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(54) Benævnelse: **FREMGANGSMÅDE TIL FREMSTILLING AF L-AMINOSYRER VED ANVENDELSE AF STAMMER FRA ENTEROBACTERIACEAE-FAMILIEN**

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Fortsættes ...

DESCRIPTION

Field of the Invention

[0001] This invention relates to a process for preparing L-amino acids, in particular L-threonine, using recombinant microorganisms of the Enterobacteriaceae family in which the open reading frame (ORF) designated yodA is overexpressed, and to these microorganisms.

Prior Art

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

[0003] It is known that L-amino acids are prepared by fermenting strains of the Enterobacteriaceae, in particular Escherichia coli (E. coli) and Serratia marcescens. Because of the great importance, efforts are constantly being made to improve the preparation methods. Methodological improvements can relate to fermentation measures of a technical nature, such as stirring or supply with oxygen, or to the composition of the nutrient media, such as the sugar concentration during the fermentation, or to the working-up into product form, for example by means of ion exchange chromatography, or to the intrinsic performance characteristics of the microorganism itself.

[0004] Methods of mutagenesis, selection and mutant choice are used for improving the performance characteristics of these microorganisms. This makes it possible to obtain strains which are resistant to antimetabolites, such as the threonine analog α-amino-β-hydroxyvaleric acid (AHV), or auxotrophic for metabolites which are of regulatory importance, and produce L-amino acids such as L-threonine. For some years now, methods of recombinant DNA technology have also been used for improving L-amino acid-producing strains of the Enterobacteriaceae family, by amplifying individual amino acid biosynthesis genes and investigating the effect this has on production. Comprehensive information on the cell biology and molecular biology of Escherichia coli and Salmonella can be found in Neidhardt (ed): Escherichia coli and Salmonella, Cellular and Molecular Biology, 2nd edition, ASM Press, Washington, D.C., USA, (1996).

Object of the Invention

[0005] The inventors have set themselves the object of providing novel measures for improving the fermentative preparation of L-amino acids, in particular L-threonine.

Summary of the Invention

[0006] The invention relates to recombinant microorganisms of the Enterobacteriaceae family which contain an overexpressed yodA ORF, which encodes a polypeptide which is annotated as being a putative metal-binding protein, and which produce L-amino acids, in particular L-threonine, in an improved manner.

[0007] The microorganisms which are not recombinant for the yodA ORF, and which do not contain any potentiated yodA ORF, are in each case used as the starting point for the comparison.

[0008] These microorganisms include, in particular, microorganisms of the Enterobacteriaceae family in which a polynucleotide, which encodes a polypeptide whose amino acid sequence is at least 95%, preferably at least 98% or at least 99%, particularly preferably 99.5% and, very particularly preferably, 100%, identical to an amino acid sequence selected from the group SEQ ID No. 4 and SEQ ID No. 6, is potentiated.

[0009] Preference is given to amino acid sequences which are identical to those from SEQ ID No. 4 and SEQ ID No. 6. Said microorganisms contain overexpressed polynucleotides selected from the group:

1. a) polynucleotide having the nucleotide sequence SEQ ID No. 3 or SEQ ID No. 5;

2. b) polynucleotide having a nucleotide sequence which corresponds to SEQ ID No. 3 or SEQ ID No. 5 within the limits of the degeneracy of the genetic code;
3. c) polynucleotide sequence having a sequence which hybridizes, under stringent conditions, with the sequence which is complementary to the sequence SEQ ID No. 3 or SEQ ID No. 5;
4. d) polynucleotide having a sequence SEQ ID No. 3 or SEQ ID No. 5 which contains functionally neutral sense mutants,

with the polynucleotides encoding a putative metal-binding protein.

[0010] The invention also relates to a process for fermentatively preparing L-amino acids, in particular L-threonine, using recombinant microorganisms of the Enterobacteriaceae family which already produce L-amino acids and in which at least the open reading frame (ORF) designated yodA, or nucleotide sequences encoding its gene product, is/are overexpressed.

[0011] Preference is given to using the described microorganisms.

Detailed Description of the Invention

[0012] The mention, in that which follows, of L-amino acids or amino acids means one or more amino acids, including their salts, selected from the group L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-proline, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan, L-arginine and L-homoserine. Particular preference is given to L-threonine.

[0013] In this connection, the term "potentiation" describes an increase in the intracellular activity or concentration of one or more enzymes or proteins, which are encoded by the corresponding DNA, in a microorganism, which increase is achieved, for example, by increasing the copy number of the gene or genes, or of the ORF or ORFs, by at least one (1) copy, by functionally linking a strong promoter to the gene, or by using a gene or allele or ORF which encodes a corresponding enzyme or protein having a high activity, or by, where appropriate, combining these measures.

[0014] A segment of a nucleotide sequence which encodes, or can encode, a protein or polypeptide or ribonucleic acid to which the prior art cannot assign any function is described as being an open reading frame (ORF). After a function has been assigned to the nucleotide sequence segment in question, the segment is generally referred to as being a gene. In general, alleles are understood as being alternative forms of a given gene. The forms are characterized by differences in their nucleotide sequences.

[0015] In general, the protein encoded by a nucleotide sequence, i.e. an ORF, a gene or an allele, or the encoded ribonucleic acid, is described as being a gene product.

[0016] In general, the measures of potentiation, in particular overexpression, increase the activity or concentration of the corresponding protein by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, at most up to 1000% or 2000%, based on that of the wild-type protein or on the activity or concentration of the protein in the microorganism or parent strain which is not recombinant for the corresponding enzyme or protein. A nonrecombinant microorganism or parent strain is understood as being the microorganism on which the measures according to the invention are implemented.

[0017] The invention relates to a process for preparing L-amino acids by fermenting recombinant microorganisms of the Enterobacteriaceae family, which process is characterized in that

1. a) the microorganisms as defined in claims 1-14 are cultured in a medium under conditions under which the desired L-amino acid is enriched in the medium or in the cells, and
2. b) the desired L-amino acid is isolated, with, where appropriate, constituents of the fermentation broth, and/or the biomass, in its entirety or in portions (≥ 0 to 100%), remaining in the isolated product or being completely removed.

[0018] The microorganisms, which are recombinant, which contain an overexpressed open reading frame (ORF) designated yodA, and which are likewise part of the subject-matter of the present invention, can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, where appropriate starch and where appropriate cellulose, or from glycerol and ethanol. The microorganisms are representatives of the Enterobacteriaceae family, selected from the genera Escherichia, Erwinia, Providencia and Serratia. The genera Escherichia and Serratia are preferred. The species Escherichia coli and Serratia marcescens are to be mentioned, in particular, in the case of the genus Escherichia and, respectively, in the case of the genus

Serratia.

