as, for example, valine hydroxamate, resistance to purine analogues, such as, for example, 6-dimethylaminopurine, a need for L-methionine, optionally a partial and compensable need for L-isoleucine, a need for meso-diaminopimelic acid, auxotrophy in respect of threonine-containing dipeptides, resistance to L-threonine, resistance to L-homoserine, resistance to L-lysine, resistance to L-methionine, resistance to L-glutamic acid, resistance to L-aspartate, resistance to L-leucine, resistance to L-phenylalanine, resistance to L-serine, resistance to L-cysteine, resistance to L-valine, sensitivity to fluoropyruvate, defective threonine dehydrogenase, optionally an ability for sucrose utilization, enhancement of the threonine operon, enhancement of homoserine dehydrogenase L-aspartate kinase I, preferably of the feed back resistant form, enhancement of homoserine kinase, enhancement of threonine synthase, enhancement of aspartate kinase, optionally of the feed back resistant form, enhancement of aspartate semialdehyde dehydrogenase, enhancement of phosphoenolpyruvate carboxylase, optionally of the feed back resistant form, enhancement of phosphoenolpyruvate synthase, enhancement of transhydrogenase, enhancement of the RhtB gene product, enhancement of the RhtC gene product,
enhancement of the YfiK gene product, enhancement of a pyruvate carboxylase, and attenuation of acetic acid formation.

[0018] It has been found that microorganisms of the Enterobacteriaceae family produce L-threonine, in an improved manner after over-expression of at least the cysB gene and optionally one or more of the genes of the cysteine biosynthesis pathway chosen from the group consisting of cysG, cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysL, cysH, cysE and sbp.

[0019] The nucleotide sequences of the genes of Escherichia coli belong to the prior art (See following text references) and can also be found in the genome sequence of Escherichia coli published by Blattner et al. (Science 277: 1453 - 1462 (1997)). The genes and activities of the cysteine biosynthesis pathway (cysteine biosynthetic pathway) are also described in summary form in Kredich (In: Neidhardt (ed), Escherichia coli and Salmonella, American Society for Microbiology, Washington, D.C., USA: 514-527 (1996)).

cysG gene:
Description: Uroporphyrinogen III C methyl-transferase; precorrin-2 oxidase; ferrochelatase
EC No.: 2.1.1.107; 1.-.-.-; 4.99.1.-
Accession No.: AE000412

ckyB gene:
Description: Positive regulator of the cys regulon, transcription activator
Accession No.: AE000225

ckyZ gene:
Description: Sulfate transporter
Accession No.: AE000329

ckyK gene:
Description: Cysteine synthase A, O-acetylserine (thiol)-lyase A
EC No.: 4.2.99.8
Accession No.: AE000329
Alternative gene name: cysZ

ckyM gene:
Description: Cysteine synthase B, O-acetylserine (thiol)-lyase B
EC No.: 4.2.99.8
Accession No.: AE000329

ckyA gene:
Description: ATP-binding protein of the sulfate transport system
Accession No.: AE000329

ckyW gene:
Description: Membrane-bound sulfate transport protein
Accession No.: AE000329, AE000330

ckyU gene:
Description: Permease protein of the sulfate transport system
(continued)

**cysP gene:**
Description: Periplasmic thiosulfate-binding protein
Accession No.: AE000330

**cysD gene:**
Description: Sub-unit 2 of ATP sulfonylase (ATP:sulfate adenylyl-transferase)
EC No.: 2.7.7.4
Accession No.: AE000358

**cysN gene:**
Description: Sub-unit 1 of ATP sulfonylase (ATP:sulfate adenylyl-transferase)
EC No.: 2.7.7.4
Accession No.: AE000358

**cysC gene:**
Description: Adenylyl sulfate kinase (APS kinase)
EC No.: 2.7.1.25
Accession No.: AE000358

**cysJ gene:**
Description: Flavoprotein of NADPH sulfite reductase
EC No.: 1.8.1.2
Accession No.: AE000360

**cysI gene:**
Description: Haemoprotein of NADPH sulfite reductase
EC No.: 1.8.1.2
Accession No.: AE000360

**cysH gene:**
Description: Phosphoadenosine phosphosulfate reductase (PAPS reductase)
EC No.: 1.8.99.4
Accession No.: AE000360
The nucleic acid sequences can be found in the databanks of the National Center for Biotechnology Information (NCBI) of the National Library of Medicine (Bethesda, MD, USA), the nucleotide sequence databank of the European Molecular Biologies Laboratories (EMBL, Heidelberg, Germany or Cambridge, UK) or the DNA databank of Japan (DDBJ, Mishima, Japan).

The genes described in the text references mentioned can be used according to the invention. Alleles of the genes which result from the degeneracy of the genetic code or due to “sense mutations” of neutral function can furthermore be used.

To achieve an enhancement, for example, expression of the genes or the catalytic properties of the proteins can be increased. The two measures can optionally be combined.

To achieve an over-expression, the number of copies of the corresponding genes can be increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is additionally possible to increase the expression in the course of fermentative L-threonine production. The expression is likewise improved by measures to prolong the life of the m-RNA. Furthermore, the enzyme activity is also enhanced by preventing the degradation of the enzyme protein. The genes or gene constructs can either be present in plasmids with a varying number of copies, or can be integrated and amplified in the chromosome. Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

Plasmid vectors which are capable of replication in Enterobacteriaceae, such as e.g. cloning vectors derived from pACYC184 (Bartolomé et al.; Gene 102: 75-78 (1991)), pTrc99A (Amann et al.; Gene 69: 301-315 (1988)) or pSC101 derivatives (Vocke and Bastia, Proceedings of the National Academy of Sciences USA 80 (21): 6557-6561 (1983)) can be used. A strain transformed with a plasmid vector, where the plasmid vector carries at least the cysB gene and optionally one or more of the genes chosen from the group consisting of cysG, cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysL, cysH, cysE and sbp or nucleotide sequences which code for these, can be employed in a process according to the invention.

Plasmid vectors which are capable of replication in Enterobacteriaceae, such as e.g. cloning vectors derived from pACYC184 (Bartolomé et al.; Gene 102: 75-78 (1991)), pTrc99A (Amann et al.; Gene 69: 301-315 (1988)) or pSC101 derivatives (Vocke and Bastia, Proceedings of the National Academy of Sciences USA 80 (21): 6557-6561 (1983)) can be used. A strain transformed with a plasmid vector, where the plasmid vector carries at least the cysB gene and optionally one or more of the genes chosen from the group consisting of cysG, cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysL, cysH, cysE and sbp or nucleotide sequences which code for these, can be employed in a process according to the invention.

It is also possible to transfer mutations which affect the expression of the particular gene into various strains by sequence exchange (Hamilton et al. (Journal of Bacteriology 171: 4617 - 4622 (1989)), conjugation or transduction.

It may furthermore be advantageous for the production of L-threonine, with strains of the Enterobacteriaceae family to enhance one or more enzymes of the known threonine biosynthesis pathway or enzymes of anaerobic metabolism or enzymes for the production of reduced nicotinamide adenine dinucleotide phosphate or enzymes of glycolysis or PTS enzymes, in addition to overexpression of the cysB gene and optionally of one or more of the genes of the cystine biosynthesis pathway chosen from the group consisting of cysG, cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysL, cysH, cysE and sbp.