[0019] In general, recombinant microorganisms are produced by transformation, transduction or conjugation, or a combination of these methods, with a vector which contains the desired ORF, the desired gene, an allele of this ORF or gene, or parts thereof, and/or a promoter which potentiates the expression of the ORF or gene. This promoter can be the promoter which has been produced, as a result of potentiating mutation, from the endogenous regulatory sequence located upstream of the gene or ORF, or an efficient promoter can have been fused to the gene or ORF.

[0020] Examples of suitable, in particular L-threonine-producing, strains of the genus Escherichia, in particular of the species Escherichia coli, are

- Escherichia coli H4581	(EP 0 301 572)
- Escherichia coli KY10935	(Bioscience Biotechnology and Biochemistry 61(11):1877-1882 (1997)
- Escherichia coli VNIIgenetika MG442	(US-A-4278 765)
- Escherichia coli VNIIgenetika M1	(US-A-4,321,325)
- Escherichia coli VNIIgenetika 472T23	(US-A-5,631,157)
- Escherichia coli BKIIM B-3996	(US-A-5,175,107)
- Escherichia coli kat 13	(WO 98/04715)
- Escherichia coli KCCM-10132	(WO 00/09660)

[0021] Examples of suitable L-threonine-producing strains of the genus Serratia, in particular of the species Serratia marcescens, are

- Serratia marcescens HNr21 (Applied and Environmental Microbiology 38(6): 1045-1051 (1979))
- Serratia marcescens TLr156 (Gene 57(2-3): 151-158 (1987))
- Serratia marcescens T-2000 (Applied Biochemistry and Biotechnology 37(3): 255-265 (1992))

[0022] L-Threonine-producing strains from the Enterobacteriaceae family preferably possess, inter alia, one or more of the genetic or phenotypic features selected from the group:

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 borrelidine, resistance to cyclopentanecarboxylic acid, resistance to rifampicin, resistance to valine analogs such as valine hydroxamate, resistance to purine analogs, such as 6-dimethylaminopurine, requirement for L-methionine, where appropriate partial and compensatable requirement for L-isoleucine, requirement for meso-diaminopimelic acid, auxotrophy with regard to threonine-containing dipeptides, resistance to L-threonine, resistance to threonine raffinate, 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 towards fluoropyruvate, defective threonine dehydrogenase, where appropriate ability to utilize sucrose, potentiation of the threonine operon, potentiation of homoserine dehydrogenase I-aspartate kinase I, preferably of the feedback-resistant form, potentiation of homoserine kinase, potentiation of threonine synthase, potentiation of aspartate kinase, where appropriate of the feedback-resistant form, potentiation of aspartate semialdehyde dehydrogenase, potentiation of phosphoenolpyruvate carboxylase, where appropriate of the feedback-resistant form, potentiation of phosphoenolpyruvate synthase, potentiation of transhydrogenase, potentiation of the RhtB gene product, potentiation of the RhtC gene product, potentiation of the YfiK gene product, potentiation of a pyruvate carboxylase and attenuation of acetic acid formation.

[0023] It has been found that, after the yodA gene or open reading frame (ORF), or its alleles, has/have been overexpressed, microorganisms of the Enterobacteriaceae family produce L-amino acids, in particular L-threonine, in an improved manner.

[0024] The nucleotide sequences of the genes or open reading frames (ORFs) of Escherichia coli belong to the prior art and can be obtained from the Escherichia coli genome sequence published by Blattner et al. (Science 277: 1453-1462 (1997)). It is known that enzymes which are endogenous to the host (methionine aminopeptidase) can cleave off the N-terminal amino acid methionine.

[0025] The nucleotide sequences for the yodA ORF are likewise known from Shigella flexneri, which also belongs to the Enterobacteriaceae family.

[0026] The Escherichia coli K12 yodA ORF is described, inter alia, by the following particulars:

Designation:	open reading frame
Function:	annotated as being a putative metal-binding protein; expression increased in connection with cadmium and hydrogen peroxide stress
Description:	the yodA open reading frame encodes a 24.8 KD protein; the isoelectric point is 5.9; being chromosomally located, it is present, for example in the case of Escherichia coli K12 MG1655, in the intergenic region between the open reading frame b1972, encoding a putative membrane protein, and the yodB ORF, encoding a putative cytochrome b
References:	Blattner et al., Science 5; 277 (5331): 1453-1474 (1997)
	Puskarova et al., Microbiology 148 (PT 12): 3801-3811 (2002),
	David et al., Acta Crystallographica Section D. Biological Crystallography 58(PT 7): 1243-1245 (2002),
	David et al., The Journal of Biological Chemistry 278(44): 43728-43735 (2003)
Accession No.:	AE000288
Alternative gene name:	b1973

[0027] The nucleic acid sequences can be obtained from the databases belonging to the National Center for Biotechnology Information (NCBI) of the National Library of Medicine (Bethesda, MD, USA), from the nucleotide sequence database belonging to the European Molecular Biologies Laboratories (EMBL, Heidelberg, Germany and Cambridge, UK) or from the Japanese DNA database (DDBJ, Mishima, Japan).

[0028] For the sake of greater clarity, the known nucleotide sequence of the Escherichia coli yodA ORF is given in SEQ ID No. 3 and the known sequence for the Shigella flexneri (AE015219) yaaU-ORF is given in SEQ ID No. 5. The amino acid sequences of the proteins encoded by these reading frames are depicted as SEQ ID No. 4 and, respectively, SEQ ID No. 6.

[0029] The open reading frames described in the given text passages can be used in accordance with the invention. It is furthermore possible to use alleles of the genes or open reading frames which ensue from the degeneracy of the genetic code or as a result of functionally neutral sense mutations. The use of endogenous genes or endogenous open reading frames is preferred.

[0030] "Endogenous genes" or "endogenous nucleotide sequences" are understood as being the genes or open reading frames, or alleles or nucleotide sequences which are present in the population of a species.

[0031] Disclosed is that the suitable alleles of the yodA ORF which contain functionally neutral sense mutations include, inter alia, those which lead to at most 20 or to at most 10, preferably to at most 5 or to at most 3, very particularly preferably to at most 2 or to at most one, conservative amino acid substitution in the protein which they encode.

[0032] In the case of the aromatic amino acids, the substitutions are said to be conservative when phenylalanine, tryptophan and tyrosine are substituted for each other. In the case of the hydrophobic amino acids, the substitutions are said to be conservative when leucine, isoleucine and valine are substituted for each other. In the case of the polar amino acids, the substitutions are said to be conservative when glutamine and asparagine are substituted for each other. In the case of the basic amino acids, the substitutions are said to be conservative when arginine, lysine and histidine are substituted for each other. In the case of the acidic amino acids, the substitutions are said to be conservative when aspartic acid and glutamic acid are substituted for each other. In the case of the hydroxyl group-containing amino acids, the substitutions are said to be conservative when serine and threonine are substituted for each other.

[0033] Also disclosed is that in the same way, it is also possible to use those nucleotide sequences which encode variants of said proteins which additionally contain, as the N terminus or C terminus, an extension or truncation by at least one (1) amino acid. This extension or truncation amounts to not more than 50, 40, 30, 20, 10, 5, 3 or 2 amino acids or amino acid residues.

[0034] The suitable alleles also include those which encode proteins in which at least one (1) amino acid has been inserted or deleted. The maximum number of such changes, designated indels, can affect 2, 3, 5, 10 or 20, in no case, however, more than 30, amino acids.

[0035] The suitable alleles furthermore include those which can be obtained by hybridization, in particular under stringent conditions, using SEQ ID No. 3 or SEQ ID No. 5, or parts thereof, in particular the coding regions, or the sequences which are complementary thereto.

[0036] The skilled person can find instructions for identifying DNA sequences by means of hybridization in, inter alia, the manual "The DIG System Users Guide for Filter Hybridization" from the company Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology 41: 255-260 (1991)). The hybridization takes place under stringent conditions; that is, the only hybrids to be formed are those in which probe and target sequence, i.e. the polynucleotides treated with the probe, are at least 80% identical. It is known that the stringency of the hybridization, including the washing steps, is influenced, or determined, by varying the buffer composition, the temperature and the salt concentration. The hybridization reaction is generally carried out at a stringency which is relatively low as compared with that of the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

[0037] For example, a buffer corresponding to 5x SSC buffer can be used, at a temperature of approx. 50°C-68°C, for the hybridization reaction. Under these conditions, probes can also hybridize with polynucleotides which exhibit less than 80% identity with the sequence of the probe. These latter hybrids are less stable and are removed by washing under stringent conditions. This can be achieved, for example, by lowering the salt concentration down to 2x SSC and, where appropriate, subsequently to 0.5× SSC (The DIG System User's Guide for Filter Hybridisation, Boehringer Mannheim, Mannheim, Germany, 1995), with the temperature being adjusted to approx. 50°C-68°C, approx. 52°C-68°C, approx. 54°C-68°C, approx. 56°C-68°C, approx. 58°C-68°C, approx. 60°C-68°C, approx. 62°C-68°C, approx. 64°C-68°C, or approx. 66°C-68°C. Temperature ranges of approx. 64°C-68°C or approx. 66°C-68°C are preferred. It is possible, where appropriate, to lower the salt concentration down to a concentration corresponding to 0.2× SSC or 0.1× SSC. Polynucleotide fragments which possess, for example, at least 80% or at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, identity with the sequence of the probe which is employed, or with the nucleotide sequences depicted in SEQ ID No. 3 or SEQ ID No. 5, can be isolated by increasing the hybridization temperature stepwise from 50°C to 68°C in steps of approx. 1-2°C. Further instructions for the hybridization can be obtained commercially in the form of kits (e.g. DIG Easy Hyb from the company Roche Diagnostics GmbH, Mannheim, Germany, Catalog No. 16035558). The nucleotide sequences which are thus obtained encode polypeptides which possess at least 90%, in particular at least 95%, preferably at least 98% or at least 99%, very particularly preferably 99.5%, identity with the amino acid sequences depicted in SEQ ID No. 4 or SEQ ID No. 6.

[0038] In order to achieve a potentiation, it is possible, for example, to increase the expression of the genes or open reading frames or alleles. Also disclosed is to increase the catalytic properties of the protein. Both measures can be combined, where appropriate.

[0039] In order to achieve overexpression, it is possible, for example, to increase the copy number of the corresponding genes or open reading frames or to mutate the promoter and regulatory region or the ribosomal binding site which is located upstream of the structural gene. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By incorporating inducible promoters, it is additionally possible to increase expression during the course of the fermentative L-amino acid, in particular L-threonine, production; it can furthermore also be advantageous to use, for the gene expression, promoters which enables a different chronological gene expression to be obtained. The expression is likewise improved by measures for extending the lifetime of the m-RNA. The enzyme activity is furthermore also potentiated by preventing the enzyme protein from being broken down. The ORFs, genes or gene constructs can either be present in plasmids having different copy numbers or be integrated, and amplified, in the chromosome. As an alternative, it is also possible to achieve overexpression of the genes concerned by altering the composition of the medium and the conduct of the culture.

[0040] Methods for achieving overexpression are adequately described in the prior art, for example in Makrides et al. (Microbiological Reviews 60 (3), 512-538 (1996)). Using vectors increases the copy number by at least one (1) copy. The vectors used can be plasmids, as described, for example, in US 5,538,873. The vectors used can likewise be phages, for example the phage Mu, as described in EP 0 332 448, or the phage lambda (λ). An increase in the copy number can also be achieved by incorporating an additional copy into another site in the chromosome, for example into the phage λ att site (Yu and Court, Gene 223, 77-81 (1998)). US 5,939,307 reports that it was possible to increase expression by incorporating expression cassettes or promoters, such as the tac promoter, the trp promoter, the lpp promoter, or the phage λ P_L promoter or P_R promoter, for example

upstream of the chromosomal threonine operon. It is possible to use the T7 phage promoters, the gearbox promoters or the nar promoter in the same way. Expression cassettes or promoters of this nature can also be used to ovrerexpress plasmid-bound genes, as described in EP 0 593 792. Using the $lacI^Q$ allele makes it possible, in turn, to control the expression of plasmid-bound genes (Glascock and Weickert, Gene 223, 221-231 (1998)). It is furthermore possible for the activities of the promoters to be increased by modifying their sequences by means of using insertion(s) and/or deletion(s) to effect one or more nucleotide substitutions. A different chronological gene expression can be achieved, for example, by using the growth phase-dependent fis promoter, as described in Walker et al. (Journal of Bacteriology 181: 1269-80 (1999)).

[0041] The skilled person can find general instructions in this regard in, inter alia, Chang and Cohen (Journal of Bacteriology 134: 1141-1156 (1978)), Hartley and Gregori (Gene 13: 347-353 (1981)), Amann and Brosius (Gene 40: 183-190 (1985)), de Broer et al. (Proceedings of the National Academy of Sciences of the United States of America 80: 21-25 (1983)), LaVallie et al. (BIO/TECHNOLOGY 11: 187-193 (1993)), PCT/US97/13359, Llosa et al. (Plasmid 26: 222-224 (1991)), Quandt and Klipp (Gene 80: 161-169 (1989)), Hamilton et al. (Journal of Bacteriology 171: 4617-4622 (1989)), and Jensen and Hammer (Biotechnology and Bioengineering 58: 191-195 (1998)), and in known text books dealing with genetics and molecular biology.