Thus, for example, at the same time one or more of the genes chosen from the group consisting of

- the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine
The pyc gene of Corynebacterium glutamicum which codes for pyruvate carboxylase (WO 99/18228),

- the pps gene which codes for phosphoenol pyruvate synthase (Molecular and General Genetics 231: 332-336 (1992)).

- the ppc gene which codes for phosphoenol pyruvate carboxylase (Gene 31: 279-283 (1984)).

- the pntA and pntB genes which code for transhydrogenase (European Journal of Biochemistry 158: 647-653 (1986)).


- the mqo gene which codes for malate:quinone oxidoreductase (WO 02/06459).


- the thrE gene of Corynebacterium glutamicum which codes for threonine export (WO 01/92545).


- the hns gene which codes for the DNA-binding protein HLP-II (Molecular and General Genetics 212: 199-202 (1988)).

- the pgm gene which codes for phosphoglucomutase (Journal of Bacteriology 176: 5847-5851 (1994)).

- the fba gene which codes for fructose biphosphate aldolase (Biochemical Journal 257: 529-534 (1989)).

- the ptsH gene of the ptsHIcrr operon which codes for the phosphohistidine protein hexose phosphotransferase of the phosphotransferase system PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)).

- the ptsI gene of the ptsHIcrr operon which codes for enzyme I of the phosphotransferase system PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)).

- the crr gene of the ptsHIcrr operon which codes for the glucose-specific IIA component of the phosphotransferase system PTS (Journal of Biological Chemistry 262: 16241-16253 (1987)).

- the ptsG gene which codes for the glucose-specific IIBC component (Journal of Biological Chemistry 261: 16398-16403 (1986)).

- the lrp gene which codes for the regulator of the leucine regulon (Journal of Biological Chemistry 266: 10768-10774 (1991)).

- the mopB gene which codes for 10 Kd chaperone (Journal of Biological Chemistry 261: 12414-12419 (1986)) and is also known by the name groES.

- the ahpC gene of the ahpCF operon which codes for the small sub-unit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences USA 92: 7617-7621 (1995))

- the ahpF gene of the ahpCF operon which codes for the large sub-unit of alkyl hydroperoxide reductase (Proceedings of the National Academy of Sciences USA 92: 7617-7621 (1995))

Can be over-expressed.

[0029] It may furthermore be advantageous for the production of L-threonine, in addition to overexpression of the cysB gene and optionally of one or more of the genes chosen from the group consisting of cysG, cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysI, cysH, cysE and sbp, for one or more of the genes chosen from the group consisting of...
• the tdh gene which codes for threonine dehydrogenase (Ravnikar and Somerville (Journal of Bacteriology 169: 4716-4721 (1987)),

• the mdh gene which codes for malate dehydrogenase (E.C. 1.1.1.37) (Vogel et al. (Archives in Microbiology 149: 36-42 (1987)),

• the gene product of the open reading frame (orf) yjIA (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),

• the gene product of the open reading frame (orf) yjFP (Accession Number AAC77179 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)),

• the pckA gene which codes for the enzyme phosphoenol pyruvate carboxykinase (Medina et al. (Journal of Bacteriology 172: 7151-7156 (1990)),

• the poxB gene which codes for pyruvate oxidase (Grabau and Cronan (Nucleic Acids Research 14 (13), 5449-5460 (1986)),

• the aceA gene which codes for the enzyme isocitrate lyase (Matsuoko and McFadden (Journal of Bacteriology 170, 4528-4536 (1988)),

• the dgsA gene which codes for the DgsA regulator of the phosphotransferase system (Hosono et al. (Bioscience, Biotechnology and Biochemistry 59: 256-251 (1995)) and is also known under the name of the mlc gene,

• the fruR gene which codes for the fructose repressor (Jahreis et al. (Molecular and General Genetics 226: 332-336 (1991)) and is also known by the name of the cra gene, and

• the rpoS gene which codes for the sigma$^{38}$ factor (WO 01/05939) and is also known under the name of the katF gene,

to be eliminated.

[0030] The term “attenuation” in this connection describes the reduction or elimination of the intracellular activity or concentration of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or a gene or allele which codes for a corresponding enzyme or protein with a low activity or inactivates the corresponding enzyme or protein or gene, and optionally combining these measures.

[0031] By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the starting microorganism.


[0033] The microorganisms produced according to the invention can be cultured in the batch process (batch culture), the fed batch (feed process) or the repeated fed batch process (repetitive feed process). A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioprozesstechnik [Bioprocess Technology 1. Introduction to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

[0034] The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1991).

[0035] Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

[0036] Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources
of nitrogen can be used individually or as a mixture.

Example 1

Preparation of L-threonine using the cysB gene

1a) Construction of the expression plasmid pTrc99AcysB

The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer, instructions with "Qiagen Genomic-tips 100/G" (QiAGEN, Hilden, Germany). A DNA fragment approx. 1000 bp in size can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with PfuDNA polymerase (Promega Corporation, Madison, USA). The PCR product is ligated according to the manufacturer's instructions with the vector pCR-Blunt II-TOPO (Zero Blunt TOPO PCR Cloning Kit, Invitrogen, Groningen, The Netherlands) and transformed into the E. coli strain TOP10.
1b) Preparation of L-threonine with the strain MG442/pTrc99AcysB

[0046] The L-threonine-producing E. coli strain MG442 is described in the patent specification US-A- 4,278,765 and deposited as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

[0047] The strain MG442 is transformed with the expression plasmid pTrc99AcysB described in example Ia and with the vector pTrc99A and plasmid-carrying cells are selected on LB agar with 50 μg/ml ampicillin. The strains MG442/pTrc99AcysB and MG442/pTrc99A are formed in this manner. Selected individual colonies are then multiplied further on minimal medium with the following composition: 3.5 g/l Na₂HPO₄·2H₂O, 1.5 g/l KH₂PO₄, 1 g/l NH₄Cl, 0.1 g/l MgSO₄·7H₂O, 2 g/l glucose, 20 g/l agar, 50 mg/ml ampicillin. The formation of L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l (NH₄)₂SO₄, 1 g/l KH₂PO₄, 0.5 g/l MgSO₄·7H₂O, 15 g/l CaCO₃, 20 g/l glucose, 50 mg/l ampicillin are inoculated and the batch is incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland).