[0042] It is possible to use plasmid vectors which can be replicated in Enterobacteriaceae, such as pACYC184-derived cloning vectors (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)). In one process according to the invention, it is possible to use a plasmid vector-transformed strain, with the plasmid vector carrying at least one nucleotide sequence or allele encoding the yodA ORF or its gene product.

[0043] The term "transformation" is understood as meaning the uptake of an isolated nucleic acid by a host (microorganism).

[0044] It is likewise possible to use sequence exchange (Hamilton et al.; Journal of Bacteriology 171: 4617-4622 (1989)), conjugation or transduction to transfer mutations, which affect the expression of the respective genes or open reading frames, into different strains.

[0045] More detailed explanations with regard to the concepts of genetics and molecular biology can be found in known text books dealing with genetics and molecular biology, such as the text book by Birge (Bacterial and Bacteriophage Genetics, 4th ed., Springer Verlag, New York (USA), 2000) or the text book by Berg, Tymoczko and Stryer (Biochemistry, 5th ed., Freeman and Company, New York (USA), 2002) or the manual of Sambrook et al. (Molecular Cloning, A Laboratory Manual, (3-Volume Set), Cold Spring Harbor Laboratory Press, Cold Spring Harbor (USA), 2001).

[0046] When using strains of the Enterobacteriaceae family to produce L-amino acids, in particular L-threonine, it can also be advantageous, in addition to overexpressing the yodA open reading frame, to potentiate one or more enzymes of the known threonine biosynthesis pathway, or enzymes of anaplerotic metabolism, or enzymes for producing reduced nicotinamide adenine dinucleotide phosphate, or enzymes of glycolysis, or PTS enzymes, or enzymes of sulfur metabolism. Preference is generally given to using endogenous genes.

[0047] Thus, it is possible, for example, to simultaneously potentiate, in particular overexpress, one or more of the genes selected from the group

- at least one gene of the thrABC operon encoding aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,278,765),
- the Corynebacterium glutamicum pyruvate carboxylase-encoding pyc gene (WO 99/18228),
- the phosphoenolpyruvate synthase-encoding pps gene (Molecular and General Genetics 231(2): 332-336 (1992)),
- the phosphoenolpyruvate carboxylase-encoding ppc gene (WO 02/064808),
- the pntA and pntB genes encoding the pyridine transhydrogenase subunits (European Journal of Biochemistry 158: 647-653 (1986)),
- the rhtB gene encoding the homoserine resistance-mediating protein (EP-A-0 994 190),
- the rhtC gene encoding the threonine resistance-mediating protein (EP-A-1 013 765),
- the Corynebacterium glutamicum thrE gene encoding the threonine export carrier protein (WO 01/92545),
- the glutamate dehydrogenase-encoding gdhA gene (Nucleic Acids Research 11: 5257-5266 (1983); Gene 23: 199-209 (1983)),
- the phosphoglucomutase-encoding pgm gene (WO 03/004598),
- the fructose biphosphate aldolase-encoding fba gene (WO 03/004664),
- the ptsHIcrr operon ptsH gene encoding the phosphohistidine protein hexose phosphotransferase of the phosphotransferase system PTS (WO 03/004674),

- the ptsHlcrr operon ptsI gene encoding the enzyme I of the phosphotransferase system PTS (WO 03/004674),
- the ptsHlcrr operon crr gene encoding the glucose-specific IIA component of the phosphotransferase system PTS (WO 03/004674),
- the ptsG gene encoding the glucose-specific IIBC component (WO 03/004670),
- the lrp gene encoding the leucine regulon regulator (WO 03/004665),
- the fadR gene encoding the fad regulon regulator (WO 03/038106),
- the iclR gene encoding the central intermediary metabolism regulator (WO 03/038106),
- the ahpCF operon ahpC gene encoding the small subunit of alkyl hydroperoxide reductase (WO 03/004663),
- the ahpCF operon ahpF gene encoding the large subunit of alkyl hydroperoxide reductase (WO 03/004663),
- the cysteine synthase A-encoding cysK gene (WO 03/006666),
- the cysB gene encoding the cys regulon regulator (WO 03/006666),
- the cysJIH operon cysJ gene encoding the NADPH sulfite reductase flavoprotein (WO 03/006666),
- the cysJIH operon cysI gene encoding the NADPH sulfite reductase hemoprotein (WO 03/006666),
- the cysJIH operon cysH gene encoding adenylyl sulfate reductase (WO 03/006666),
- the rseABC operon rseA gene encoding a membrane protein possessing anti-sigmaE activity (WO 03/008612),
- the rseABC operon rseC gene encoding a global regulator of the SigmaE factor (WO 03/008612),
- the sucABCD operon sucA gene encoding the decarboxylase subunit of 2-ketoglutarate dehydrogenase (WO 03/008614),
- the sucABCD operon sucB gene encoding the dihydrolipoyl transsuccinase E2 subunit of 2-ketoglutarate dehydrogenase (WO 03/008614),
- the suc ABCD operon sucC gene encoding the β subunit of succinyl-CoA synthetase (WO 03/008615),
- the sucABCD operon sucD gene encoding the α subunit of succinyl-CoA synthetase (WO 03/008615),
- the aceE gene encoding the E1 component of the pyruvate dehydrogenase complex (WO 03/076635),
- the aceF gene encoding the E2 component of the pyruvate dehydrogenase complex (WO 03/076635),
- the rseB gene encoding the SigmaE factor activity regulator (Molecular Microbiology 24(2): 355-371 (1997)),
- the gene product of the Escherichia coli yaaU open reading frame (ORF) (Accession Number AE005181 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA, (DE10361268.8)).

[0048] For the purpose of producing L-amino acids, in particular L-threonine, it can also be advantageous, in addition to overexpressing the yodA open reading frame, to attenuate, in particular eliminate or reduce, the expression of one or more of the genes selected from the group

- the threonine dehydrogenase-encoding tdh gene (Journal of Bacteriology 169: 4716-4721 (1987)),
- the malate dehydrogenase (E.C. 1.1.1.37)-encoding mdh gene (Archives in Microbiology 149: 36-42 (1987)),
- the gene product of the Escherichia coli yjfA open reading frame (ORF) (Accession Number AAC77180 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA, (WO 02/29080)),
- the gene product of the Escherichia coli ytfP open reading frame (ORF) (Accession Number AAC77179 of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA, (WO 02/29080)),
- the pckA gene encoding the enzyme phosphoenolpyruvate carboxykinase (WO 02/29080),
- the pyruvate oxidase-encoding poxB gene (WO 02/36797),
- the dgsA gene (WO 02/081721), which is also known under the designation mlc gene, encoding the DgsA regulator of the phosphotransferase system,
- the fructose repressor-encoding fruR gene (WO 02/081698), which is also known under the designation cra gene,
- the Sigma^{38} factor-encoding rpoS gene (WO 01/05939), which is also known under the designation katF gene, and
- the aspartate ammonium lyase-encoding aspA gene (WO 03/008603).

[0049] In this connection, the term "attenuation" describes the reduction or elimination of the intracellular activity or concentration of one or more enzymes or proteins, which are encoded by the corresponding DNA, in a microorganism, with, for example, use being made of a promoter which is weaker than in the microorganism or parent strain which is not recombinant for the corresponding enzyme or protein, or of a gene or allele which encodes a corresponding enzyme or protein having a lower activity, or the corresponding enzyme or protein, or the open reading frame or the gene, being inactivated, and, where appropriate, these measures being combined.

[0050] The measures of attenuation in general lower the activity or concentration of the corresponding protein down to from 0 to 75%, from 0 to 50%, from 0 to 25%, from 0 to 10%, or from 0 to 5%, of the activity or concentration of the wild-type protein or of

the activity or concentration of the protein in the microorganism or parent strain which is for the corresponding enzyme or protein. The nonrecombinant microorganism or parent strain is understood as being the microorganism on which the measures according to the invention are implemented.

[0051] In order to achieve an attenuation, it is possible, for example, for the expression of the genes or open reading frames, or the catalytic properties of the enzyme proteins, to be reduced or eliminated. Both measures can be combined, where appropriate.

[0052] Gene expression can be reduced by means of conducting the culture in a suitable manner, by means of genetically altering (mutating) the signal structures for gene expression or else by means of antisense RNA technology. Examples of signal structures for gene expression are repressor genes, activator genes, operators, promoters, attenuators, ribosome binding sites, the start codon and terminators. The skilled person can, for example, find information in this regard in, inter alia, Jensen and Hammer (Biotechnology and Bioengineering 58: 191-195 (1998)), Carrier and Keasling (Biotechnology Progress 15: 58-64 (1999)), Franch and Gerdes (Current Opinion in Microbiology 3: 159-164 (2000)) and known text books dealing with genetics and molecular biology, such as the text book by Knippers ("Molekulare Genetik [Molecular Genetics]", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or that by Winnacker ("Gene und Klone [Genes and Clones]", VCH Verlagsgesellschaft, Weinheim, Germany, 1990).

[0053] Mutations which lead to a change or reduction in the catalytic properties of enzyme proteins are known from the prior art. Examples which may be mentioned are the studies by Qiu and Goodman (Journal of Biological Chemistry 272: 8611-8617 (1997)), Yano et al. (Proceedings of the National Academy of Sciences of the United States of America 95: 5511-5515 (1998)), Wente and Schachmann (Journal of Biological Chemistry 266: 20833-20839 (1991)). Comprehensive accounts can be obtained from known text books dealing with genetics and molecular biology, such as that by Hagemann ("Allgemeine Genetik [General genetics]", Gustav Fischer Verlag, Stuttgart, 1986).

[0054] Mutations which come into consideration are transitions, transversions, insertions and deletions of at least one (1) base pair or nucleotide. Depending on the effect which the amino acid substitution produced by the mutation has on the enzyme activity, reference is made to missense mutations or to nonsense mutations. The missense mutation leads to the substitution of a given amino acid in a protein with another amino acid, with the amino acid substitution being, in particular, a nonconservative amino acid substitution. This substitution impairs the functional ability or activity of the protein and reduces it down to a value of from 0 to 75%, from 0 to 50%, from 0 to 25%, from 0 to 10% or from 0 to 5%. The nonsense mutation leads to a stop codon being formed in the coding region of the gene and, as a result, to translation being terminated prematurely. Insertions or deletions of at least one base pair in a gene lead to frame shift mutations, which in turn result in incorrect amino acids being incorporated or in the translation being terminated prematurely. If the mutation results in a stop codon being formed in the coding region, this then likewise leads to translation being terminated prematurely. Deletions of at least one (1) or more codons typically also lead to complete loss of the enzyme activity.

[0055] Instructions for generating these mutations belong to the prior art and can be obtained from known text books dealing with genetics and molecular biology, such as the text book by Knippers ("Molekulare Genetik [Molecular genetics]", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone [Genes and clones]", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik [General genetics]", Gustav Fischer Verlag, Stuttgart, 1986).

[0056] Suitable mutations in the genes can be incorporated into suitable strains by means of gene or allele substitution.

[0057] A common method is the method, described by Hamilton et al. (Journal of Bacteriology 171: 4617-4622 (1989)), of gene substitution using a conditionally replicating pSC101 derivate, pMAK705. Other methods described in the prior art, such as that of Martinez-Morales et al. (Journal of Bacteriology 181: 7143 -7148 (1999)) or that of Boyd et al. (Journal of Bacteriology 182: 842-847 (2000)), can also be used.

[0058] It is likewise possible to transfer mutations in the respective genes, or mutations which affect the expression of the respective genes or open reading frames, into different strains by means of conjugation or transduction.

[0059] For the purpose of producing L-amino acids, in particular L-threonine, it can also be advantageous, in addition to overexpressing the yodA open reading frame, to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

[0060] The microorganisms which have been prepared in accordance with the invention can be cultured in a batch process, in a

fed-batch process, in a repeated fed-batch process or in a continuous process (DE102004028859.3 or US 5,763,230). A summary of known culture methods is given in the text book by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess technology 1. Introduction to bioprocess technology] (Gustav Fischer Verlag, Stuttgart, 1991)) or in the text book by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and peripheral installations] (Vieweg Verlag, Braunschweig/ Wiesbaden, 1994)).

[0061] The culture medium which is to be used must adequately satisfy the requirements of the given strains. The manual "Manual of Methods for General Bacteriology" from the American Society for Bacteriology (Washington D.C., USA, 1981) contains descriptions of culture media for different microorganisms.

[0062] Sugars and carbohydrates, such as glucose, sucrose, lactose, fructose, maltose, molasses, starch and, where appropriate, cellulose, oils and fats, such as soybean oil, sunflower oil, peanut oil and coconut fat, fatty acids, such as palmitic acid, stearic acid and linoleic acid, alcohols, such as glycerol and ethanol, and organic acids, such as acetic acid, can be used as the carbon source. These substances can be used individually or as a mixture.

[0063] Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, cornsteep liquor, soybean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the nitrogen source. The nitrogen sources can be used individually or as mixtures.

[0064] Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate, or the corresponding sodium-containing salts, can be used as the phosphorus source. In addition, the culture medium has to contain metal salts, such as magnesium sulfate or iron sulfate, which are required for growth. Finally, it is possible to use essential growth-promoting substances, such as amino acids and vitamins, in addition to the above-mentioned substances. Moreover, suitable precursors can be added to the culture medium. The above-mentioned addition substances can be added to the culture in the form of a once-only mixture or else fed in, in a suitable manner, as the culture proceeds.