[0048] 250 μl portions of this preculture are transinoculated into 10 ml of production medium (25 g/l (NH₄)₂SO₄, 2 g/l KH₂PO₄, 1 g/l MgSO₄·7H₂O, 0.03 g/l FeSO₄·7H₂O, 0.018 g/l MnSO₄·1H₂O, 30 g/l CaCO₃, 20 g/l glucose, 50 mg/l ampicillin) and the batch is incubated for 48 hours at 37°C. For complete induction of the expression of the cysB gene, 100 mg/l isopropyl β-D-thiogalactopyranoside (IPTG) are added in parallel batches. The formation of L-threonine by the starting strain MG442 is investigated in the same manner, but no addition of ampicillin to the medium takes place. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

[0049] The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

[0050] The result of the experiment is shown in Table 1.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Additives</th>
<th>OD (660 nm)</th>
<th>L-Threonine g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG442</td>
<td>-</td>
<td>5.6</td>
<td>1.4</td>
</tr>
<tr>
<td>MG442/pTrc99A</td>
<td>-</td>
<td>3.8</td>
<td>1.3</td>
</tr>
<tr>
<td>MG442/pTrc99AcysB</td>
<td>-</td>
<td>4.4</td>
<td>1.7</td>
</tr>
<tr>
<td>MG442/pTrc99AcysB</td>
<td>IPTG</td>
<td>5.4</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Example 2

Preparation of L-threonine using the cysK gene

2a) Construction of the expression plasmid pTrc99AcysK

[0051] The cysK gene from E. coli K12 is amplified using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence of the cysK gene in E. coli K12 MG1655 (Accession Number AE000329, Blattner et al. (Science 277: 1453-1462 (1997)), PCR primers are synthesized (MWG Biotech, Ebersberg; Germany):

```plaintext
cysK1: 5' - CAGTTAAGGACAGCCATGAG - 3' (SEQ ID No. 3)
cysK2: 5' - GCTGGCATTACTGTTGCAATTC - 3' (SEQ ID No. 4)
```

[0052] The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer, s instructions with “Qiagen Genomic-tips 100/G” (QIAGEN, Hilden, Germany). A DNA fragment approx. 1000 bp in size can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with Pfu-DNA polymerase (Promega Corporation, Madison, USA). The PCR product is ligated according to the manufacturer’s instructions with the vector pCR-Blunt II-TOPO (Zero Blunt TOPO PCR Cloning Kit, Invitrogen, Groningen, The Netherlands) and transformed into the E. coli strain TOP10.

[0053] Selection of plasmid-carrying cells takes place on LB agar, to which 50 μg/ml kanamycin are added. After isolation of the plasmid DNA, the vector pCR-Blunt II-TOPO-cysK is cleaved with the restriction enzymes Spel and XbaI.
and, after separation in 0.8% agarose gel, the cysK fragment is isolated with the aid of the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden) is cleaved with the enzyme XbaI and ligation is carried out with the cysK fragment isolated. The E. coli strain XL1-Blue MRF' (Stratagene, La Jolla, USA) is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 μg/ml ampicillin are added. Successful cloning can be demonstrated after plasmid DNA isolation by control cleavage with the enzymes HindIII and PvuII. The plasmid is called pTrc99AcysK (Figure 2).

2b) Preparation of L-threonine with the strain MG442/pTrc99AcysK


[0055] The strain MG442 is transformed with the expression plasmid pTrc99AcysK described in example 2a and with the vector pTrc99A and plasmid-carrying cells are selected on LB agar with 50 μg/ml ampicillin. The strains MG442/pTrc99AcysK and MG442/pTrc99A are formed in this manner. Selected individual colonies are then multiplied further on minimal medium with the following composition: 3.5 g/l Na₂HPO₄·2H₂O, 1.5 g/l KH₂PO₄, 1 g/l NH₄Cl, 0.1 g/l MgSO₄·7H₂O, 2 g/l glucose, 20 g/l agar, 50 mg/l ampicillin. The formation of L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l (NH₄)₂SO₄, 1 g/l KH₂PO₄, 0.5 g/l MgSO₄·7H₂O, 15 g/l CaCO₃, 20 g/l glucose, 50 mg/l ampicillin are inoculated and the batch is incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland).

[0056] 250 μl portions of this preculture are transinoculated into 10 ml of production medium (25 g/l (NH₄)₂SO₄, 2 g/l KH₂PO₄, 1 g/l MgSO₄·7H₂O, 0.03 g/l FeSO₄·7H₂O, 0.018 g/l MnSO₄·H₂O, 30 g/l CaCO₃, 20 g/l glucose, 50 mg/l ampicillin) and the batch is incubated for 48 hours at 37°C. The formation of L-threonine by the starting strain MG442 is investigated in the same manner, but no addition of ampicillin to the medium takes place. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

[0057] The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

[0058] The result of the experiment is shown in Table 2.

<table>
<thead>
<tr>
<th>Strain</th>
<th>OD (660 nm)</th>
<th>L-Threonine g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG442</td>
<td>5.6</td>
<td>1.4</td>
</tr>
<tr>
<td>MG442/pTrc99A</td>
<td>3.8</td>
<td>1.3</td>
</tr>
<tr>
<td>MG442/pTrc99AcysK</td>
<td>5.6</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Example 3

Preparation of L-threonine using the cysM gene

3a) Construction of the expression plasmid pTrc99AcysM

[0059] The cysM gene from E. coli K12 is amplified using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence of the cysM gene in E. coli K12 MG1655 (Accession Number AE000329, Blattner et al. (Science 277: 1453-1462 (1997)), PCR primers are synthesized (MWG Biotech, Ebersberg, Germany). The sequences of the primers are modified such that recognition sites for restriction enzymes are formed. The recognition sequence for XbaI is chosen for the cysM1 primer and the recognition sequence for HindIII for the cysM2 primer, which are marked by underlining in the nucleotide sequence shown below:

- cysM1: 5' - CGCATCAGTCTAGACCACGTTAGGATAG - 3' (SEQ ID No. 5)
- cysM2: 5' - CATCAGTCTCAGGACTTTTAATCC - 3' (SEQ ID No. 6)

[0060] The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer's
instructions with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment approx. 950 bp in size can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with Pfu-DNA polymerase (Promega Corporation, Madison, USA). The PCR product is ligated according to the manufacturer’s instructions with the vector pCR-Blunt II-TOPO (Zero Blunt TOPO PCR Cloning Kit, Invitrogen, Groningen, The Netherlands) and transformed into the E. coli strain TOP10.

[0061] Selection of plasmid-carrying cells takes place on LB agar, to which 50 μg/ml kanamycin are added. After isolation of the plasmid DNA, the vector pCR-Blunt II-TOPO-cysM is cleaved with the restriction enzymes HindIII and XbaI and, after separation in 0.8% agarose gel, the cysM fragment is isolated with the aid of the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden) is cleaved with the enzymes HindIII and XbaI and ligation is carried out with the cysM fragment isolated. The E. coli strain XL1-Blue MRF' (Strategene, La Jolla, USA) is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 μg/ml ampicillin are added. Successful cloning can be demonstrated after plasmid DNA isolation by control cleavage with the enzymes EcoRV, Eco91I, Paul and SspI. The plasmid is called pTrc99AcysM (Figure 3).

3b) Preparation of L-threonine with the strain MG442/pTrc99AcysM

[0062] The L-threonine-producing E. coli strain MG442 is described in the patent specification US-A- 4,278,765 and deposited as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

[0063] The strain MG442 is transformed with the expression plasmid pTrc99AcysM described in example 3a and with the vector pTrc99A and plasmid-carrying cells are selected on LB agar with 50 μg/ml ampicillin. The strains MG442/pTrc99AcysM and MG442/pTrc99A are formed in this manner. Selected individual colonies are then multiplied further on minimal medium with the following composition: 3.5 g/l Na₂HPO₄*2H₂O, 1.5 g/l KH₂PO₄, 1 g/l NH₄Cl, 0.1 g/l MgSO₄*7H₂O, 2 g/l glucose, 20 g/l agar, 50 mg/l ampicillin. The formation of L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l (NH₄)₂SO₄, 1 g/l KH₂PO₄, 0.5 g/l MgSO₄*7H₂O, 15 g/l CaCO₃, 20 g/l glucose, 50 mg/l ampicillin are inoculated and the batch is incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland).

[0064] 250 μ portions of this preculture are transinoculated into 10 ml of production medium (25 g/l (NH₄)₂SO₄, 2 g/l KH₂PO₄, 1 g/l MgSO₄*7H₂O, 0.03 g/l FeSO₄*7H₂O, 0.018 g/l MnSO₄*H₂O, 30 g/l CaCO₃, 20 g/l glucose, 50 mg/l ampicillin) and the batch is incubated for 48 hours at 37°C. The formation of L-threonine by the starting strain MG442 is investigated in the same manner, but no addition of ampicillin to the medium takes place. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

[0065] The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

[0066] The result of the experiment is shown in Table 3.

<table>
<thead>
<tr>
<th>Strain</th>
<th>OD (660 nm)</th>
<th>L-Threonine g/l</th>
</tr>
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<tbody>
<tr>
<td>MG442</td>
<td>5.6</td>
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<tr>
<td>MG442/pTrc99A</td>
<td>3.8</td>
<td>1.3</td>
</tr>
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<td>MG442/pTrc99AcysM</td>
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</table>

Example 4
Preparation of L-threonine using the cysP, cysU, cysW and cysA genes

4a) Construction of the expression plasmid pTrc99AcysPUWA

[0067] The cysP, cysU, cysW and cysA genes from E. coli K12 are amplified using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence of the cysP, cysU, cysW and cysA genes in E. coli K12 MG1655 (Accession Number AE000329 and AE000330, Blattner et al. (Science 277: 1453-1462 (1997))). PCR primers are synthesized (MWG Biotech, Ebersberg, Germany). The sequences of the primers are modified such that recognition sites for restriction enzymes are formed. The recognition sequence for XbaI is chosen for the cysPUWA1
primer and the recognition sequence for HindIII for the cysPUWA2 primer, which are marked by underlining in the nucleotide sequence shown below:

\[
\text{cysPUWA1: } 5' - \text{GTCTCTAGATAAATAAGGGTGCGCAATGGC} - 3' \text{ (SEQ ID No. 7)}
\]

\[
\text{cysPUWA2: } 5' - \text{CCGGGCGTTAAAAGCTTCACTCAACC} - 3' \text{ (SEQ ID No. 8)}
\]

[0068] The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer’s instructions with “Qiagen Genomic-tips 100/G” (QIAGEN, Hilden, Germany). A DNA fragment approx. 3900 bp in size can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with Pfu-DNA polymerase (Promega Corporation, Madison, USA). The PCR product is cleaved with the restriction enzymes XbaI and HindIII and ligated with the vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden), which has been digested with the enzymes XbaI and HindIII. The E. coli strain XL1-Blue MRF’ (Stratagene, La Jolla, USA) is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 μg/ml ampicillin are added. Successful cloning can be demonstrated after plasmid DNA isolation by control cleavage with the enzymes BamHI, EcoRV, MluI, NdeI and SspI. The plasmid is called pTrc99AcysPUWA (Figure 4).

4b) Preparation of L-threonine with the strain MG442/pTrc99AcysPUWA

[0069] The L-threonine-producing E. coli strain MG442 is described in the patent specification US-A- 4,278,765 and deposited as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

[0070] The strain MG442 is transformed with the expression plasmid pTrc99AcysPUWA described in example 4a and with the vector pTrc99A and plasmid-carrying cells are selected on LB agar with 50 μg/ml ampicillin. The strains MG442/pTrc99AcysPUWA and MG442/pTrc99A are formed in this manner. Selected individual colonies are then multiplied further on minimal medium with the following composition: 3.5 g/l Na₂HPO₄·2H₂O, 1.5 g/l KH₂PO₄, 1 g/l NH₄Cl, 0.1 g/l MgSO₄·7H₂O, 2 g/l glucose, 0.03 g/l FeSO₄·7H₂O, 15 g/l CaCO₃, 20 g/l glucose, 50 mg/l ampicillin. The formation of L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l (NH₄)₂SO₄, 1 g/l KH₂PO₄, 0.5 g/l MgSO₄·7H₂O, 15 g/l CaCO₃, 20 g/l glucose, 50 mg/l ampicillin are inoculated and the batch is incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland).

[0071] 250 μl portions of this preculture are transinoculated into 10 ml of production medium (25 g/l (NH₄)₂SO₄, 2 g/l KH₂PO₄, 1 g/l MgSO₄·7H₂O, 0.03 g/l FeSO₄·7H₂O, 0.018 g/l MnSO₄·H₂O, 30 g/l CaCO₃, 20 g/l glucose, 50 mg/l ampicillin) and the batch is incubated for 48 hours at 37°C. For complete induction of the expression of the cysPUWA genes, 100 mg/l isopropyl β-D-thiogalactopyranoside (IPTG) are added in parallel batches. The formation of L-threonine by the starting strain MG442 is investigated in the same manner, but no addition of ampicillin to the medium takes place. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

[0072] The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

[0073] The result of the experiment is shown in Table 4.

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</table>
Example 5

Preparation of L-threonine using the cysD, cysN and cysC genes

5a) Construction of the expression plasmid pTrc99AcysDNC

[0074] The cysD, cysN and cysC genes from E. coli K12 are amplified using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence of the cysD, cysN and cysC genes in E. coli K12 MG1655 (Accession Number AE000358, Blattner et al. (Science 277: 1453-1462 (1997))), PCR primers are synthesized (MWG Biotech, Ebersberg, Germany). The sequences of the primers are modified such that recognition sites for restriction enzymes are formed. The recognition sequence for XbaI is chosen for the cysDNC1 primer and the recognition sequence for HindIII for the cysDNC2 primer, which are marked by underlining in the nucleotide sequence shown below:

\[
\text{cysDNC1: 5'} - \text{GCAAGAAAATAGCGGTCTAGA} \text{TAAGGAACG} - 3' \text{ (SEQ ID No. 9)}
\]

\[
\text{cysDNC2: 5'} - \text{CATGGAAAGCTT} \text{GTGGTGTCTCAGG} - 3' \text{ (SEQ ID No. 10)}
\]

[0075] The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer, s instructions with “Qiagen Genomic-tips 100/G” (QIAGEN, Hilden, Germany). A DNA fragment approx. 3000 bp in size can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with Pfu-DNA polymerase (Promega Corporation, Madison, USA). The PCR product is cleaved with the restriction enzymes XbaI and HindIII and ligated with the vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden), which has been digested with the enzymes XbaI and HindIII. The E. coli strain XL1-Blue MRF’ (Stratagene, La Jolla, USA) is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 \( \mu \)g/ml ampicillin are added. Successful cloning can be demonstrated after plasmid DNA isolation by control cleavage with the enzymes EcoRV, HincII, NruI, PvuI ans SeaI. The plasmid is called pTrc99AcysDNC (Figure 5).