[0065] In general, the fermentation is carried out at a pH of from 5.5 to 9.0, in particular from 6.0 to 8.0. Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or ammonia water, or acidic compounds, such as phosphoric acid or sulfuric acid, are used, in a suitable manner, for controlling the pH of the culture. Antifoamants, such as fatty acid polyglycol esters, can be used for controlling foaming. Suitable substances having a selective action, for example antibiotics, can be added to the medium in order to maintain the stability of plasmids. Oxygen or oxygen-containing gas mixtures, such as air, is/are introduced into the culture in order to maintain aerobic conditions. The temperature of the culture is normally from 25°C to 45°C and preferably from 30°C to 40°C. The culturing is continued until a maximum of L-amino acids or L-threonine has been formed. This objective is normally achieved within 10 to 160 hours.

[0066] L-Amino acids can be analyzed by means of anion exchange chromatography followed by ninhydrin derivatization, as described in Spackman et al. (Analytical Chemistry 30: 1190-1206 (1958)); otherwise, they can be analyzed by reversed phase HPLC as described in Lindroth et al. (Analytical Chemistry 51: 1167-1174 (1979)).

[0067] The process according to the invention is used for fermentatively preparing L-amino acids, such as L-threonine, L-isoleucine, L-valine, L-methionine, L-homoserine, L-tryptophan and L-lysine, in particular L-threonine.

[0068] The following microorganism has been deposited in the Deutsche Sammlung für Mikroorganismen und Zellkulturen [German collection of microorganisms and cell cultures] (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty:

- Escherichia coli strain E. coli MG442, as DSM 16574.

[0069] The present invention is clarified below with the aid of implementation examples.

[0070] The minimal (M9) and complete (LB) media for Escherichia coli which are used are described by J.H. Miller (A short course in bacterial genetics (1992), Cold Spring Harbor Laboratory Press). The isolation of plasmid DNA from Escherichia coli, and all the techniques for restriction, ligation and treatment with Klenow phosphatase and alkali phosphatase, are carried out in accordance with Sambrook et al. (Molecular Cloning - A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press). Unless otherwise described, Escherichia coli is transformed in accordance with Chung et al. (Proceedings of the National Academy of

Sciences of the United States of America 86: 2172-2175 (1989)).

[0071] The incubation temperature when preparing strains and transformants is 37°C.

Example 1

Constructing the expression plasmid pTrc99AyodA

[0072] The E. coli K12 yodA open reading frame is amplified using the polymerase chain reaction (PCR) and synthetic oligonucleotides. PCR primers are synthesized (MWG Biotech, Ebersberg, Germany) on the basis of the nucleotide sequence of the yodA open reading frame in E. coli K12 MG1655 (Accession Number AE000288, Blattner et al. (Science 277: 1453-1474 (1997)). The primers contain sequences for restriction enzymes, with these sequences being marked by underlining in the nucleotide sequences which are depicted below. The primer yodA_5' contains the restriction cleavage site for NcoI, while the primer yodA_3' contains that for HincII.

yodA_5' 5'- GGTCCATGGCGATTCGTCTTTACAAACTGG-3' (SEQ ID No. 1)

yodA_3' 5'- GGAATCCGTTATTGGTTAACTC - 3' (SEQ ID No. 2)

[0073] The chromosomal E. coli K12 MG1655 DNA which is employed for the PCR is isolated, in accordance with the manufacturer's instructions, using "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany). The specific primers are able to amplify a DNA fragment of approx. 830 bp in size (SEQ ID No. 3) under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) using Pfu DNA polymerase (Promega Corporation, Madison, USA).

[0074] The PCR product is ligated, in accordance with the manufacturer's instructions, to the vector pCR-Blunt II-TOPO (Zero Blunt TOPO cloning kit, Invitrogen, Groningen, the Netherlands), in accordance with the manufacturer's instructions, and the latter is transformed into the E. coli strain TOP10. Plasmid-harboring cells are selected from LB agar to which kanamycin has been added to a concentration of 50 µg/ml. After the plasmid DNA has been isolated, the vector is cleaved with the restriction enzymes EcoRI and EcoRV and, after having been checked by being separated in an 0.8% agarose gel, is designated pCRBluntyodA.

[0075] The vector pCRBluntyodA is then restricted with the restriction enzymes NcoI and HincII and the yodA fragment is isolated using a QIAquick gel extraction kit (QIAGEN, Hilden, Germany) after it has been separated in an 0.8% agarose gel. The vector pTrc99A (Pharmacia Biotech, Uppsala, Sweden) is cleaved with the enzymes NcoI and SmaI and ligated to the isolated yodA fragment. The E. coli strain XL1-Blue MRF' (Stratagene, La Jolla, USA) is transformed with the ligation mixture and plasmid-harboring cells are selected on LB agar to which ampicillin has been added to a concentration of 50 µg/ml. After the plasmid DNA had been isolated, the fact that the cloning has been successful can be demonstrated by carrying out a control cleavage with the enzyme EcoRV.

[0076] The plasmid is designated pTrc99AyodA (Figure 1).

Example 2

Preparing L-threonine using the strain MG442/pTrc99AyodA

[0077] The L-threonine-producing E. coli strain MG442 is described in the patent specification US-A-4,278,765 and has been deposited in the Russian national collection of industrial microorganisms (VKPM, Moscow, Russia) as CMIM B-1628 and in the Deutsche Sammlung fur Mikroorganisms und Zellkulturen [German collection of microorganisms and cell cultures] (DSMZ, Braunschweig, Germany), in accordance with the Budapest Treaty, as DSM 16574.

[0078] The strain MG442 is transformed with the expression plasmid pTrc99AyodA, as described in example 1, and with the

vector pTrc99A, and plasmid-harboring cells are selected on LB agar containing 50 µg of ampicillin/ml. This results in the strains MG442/pTrc99AyodA and MG442/pTrc99A. Selected individual colonies are then propagated further on minimal medium having the following composition: 3.5 g of $Na_2HPO_4*2H_2O$/l, 1.5 g of KH_2PO_4/l, 1 g of NH_4Cl/l, 0.1 g of $MgSO_4*7H_2O$/l, 2 g of glucose/l, 20 g of agar/l and 50 mg of ampicillin/l.