5b) Preparation of L-threonine with the strain MG442/pTrc99AcysDNC

[0076] The L-threonine-producing E. coli strain MG442 is described in the patent specification US-A- 4,278,765 and deposited as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

[0077] The strain MG442 is transformed with the expression plasmid pTrc99AcysDNC described in example 5a and with the vector pTrc99A and plasmid-carrying cells are selected on LB agar with 50 \( \mu \)g/ml ampicillin. The strains MG442/pTrc99AcysDNC and MG442/pTrc99A are formed in this manner. Selected individual colonies are then multiplied further on minimal medium with the following composition: 3.5 g/l \( \text{Na}_2\text{HPO}_4\cdot2\text{H}_2\text{O} \), 1.5 g/l \( \text{KH}_2\text{PO}_4 \), 1 g/l \( \text{NH}_4\text{Cl} \), 0.1 g/l \( \text{MgSO}_4\cdot7\text{H}_2\text{O} \), 2 g/l glucose, 20 g/l agar, 50 mg/l ampicillin. The formation of L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium with the following composition: 2 g/l yeast extract, 10 g/l (\( \text{NH}_4\)\(_2\)\(_4\)\(_2\)\(_2\)\(_2\)\(_2\)\(_2\)), 1 g/l \( \text{KH}_2\text{PO}_4 \), 0.5 g/l \( \text{MgSO}_4\cdot7\text{H}_2\text{O} \), 15 g/l \( \text{CaCO}_3 \), 20 g/l glucose, 50 \( \mu \)g/ml ampicillin are inoculated and the batch is incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland).

[0078] 250 \( \mu \)l portions of this preculture are transinoculated into 10 ml of production medium (25 g/l (\( \text{NH}_4\)\(_2\)\(_2\)\(_2\)\(_2\)\(_2\)), 2 g/l \( \text{KH}_2\text{PO}_4 \), 1 g/l \( \text{MgSO}_4\cdot7\text{H}_2\text{O} \), 0.03 g/l \( \text{FeSO}_4\cdot7\text{H}_2\text{O} \), 0.018 g/l \( \text{MnSO}_4\cdot1\text{H}_2\text{O} \), 30 g/l \( \text{CaCO}_3 \), 20 g/l glucose, 50 mg/l ampicillin) and the batch is incubated for 48 hours at 37°C. The formation of L-threonine by the starting strain MG442 is investigated in the same manner, but no addition of ampicillin to the medium takes place. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

[0079] The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

[0080] The result of the experiment is shown in Table 5.

<table>
<thead>
<tr>
<th>Strain</th>
<th>OD (660 nm)</th>
<th>L-Threonine g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG442</td>
<td>5.6</td>
<td>1.4</td>
</tr>
<tr>
<td>MG442/pTrc99A</td>
<td>3.8</td>
<td>1.3</td>
</tr>
<tr>
<td>MG442/pTrc99AcysDNC</td>
<td>5.1</td>
<td>2.5</td>
</tr>
</tbody>
</table>
Example 6

Preparation of L-threonine using the cysJ and cysI genes

6a) Construction of the expression plasmid pTrc99AcysJI

[0081] The cysJ and cysI genes from E. coli K12 are amplified using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence of the cysJ and cysI genes in E. coli K12 MG1655 (Accession Number AE000360, Blattner et al. (Science 277: 1453-1462 (1997))), PCR primers are synthesized (MWG Biotech, Ebersberg, Germany):

\[
\text{cysJ1: 5'} - \text{CTGGAACATAACGACGATGAC} - 3' \quad (\text{SEQ ID No. 11})
\]

\[
\text{cysJ2: 5'} - \text{GACCGGGCTGATGGTTAATCC} - 3' \quad (\text{SEQ ID No. 12})
\]

[0082] The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer, s instructions with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment approx. 3550 bp in size can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with Pfu-DNA polymerase (Promega Corporation, Madison, USA). The PCR product is ligated according to the manufacturer’s instructions with the vector pCR-Blunt II-TOPO (Zero Blunt TOPO PCR Cloning Kit, Invitrogen, Groningen, The Netherlands) and transformed into the E. coli strain TOP10.

[0083] Selection of plasmid-carrying cells takes place on LB agar, to which 50 μg/ml kanamycin are added. After isolation of the plasmid DNA, the vector pCR-Blunt II-TOPO-cysJI is cleaved with the restriction enzymes HindIII and XbaI and, after separation in 0.8% agarose gel, the cysJI fragment is isolated with the aid of the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden) is cleaved with the enzymes HindIII and XbaI and ligation is carried out with the cysJI fragment isolated. The E. coli strain XL1-Blue MRF' (Stratagene, La Jolla, USA) is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 μg/ml ampicillin are added. Successful cloning can be demonstrated after plasmid DNA isolation by control cleavage with the enzymes Accl, Cia1 and Sphl. The plasmid is called pTrc99AcysJI (Figure 6).

6b) Preparation of L-threonine with the strain MG442/pTrc99AcysJI

[0084] The L-threonine-producing E. coli strain MG442 is described in the patent specification US-A- 4,278,765 and deposited as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

[0085] The strain MG442 is transformed with the expression plasmid pTrc99AcysJI described in example 6a and with the vector pTrc99A and plasmid-carrying cells are selected on LB agar with 50 μg/ml ampicillin. The strains MG442/pTrc99AcysJI and MG442/pTrc99A are formed in this manner. Selected individual colonies are then multiplied further on minimal medium with the following composition: 3.5 g/l NaHPO\textsubscript{4}·2H\textsubscript{2}O, 1.5 g/l KH\textsubscript{2}PO\textsubscript{4}, 1 g/l NH\textsubscript{4}Cl, 0.1 g/l MgSO\textsubscript{4}·7H\textsubscript{2}O, 2 g/l glucose, 20 g/l agar, 50 mg/l ampicillin. The formation of L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 1 g/l KH\textsubscript{2}PO\textsubscript{4}, 0.5 g/l MgSO\textsubscript{4}·7H\textsubscript{2}O, 15 g/l CaCO\textsubscript{3}, 20 g/l glucose, 50 mg/l ampicillin are inoculated and the batch is incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland).

[0086] 250 μl portions of this preculture are transinoculated into 10 ml of production medium (25 g/l (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 2 g/l KH\textsubscript{2}PO\textsubscript{4}, 1 g/l MgSO\textsubscript{4}·7H\textsubscript{2}O, 0.03 g/l FeSO\textsubscript{4}·7H\textsubscript{2}O, 0.018 g/l MnSO\textsubscript{4}·H\textsubscript{2}O, 30 g/l CaCO\textsubscript{3}, 20 g/l glucose, 50 mg/l ampicillin) and the batch is incubated for 48 hours at 37°C. The formation of L-threonine by the starting strain MG442 is investigated in the same manner, but no addition of ampicillin to the medium takes place. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

[0087] The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

[0088] The result of the experiment is shown in Table 6.
Example 7

Preparation of L-threonine using the cysH gene

7a) Construction of the expression plasmid pTrc99AcysH

[0089] The cysH gene from E. coli K12 is amplified using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence of the cysH gene in E. coli K12 MG1655 (Accession Number AE000360, Blattner et al. (Science 277: 1453-1462 (1997)), PCR primers are synthesized (MWG Biotech, Ebersberg, Germany):

- **cysH1**: 5’ - GGCAAACAGTGAGGAATCTATG - 3’ (SEQ ID No. 13)
- **cysH2**: 5’ - GTCCGGCAATATTTACCCTC - 3’ (SEQ ID No. 14)

[0090] The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturer’s instructions with “Qiagen Genomic-tips 100/G” (QIAGEN, Hilden, Germany). A DNA fragment approx. 800 bp in size can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with Pfu-DNA polymerase (Promega Corporation, Madison, USA). The PCR product is ligated according to the manufacturer’s instructions with the vector pCR-Blunt II-TOPO (Zero Blunt TOPO PCR Cloning Kit, Invitrogen, Groningen, The Netherlands) and transformed into the E. coli strain TOP10.

[0091] Selection of plasmid-carrying cells takes place on LB agar, to which 50 μg/ml kanamycin are added. After isolation of the plasmid DNA, the vector pCR-Blunt II-TOPO-cysH is cleaved with the restriction enzymes HindIII and XbaI and, after separation in 0.8% agarose gel, the cysH fragment is isolated with the aid of the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden) is cleaved with the enzymes HindIII and XbaI and ligation is carried out with the cysH fragment isolated. The E. coli strain XL1-Blue MRF’ (Stratagene, La Jolla, USA) is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar, to which 50 μg/ml ampicillin are added. Successful cloning can be demonstrated after plasmid DNA isolation by control cleavage with the enzymes HincII and MluI. The plasmid is called pTrc99AcysH (Figure 7).

7b) Preparation of L-threonine with the strain MG442/pTrc99AcysH

[0092] The L-threonine-producing E. coli strain MG442 is described in the patent specification US-A- 4,278,765 and deposited as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

[0093] The strain MG442 is transformed with the expression plasmid pTrc99AcysH described in example 7a and with the vector pTrc99A and plasmid-carrying cells are selected on LB agar with 50 μg/ml ampicillin. The strains MG442/pTrc99AcysH and MG442/pTrc99A are formed in this manner. Selected individual colonies are then multiplied further on minimal medium with the following composition: 3.5 g/l Na₂HPO₄·2H₂O, 1.5 g/l KH₂PO₄, 1 g/l NH₄Cl, 0.1 g/l MgSO₄·7H₂O, 2 g/l glucose, 20 g/l agar, 50 mg/l ampicillin. The formation of L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 25 g/l (NH₄)₂SO₄, 1 g/l KH₂PO₄, 0.5 g/l MgSO₄·7H₂O, 15 g/l CaCO₃, 20 g/l glucose, 50 mg/l ampicillin are inoculated and the batch is incubated for 48 hours at 37°C. The formation of L-threonine by the starting strain MG442 is investigated in the same manner, but no addition of ampicillin to the medium takes place. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Düsseldorf, Germany) at a measurement wavelength of 660 nm.

<table>
<thead>
<tr>
<th>Strain</th>
<th>OD (660 nm)</th>
<th>L-Threonine g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG442</td>
<td>5.6</td>
<td>1.4</td>
</tr>
<tr>
<td>MG442/pTrc99A</td>
<td>3.8</td>
<td>1.3</td>
</tr>
<tr>
<td>MG442/pTrc99AcysJI</td>
<td>6.3</td>
<td>2.5</td>
</tr>
</tbody>
</table>
The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

The result of the experiment is shown in Table 7.

<table>
<thead>
<tr>
<th>Strain</th>
<th>OD (660 nm)</th>
<th>L-Threonine g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG442</td>
<td>5.6</td>
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</tr>
<tr>
<td>MG442/pTrc99A</td>
<td>3.8</td>
<td>1.3</td>
</tr>
<tr>
<td>MG442/pTrc99AcysH</td>
<td>4.1</td>
<td>2.7</td>
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</tbody>
</table>

Brief Description of the Figures:

Figure 1: Map of the plasmid pTrc99AcysB containing the cysB gene.

Figure 2: Map of the plasmid pTrc99AcysK containing the cysK gene.

Figure 3: Map of the plasmid pTrc99AcysM containing the cysM gene.

Figure 4: Map of the plasmid pTrc99AcysPUWA containing the cysP, cysU, cysW and cysA genes.

Figure 5: Map of the plasmid pTrc99AcysDNC containing the cysD, cysN and cysC genes.

Figure 6: Map of the plasmid pTrc99AcysJI containing the cysJ and cysI genes.

Figure 7: Map of the plasmid pTrc99AcysH containing the cysH gene.

The length data are to be understood as approx. data. The abbreviations and designations used have the following meaning:

- Amp: Ampicillin resistance gene
- lacI: Gene for the repressor protein of the trc promoter
- Ptrc: trc promoter region, IPTG-inducible
- cysB: Coding region of the cysB gene
- cysK: Coding region of the cysK gene
- cysM: Coding region of the cysM gene
- cysP: Coding region of the cysP gene
- cysU: Coding region of the cysU gene
- cysW: Coding region of the cysW gene
- cysA: Coding region of the cysA gene
- cysD: Coding region of the cysD gene
- cysN: Coding region of the cysN gene
• cysC: Coding region of the cysC gene
• cysJ: Coding region of the cysJ gene
• cysI: Coding region of the cysI gene
• cysH: Coding region of the cysH gene
• 5S: 5S rRNA region
• rrnBT: rRNA terminator region

[0099] The abbreviations for the restriction enzymes have the following meaning
• AccI: Restriction endonuclease from Acinetobacter calcoaceticus
• BamHI: Restriction endonuclease from Bacillus amyloliquefaciens H
• BstEII: Restriction endonuclease from Bacillus stearothermophilus ATCC 12980
• ClaI: Restriction endonuclease from Caryophannon latum
• EcoRI: Restriction endonuclease from Escherichia coli RY13
• EcoRV: Restriction endonuclease from Escherichia coli B946
• HindII: Restriction endonuclease from Haemophilus influenzae Rc
• HindIII: Restriction endonuclease from Haemophilus influenzae
• MluI: Restriction endonuclease from Micrococcus luteus IFO 12992
• NdeI: Restriction endonuclease from Neisseria dentificans
• NruI: Restriction endonuclease from Norcadia ruba (ATCC 15906)
• Paul: Restriction endonuclease from Paracoccus alcaliphilus
• PvuI: Restriction endonuclease from Proteus vulgaris (ATCC 13315)
• PvuII: Restriction endonuclease from Proteus vulgaris (ATCC 13315)
• ScaI: Restriction endonuclease from Streptomyces caespitosus
• SmaI: Restriction endonuclease from Serratia marcescens
• SpeI: Restriction endonuclease from Sphaerotilus species ATCC 13923
• Sphi: Restriction endonuclease from Streptomyces phaeochromogenes
• SspI: Restriction endonuclease from Sphaerotilus species ATCC 13925
• XbaI: Restriction endonuclease from Xanthomonas campestris

SEQUENCE PROTOCOL

[0100]
Degussa AG

Process for the preparation of L-amino acids using strains of the Enterobacteriaceae family

010275 BT

14

PatentIn version 3.1

1

22

DNA

artificial sequence

Primer

(1)..(22)

cysB1

1

gcgctaaagt ggatggtta ac 22

1

20

DNA

artificial sequence

Primer

(1)..(20)

cysB2

2

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2

21

DNA

artificial sequence

Primer

(1)..(21)

cysK1

3

cagtttaagga caggccatga g 21

4

22

DNA

artificial sequence

Primer

(1)..(22)

cysK2

4

gcgtgcatta ctgtgcaat tc 22

5

28

DNA

artificial sequence

Primer

(1)..(28)

cysM1

5

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6

25

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Primer (1)...