[0079] The formation of L-threonine is examined in 10 ml batch cultures which are contained in 100 ml Erlenmeier flasks. For this, 10 ml of preliminary culture medium of the following composition: 2 g of yeast extract/l, 10 g of $(NH_4)_2SO_4$/l, 1 g of KH_2PO_4/l, 0.5 g of $MgSO_4*7H_2O$/l, 15 g of $CaCO_3$/l, 20 g of glucose/l and 50 mg of ampicillin/l are inoculated and incubated, at 37°C and 180 rpm for 16 hours, on an ESR incubator supplied by Kühner AG (Birsfelden, Switzerland). In each case, 250 µl of this preliminary culture are inoculated into 10 ml of production medium (25 g of $(NH_4)_2SO_4$/l, 2 g of KH_2PO_4/l, 1 g of $MgSO_4*7H_2O$/l, 0.03 g of $FeSO_4*7H_2O$/l, 0.018 g of $MnSO_4*1H_2O$/l, 30 g of $CaCO_3$/l, 20 g of glucose/l and 50 mg of ampicillin/l), and the latter medium is incubated at 37°C for 48 hours. The formation of L-threonine by the parent strain MG442 is examined in the same way, but without any ampicillin being added to the medium. After the incubation, the optical density (OD) of the culture suspension is determined, at a measurement wavelength of 660 nm, using a Dr. Lange LP2W photometer (Düsseldorf, Germany).

[0080] The concentration of L-threonine which has been formed is then determined, in the culture supernatant, which has been sterilized by filtration, by means of ion exchange chromatography using an Eppendorf-BioTronik amino acid analyzer (Hamburg, Germany), and post column reaction involving detection with ninhydrin.

[0081] The result of the experiment is shown in Table 1.

Table 1

Strain	OD (660 nm)	L-Threonine g/l
MG442	5.6	1.4
MG442/pTrc99A	3.8	1.3
MG442/pTrc99AyodA	4.7	2.1

Brief Description of the Figure:

[0082] The length values are to be regarded as being approximate. The abbreviations and terms employed have the following meanings:

- Amp: ampicillin resistance gene
- lacI: gene for the repressor protein of the trc promoter
- Ptrc: trc promoter region, IPTG-inducible
- yodA: coding region of the yodA open reading frame
- 5S: 5S rRNA region
- rrnBT: rRNA terminator region

[0083] The abbreviations for the restriction enzymes have the following meanings:

- EcoRI: Escherichia coli RY13 restriction endonuclease
- EcoRV: Escherichia coli B946 restriction endonuclease
- HincII: Haemophilus influenzae R_c restriction endonuclease
- NcoI: Rhodococcus sp. ATCC 19070 restriction endonuclease

SEQUENCE LISTING

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Tyr Arg Lys Gly Tyr Pro Thr Asp Ile Glu Met Ile Gly Ile Glu Asp
            100                 105                 110

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Val Val Glu Glu Met Met Ser His
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REFERENCES CITED IN THE DESCRIPTION

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Patentkrav

1. Rekombinant mikroorganisme fra *Enterobacteriaceae*-familien, som allerede fremstiller L-aminosyrer, og som indeholder en overudtrykt yodA-ORF, og som fremstiller L-aminosyrer på en forbedret måde i sammenligning med mikroorganismer, som ikke er rekombinante for nævnte yodA-ORF, og som ikke indeholder nogen forstærket yodA-ORF.

2. Mikroorganisme fra *Enterobacteriaceae*-familien ifølge krav 1, hvori et polynukleotid, som svarer til nævnte yodA-ORF, og som koder for et polypeptid, hvis aminosyresekvens er mindst 90% identisk med en aminosyresekvens valgt blandt SEQ ID NO: 4 og SEQ ID NO: 6, overudtrykkes.

3. Mikroorganisme ifølge krav 2, hvor det overudtrykte polynukleotid, som svarer til nævnte yodA-ORF, er valgt fra følgende gruppe:
a) polynukleotid, som har nukleotidsekvensen ifølge SEQ ID NO: 3 eller SEQ ID NO: 5;
b) polynukleotid, som har en nukleotidsekvens svarende til SEQ ID NO: 3 eller SEQ ID NO: 5 inden for grænserne for degenerering af den genetiske kode;
c) polynukleotidsekvens, som har en sekvens, der under stringente betingelser hybridiserer med den sekvens, som er komplementær med sekvensen SEQ ID NO: 3 eller SEQ ID NO: 5.

4. Mikroorganisme ifølge krav 2, hvor polypeptidet har en aminosyresekvens, som er mindst 95% identisk med en af sekvenserne valgt fra gruppen bestående af SEQ ID NO: 4 og SEQ ID NO: 6.

5. Mikroorganisme ifølge krav 2, hvor polypeptidet har aminosyresekvensen, som er identisk med den ifølge SEQ ID NO: 4 eller SEQ ID NO: 6.

6. Mikroorganisme ifølge kravene 2-5, hvor den er fremstillet ved transformation, transduktion eller

konjugation, eller en kombination af disse fremgangsmåder, med en vektor, som indeholder nævnte yodA-ORF eller en allel af denne yodA-ORF og eller en promotor, som forstærker ekspressionen af nævnte yodA-ORF eller allellen af nævnte yodA-ORF.

7. Mikroorganisme ifølge krav 1 eller 6, hvor kopiantallet af nævnte yodA-ORF eller af allellerne er forøget med mindst 1.

8. Mikroorganisme ifølge krav 7, hvor forøgelsen af kopiantallet af nævnte yodA-ORF med mindst 1 er forårsaget af integration af nævnte ORF eller alleller i mikroorganismens kromosom.

9. Mikroorganisme ifølge krav 7, hvor forøgelsen af kopiantallet af nævnte yodA-ORF med mindst 1 er opnået ved hjælp af en ekstrakromosomalt replikerende vektor.

10. Mikroorganisme ifølge krav 1 eller 2, hvor, for at opnå overekspressionen,
a) promotor- og regulatorregionen, eller ribosombindingsstedet opstrøms for nævnte yodA-ORF, er muteret, eller
b) ekspressionskassetter eller promotorer er inkorporeret opstrøms for nævnte yodA-ORF.

11. Mikroorganisme ifølge krav 1 eller 2, hvor nævnte yodA-ORF er under kontrol af en promotor, som forstærker ekspression af genet.

12. Mikroorganisme ifølge kravene 1-11, hvor overekspressionen af nævnte yodA-ORF forøger koncentrationen eller aktiviteten af yodA-genproduktet (protein) med mindst 10% baseret på aktiviteten eller koncentrationen af genproduktet i mikroorganismen eller forælderstammen, som ikke er rekombinant for nævnte yodA-ORF.

13. Mikroorganisme ifølge krav 1-12, hvor mikroorganismen er

valgt blandt slægterne *Escherichia*, *Erwinia*, *Providencia* og *Serratia*.

14. Mikroorganisme ifølge kravene 1-13, hvor den fremstiller L-threonin.

15. Fremgangsmåde til fremstilling af L-aminosyrer ved fermentering af rekombinante mikroorganismer fra *Enterobacteriaceae*-familien, hvor
a) rekombinante mikroorganismer ifølge et hvilket som helst af kravene 1-14 dyrkes i et medium under betingelser, hvorunder den ønskede L-aminosyre beriges i mediet eller i cellerne, og
b) den ønskede L-aminosyre isoleres, idet bestanddele af fermenteringsmediet og/eller biomassen i sin helhed eller dele deraf (≥ 0 til 100%), hvor det er pasende, bliver i det isolerede produkt eller fjernes helt.