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Primer (1)...

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Primer (1)...

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Claims

1. A process for the preparation of L-threonine, which comprises carrying out the following steps:

   a) fermentation of microorganisms of the Enterobacteriaceae family which produce L-threonine and in which at least the cysB gene of the cysteine biosynthesis pathway, or nucleotide sequences which code for the gene product of said gene, is (are) over-expressed,
   b) concentration of L-threonine in the medium or in the cells of the microorganisms, and
   c) isolation of said L-amino acid, constituents of the fermentation broth and/or the biomass in its entirety or portions (> 0 to 100%) thereof optionally remaining in the product.

2. A process as claimed in claim 1, wherein the polynucleotide(s) which code(s) for one or more of the gene products of cysteine biosynthesis genes chosen from the group consisting of cysG, cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysH, cysE and sbp is (are) over-expressed.

3. A process as claimed in claim 1, wherein, for the preparation of L-threonine, microorganisms of the Enterobacteriaceae family are fermented, in which in addition at the same time one or more of the genes chosen from the group consisting of:

   a) the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase,
   b) the pyc gene which codes for pyruvate carboxylase,
   c) the pps gene which codes for phosphoenolpyruvate synthase,
   d) the ppc gene which codes for phosphoenolpyruvate carboxylase,
   e) the pntA and pntB genes which code for transhydrogenase,
   f) the rhtB gene which imparts homoserine resistance,
   g) the mqo gene which codes for malate:quinone oxidoreductase,
h) the rhtC gene which imparts threonine resistance,
i) the thrE gene which codes for the threonine export protein
j) the gdhA gene which codes for glutamate dehydrogenase
k) the hns gene which codes for the DNA-binding protein HLP-II,
l) the pgm gene which codes for phosphoglucomutase,
m) the fba gene which codes for fructose biphosphate aldolase,
n) the ptsH gene which codes for the phosphohistidine protein hexose phosphotransferase,
o) the ptsI gene which codes for enzyme I of the phosphotransferase system,
p) the crr gene which codes for the glucose-specific II component,
q) the ptsG gene which codes for the glucose-specific IIBC component,
r) the lrp gene which codes for the regulator of the leucine regulon,
s) the mopB gene which codes for 10 Kd chaperone,
t) the ahpC gene which codes for the small sub-unit of alkyl hydroperoxide reductase,
u) the ahpF gene which codes for the large sub-unit of alkyl hydroperoxide reductase,
is or are over-expressed.

4. A process as claimed in claim 1, wherein, for the preparation of L-threonine, microorganisms of the Enterobacteriaceae family are fermented, in which in addition at the same time one or more of the genes chosen from the group consisting of:

   a) the tdh gene which codes for threonine dehydrogenase,
   b) the mdh gene which codes for malate dehydrogenase,
   c) the gene product of the open reading frame (orf) yfIA,
   d) the gene product of the open reading frame (orf) yfIP,
   e) the pckA gene which codes for phosphoenol pyruvate carboxykinase,
   f) the poxB gene which codes for pyruvate oxidase,
   g) the aceA gene which codes for isocitrate lyase,
   h) the dgsA gene which codes for the DgsA regulator of the phosphotransferase system,
   i) the fruR gene which codes for the fructose repressor,
   j) the rpoS gene which codes for the sigma\textsuperscript{38} factor

   is or are eliminated.

5. L-threonine producing microorganisms of the enterobacteriaceae family, wherein at least the thrABC operon, which genes code for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase, and the cysB gene are over-expressed.

6. Microorganisms according to claim 5, wherein the microorganisms originate from the genus Escherichia.

7. Microorganisms according to claim 5, wherein the microorganisms originate from the species E. coli.

Patentansprüche

1. Verfahren für die Herstellung von L-Threonin, das das Durchführen der folgenden Schritte umfasst:

   a) Fermentieren von Mikroorganismen der Familie der Enterobacteriaceae, die L-Threonin erzeugen, und wobei mindestens das cysB-Gen des Cysteinbiosynthesewegs oder die Nukleotidsequenzen, die für das Genprodukt des Gens kodieren, überexprimiert ist bzw. sind,

   b) Konzentrieren von L-Threonin im Medium oder in den Zellen der Mikroorganismen und
c) Isolieren der L-Aminosäure, der Bestandteile der Fermentationsbrühe und/oder der Biomasse insgesamt oder von Teilen (> 0 bis 100 %) derselben, die wahlweise im Produkt verbleiben.

3. Verfahren nach Anspruch 1, wobei für die Herstellung von L-Threonin Mikroorganismen der Familie der Enterobacteriaceae fermentiert werden, wobei zusätzlich gleichzeitig ein oder mehrere der Gene, die aus der Gruppe ausgewählt werden bestehend aus:

a) dem thrABC-Operon, das für Aspartatkinase, Homoserindehydrogenase, Homoserinkinase und Threoninsynthase kodiert,
b) dem pyc-Gen, das für Pyruvatscarboxylase kodiert,
c) dem psps-Gen, das für Phosphoenolpyruvatsynthase kodiert,
d) dem ppc-Gen, das für Phosphoenolpyruvatscarboxylase kodiert,
e) den pnta- und pntB-Genen, die für Transhydrogenase kodieren,
f) dem rhtB-Gen, das Homoserin Resistenz verleiht,
g) dem mqo-Gen, das für Malat:Chinonoxidoreduktase kodiert,
h) dem rhtC-Gen, das Threonin Resistenz verleiht,
i) dem thrE-Gen, das für Threoninexportprotein kodiert,
j) dem gdhA-Gen, das für Glutamatdehydrogenase kodiert,
k) dem hns-Gen, das für das DNA-Bindungsprotein HLP-II kodiert,
l) dem pgm-Gen, das für Phosphoglukomutase kodiert,
m) dem fba-Gen, das für Fruktosebiphosphataldolase kodiert,
n) dem ptsH-Gen, das für das Phosphohistidinprotein Hexosephosphotransferase kodiert,
o) dem pstl-Gen, das für das Enzym I des Phosphotransferasesystems kodiert,
p) dem crr-Gen, das für die glukosespezifische IIA-Komponente kodiert,
q) dem ptsG-Gen, das für die glukosespezifische IIBC-Komponente kodiert,
r) dem lpr-Gen, das für den Regulator des Leucinregulons kodiert,
s) dem mopB-Gen, das für 10 Kd-Chaperon kodiert,
t) dem ahpC-Gen, das für die kleine Untereinheit von Alkylhydroperoxidreduktase kodiert,
u) dem ahpF-Gen, das für die große Untereinheit der Alkylhydroperoxidreduktase kodiert,
überexprimiert ist bzw. sind.

4. Verfahren nach Anspruch 1, wobei für die Herstellung von L-Threonin Mikroorganismen der Familie der Enterobacteriaceae fermentiert werden, wobei zusätzlich gleichzeitig ein oder mehrere der Gene, die aus der Gruppe ausgewählt werden bestehend aus:

a) dem tdh-Gen, das für Threoninhydrogenase kodiert,
b) dem mdh-Gen, das für Malatdehydrogenase kodiert,
c) dem Genprodukt des offenen Leserasters (orf) yjjA,
d) dem Genprodukt des offenen Leserasters (orf) yjjP,
e) dem pckA-Gen, das für Phosphoenolpyruvatcarboxykinase kodiert,
f) dem poxB-Gen, das für Pyruvatoxidase kodiert,
g) dem aceA-Gen, das für Isocitratlyase kodiert,
h) dem dgsA-Gen, das für den DgsA-Regulator des Phosphotransferasesystems kodiert,
i) dem fruR-Gen, das für den Fruktoserepressor kodiert,
j) dem rpoS-Gen, das für den Sigma38-Faktor kodiert,
eliminiert wird oder werden.

5. L-Threonin erzeugende Mikroorganismen der Familie der Enterobacteriaceae, wobei mindestens das thrABC-Operon, welche Gene für Aspartatkinase, Homoserindehydrogenase, Homoserinkinase und Threoninsynthase kodieren, und das cysB-Gen überexprimiert sind.

6. Mikroorganismen nach Anspruch 5, wobei die Mikroorganismen aus der Gattung Escherichia stammen.

7. Mikroorganismen nach Anspruch 5, wobei die Mikroorganismen aus der Spezies E. coli stammen.

Revendications

1. Procédé de préparation de L-théonine, comprenant la réalisation des étapes suivantes:
a) la fermentation de micro-organismes de la famille des Entérobactéries qui produisent de la L-thrétiqueine et dans lesquels au moins le gène cysB de la voie de biosynthèse de la cystéine, ou les séquences nucléotidiques codant pour le produit génétique dudit gène, est (sont) surexprimé(s),
b) la concentration de la L-thrétiqueine dans le milieu ou dans les cellules des micro-organismes, et
c) l'isolement dudit L-acide aminé, des constituants du bouillon de fermentation et/ou de la biomasse dans son ensemble ou en des portions (> 0 à 100%) de celle-ci restant éventuellement dans le produit.

2. Procédé selon la revendication 1, caractérisé en ce que le ou les polynucléotide(s) codant pour un ou plusieurs des produits génétiques des gènes de biosynthèse de la cystéine choisis parmi le groupe constitué par cysG, cysZ, cysK, cysM, cysA, cysW, cysU, cysP, cysD, cysN, cysC, cysJ, cysI, cysH, cysE et sbp est (sont) surexprimé(s).

3. Procédé selon la revendication 1, caractérisé en ce que pour la préparation de la L-thrétiqueine, des micro-organismes de la famille des Entérobactéries sont fermentés, dans lesquels outre en même temps, un ou plusieurs des gènes choisis parmi le groupe constitué par :

a) l’opéron thrABC qui code pour l’aspartate kinase, l’homosérine déshydrogénase, l’homosérine kinase et la thrétiqueine synthétase,
b) le gène pyc qui code pour la pyruvate carboxylase,
c) le gène pps qui code pour la phosphoénol pyruvate synthétase,
d) le gène ppc qui code pour la phosphoénol pyruvate carboxylase,
e) les gènes pntA et pntB qui codent pour la transhydrogénase,
f) le gène rhs qui confère une résistance à l’homosérine,
g) le gène moq qui code pour la malate:quinone oxydoréductase,
h) le gène rhs qui confère une résistance à la thrétiqueine,
i) le gène thrE qui code pour la protéine d’exportation de thrétiqueine,
j) le gène gdhA qui code pour la glutamate déshydrogénase,
k) le gène hns qui code pour la protéine HLP-II fixant l’ADN,
l) le gène pgm qui code pour la phosphoglucomutase,
m) le gène fba qui code pour la fructose biphosphate aldolase,
n) le gène psh qui code pour la phosphohistidine protéine hexose phosphotransférase,
o) le gène pslI qui code pour l’enzyme I du système de phosphotransférase,
p) le gène crr qui code pour le composant IIa spécifique du glucose,
q) le gène ptsG qui code pour le composant IIBC spécifique du glucose,
r) le gène irp qui code pour le régulateur du régulon de la leucine,
s) le gène mop qui code pour la chaperone de 10 kD,
t) le gène ahpC qui code pour la petite sous-unité de l’alkyl hydroperoxyde réductase,
u) le gène ahpF qui code pour la grande sous-unité de l’alkyl hydroperoxyde réductase,
est (sont) surexprimé(s).

4. Procédé selon la revendication 1, caractérisé en ce que pour la préparation de la L-thrétiqueine, des micro-organismes de la famille des Entérobactéries sont fermentés, dans lesquels outre en même temps, un ou plusieurs des gènes choisis parmi le groupe constitué par :

a) le gène tdh qui code pour la thrétiqueine déshydrogénase,
b) le gène mdh qui code pour la malate déshydrogénase,
c) le produit génétique du cadre ouvert de lecture (orf) yifA,
d) le produit génétique du cadre ouvert de lecture (orf) yifP,
e) le gène pckA qui code pour la phosphoénol pyruvate carboxykinase,
f) le gène poxB qui code pour la pyruvate oxydase,
g) le gène aceA qui code pour l’isocitrate lyase,
h) le gène dgsA qui code pour le régulateur DgsA du système de phosphotransférase,
i) le gène fruR qui code pour le represseur de fructose,
j) le gène rpoS qui code pour le facteur sigma38,
est ou sont éliminé(s).

5. Micro-organismes producteurs de L-thrétiqueine de la famille des Entérobactéries, caractérisés en ce qu’au moins
l’opéron thrABC, lesquels gènes codent pour l’aspartate kinase, l’homosérine déshydrogénase, l’homosérine kinase et la thréonine synthétase, et le gène cysB sont surexprimés.

6. Micro-organismes selon la revendication 5, **caractérisés en ce que** les micro-organismes proviennent du genre Escherichia.

7. Micro-organismes selon la revendication 5, **caractérisés en ce que** les micro-organismes proviennent de l’espèce E. coli.
Figure 1:

pTrc99AcysB
5278 bps
Figure 2:

pTrc99AcysK
5253 bps

lacI

Ptrc

cysK

5S

rrmB

Amp

PvuI

XbaI

PvuI

HindIII
Figure 3:

![Diagram of a plasmid with various restriction sites, genes, and markers labeled. The plasmid is labeled as pTrc99AcysM with 5096 bps.]
Figure 4:

pTrc99AcysPUWA

7997 bps
Figure 5:

![Diagram of pTrc99AcysDNC](image-url)
Figure 6: pTrc99AcysJl

7801 bps
Figure 7:

pTrc99AcysH
5024 bps

HindIII
MluI
lacI
Ptrc
CysH
5S
rmBT
Amp
HindIII
MluI