16. Fremgangsmåde ifølge krav 15, hvor der med det formål at fremstille L-threonin fermenteres mikroorganismer fra *Enterobacteriaceae*-familien, hvor et eller flere af generne valgt fra gruppen:
16.1 mindst ét gen fra den thrABC-operon, som koder for aspartatkinase, homoserindehydrogenase, homoserinkinase og threoninsyntase,
16.2 det pyruvatcarboxylase-kodende pyc-gen fra *Corynebacterium glutamicum*,
16.3 det phosphoenolpyruvatsyntase-kodende pps-gen,
16.4 det phosphoenolpyruvatcarboxylase-kodende ppc-gen,
16.5 pntA- og pntB-generne, som koder for pyridintranshydrogenasesubunitterne,
16.6 rhtB-genet, som koder for det homoserinresistensmedierende protein,
16.7 rhtC-genet, som koder for det threoninresistensmedierende protein,
16.8 det threonineksportbærerprotein-kodende thrE-gen fra *Corynebacterium glutamicum*,
16.9 det glutamatdehydrogenase-kodende gdhA-gen,
16.10 det phosphoglucomutase-kodende pgm-gen,

16.11 det fructosebiphosphataldolase-kodende fba-gen,
16.12 ptsH-genet, som koder for phosphohistidinprotein-hexose-phospho-transferase,
16.13 ptsI-genet, som koder for enzymet I fra phosphotransferasesystemet,
16.14 crr-genet, som koder for den glucosespecifikke IIA-komponent,
16.15 ptsG-genet, som koder for den glucosespecifikke IIBC-komponent,
16.16 lrp-genet, som koder for leucinregulon-regulatoren,
16.17 fadR-genet, som koder for fad-regulonregulatoren,
16.18 iclR-genet, som koder for regulatoren for den centrale intermediær-metabolisme,
16.19 ahpC-genet, som koder for den lille subunit fra alkylhydro-peroxidreduktase,
16.20 ahpF-genet, som koder for den store subunit fra alkylhydro-peroxidreduktase,
16.21 det cysteinsyntase A-kodende cysK-gen,
16.22 cysB-genet, som koder for cys-regulonregulatoren,
16.23 cysJ-genet, som koder for NADPH-sulfit-reduktase-flavoproteinet,
16.24 cysI-genet, som koder for NADPH-sulfit-reduktase-hemoproteinet,
16.25 cysH-genet, som koder for adenylylsulfatreduktase,
16.26 rseA-genet, som koder for et membranprotein, der har anti-sigmaE-aktivitet,
16.27 rseC-genet, som koder for en global regulator af sigmaE-faktoren,
16.28 sucA-genet, som koder for decarboxylasesubunitten fra 2-ketoglutaratdehydrogenase,
16.29 sucB-genet, som koder for dihydrolipoyltranssuccinase-E2-subunitten fra 2-ketoglutaratdehydrogenase,
16.30 sucC-genet, som koder for β-subunitten fra succinyl-CoA-syntetase,
16.31 sucD-genet, som koder for α-subunitten fra succinyl-CoA-syntetase,
16.32 aceE-genet, som koder for E1-komponenten fra pyruvat-dehydrogenasekomplekset,

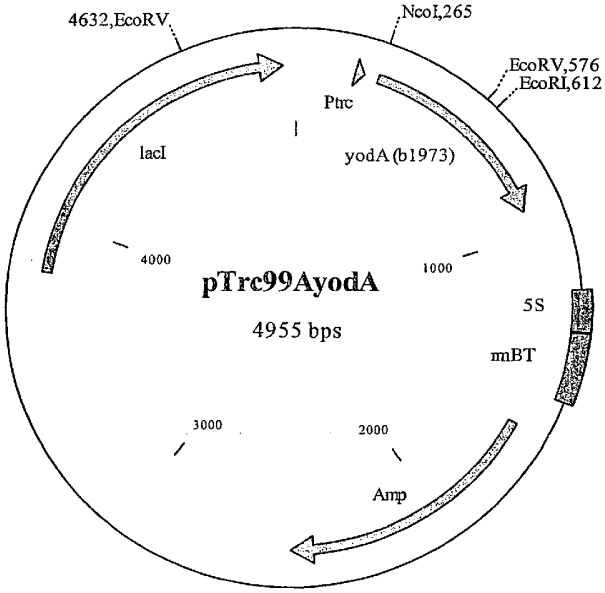
16.33 aceF-genet, som koder for E2-komponenten fra pyruvat-dehydrogenasekomplekset,
16.34 rseB-genet, som koder for SigmaE-faktoraktivitetsregulatoren og
16.35 genproduktet fra den åbne yaaU-læseramme (yaaU-ORF) fra *Escherichia coli*,
endvidere overudtrykkes samtidig.

17. Fremgangsmåde ifølge krav 15 eller 16, hvor der med det formål at fremstille L-threonin fermenteres mikroorganismer fra *Enterobacteriaceae*-familien, hvor et eller flere af generne valgt fra gruppen:
17.1 det threonindehydrogenase-kodende tdh-gen,
17.2 det malatdehydrogenase-kodende mdh-gen,
17.3 genproduktet fra den åbne yjfA-læseramme (yjfA-ORF) fra *Escherichia coli*,
17.4 genproduktet fra den åbne ytfP-læseramme (ytfP-ORF) fra *Escherichia coli*,
17.5 det phosphoenolpyruvatcarboxykinase-kodende pckA-gen,
17.6 det pyruvatoxidase-kodende poxB-gen,
17.7 dgsA-genet, som koder for DgsA-regulatoren fra phospho-transferasesystemet,
17.8 det fructoserepressor-kodende fruR-gen,
17.9 det $Sigma^{38}$-faktor-kodende rpoS-gen og
17.10 det aspartatammoniumlyase-kodende aspA-gen,
samtidig endvidere svækkes, navnlig elimineres, eller deres ekspression reduceres.

18. Fremgangsmåde ifølge kravene 15-17, hvor der fremstilles L-aminosyrer valgt fra gruppen bestående af L-asparagin, L-serin, L-glutamat, L-glycin, L-alanin, L-cystein, L-valin, L-methionin, L-prolin, L-isoleucin, L-leucin, L-tyrosin, L-phenylalanin, L-histidin, L-lysin, L-tryptophan, L-arginin og L-homoserin.

19. Fremgangsmåde ifølge krav 18, hvor der fremstilles L-aminosyrer valgt fra gruppen bestående af L-isoleucin, L-valin, L-methionin, L-homoserin, L-tryptophan og L-lysin.

DRAWINGS


4632,EcoRV
NcoI,265
EcoRV,576
EcoRI,612
Ptrc
lacI
yodA (b1973)
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