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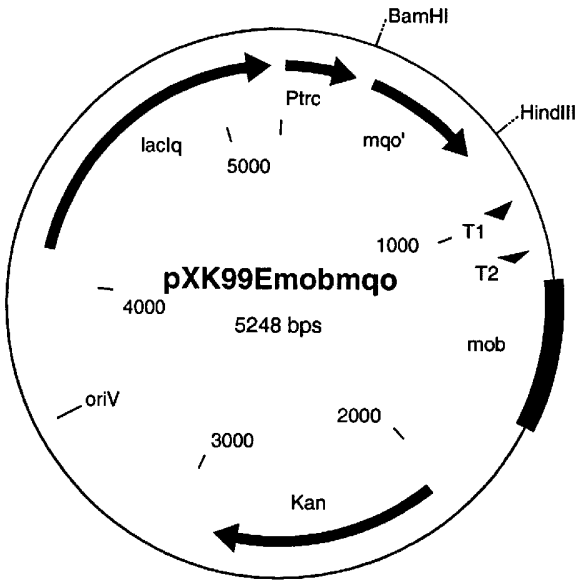
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(54) Title: PROCESS FOR THE PRODUCTION OF L-AMINO ACIDS BY FERMENTATION USING CORYNEFORM BACTERIA




(57) Abstract: The invention relates to a process for the production of L-amino acids, in which the following steps are carried out: a) fermentation of the coryneform bacteria producing the desired L-amino acid, in which at least the mqo gene is attenuated, b) concentration of the desired L-amino acid in the medium or in the cells of the bacteria, and c) isolation of the L-amino acid, and optionally, bacteria are used in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced, or bacteria are used in which at least some of the metabolic pathways reducing formation of the desired L-amino acid are excluded.

WO 02/086137 A2

# Process for the Production of L-Amino Acids by Fermentation using Coryneform Bacteria

## Field of the Invention

The invention provides a process for the production of L-amino acids, especially L-lysine, by fermentation using coryneform bacteria in which the mqo gene, which codes for malate quinone oxidoreductase, has been attenuated.

## Prior Art

L-amino acids, especially L-lysine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and, very especially, in the feeding of animals.

It is known that amino acids are produced by fermentation of strains of coryneform bacteria, especially Corynebacterium glutamicum. Because of their great importance, attempts are continuously being made to improve the production processes. Improvements to the processes may concern measures relating to the fermentation, such as, for example, stirring and oxygen supply, or the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or working up to the product form by, for example, ion-exchange chromatography, or the intrinsic performance properties of the microorganism itself.

In order to improve the performance properties of such microorganisms, methods of mutagenesis, selection and mutant selection are employed. Such methods yield strains which are resistant to antimetabolites, such as, for example, the lysine analogue S-(2-aminoethyl)-cysteine, or are auxotrophic for metabolites that are important in terms of regulation, and which produce L-amino acids.

For a number of years, methods of recombinant DNA technology have also been used for improving the strain of L-amino acid-producing strains of Corynebacterium glutamicum, by amplifying individual amino acid biosynthesis genes and studying the effect on L-amino acid production.

Object of the Invention

In EP-A-1038969 it is described that an improvement in the production of L-amino acids by fermentation can be achieved by enhancement, especially overexpression, of the mqo gene.

The inventors have set themselves the object of providing novel bases for improved processes for the production of L-amino acids, especially L-lysine, by fermentation using coryneform bacteria.

Description of the invention

Where L-amino acids or amino acids are mentioned hereinbelow, they are to be understood as meaning 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-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-lysine is particularly preferred.

Where L-lysine or lysine is mentioned hereinbelow, it is to be understood as meaning not only the bases but also the salts, such as, for example, lysine monohydrochloride or lysine sulfate.

The invention provides a process for the production of L-amino acids by fermentation using coryneform bacteria in which at least the nucleotide sequence coding for malate quinone oxidoreductase (mqo gene) is attenuated, especially excluded or expressed at a low level.

This invention also provides a process for the production of L-amino acids by fermentation in which the following steps are carried out:

a) fermentation of the L-amino acid-producing coryneform bacteria in which at least the nucleotide sequence coding for malate quinone oxidoreductase is attenuated, especially excluded or expressed at a low level;

b) concentration of the L-amino acids in the medium or in the cells of the bacteria; and

c) isolation of the desired L-amino acids, in which optionally portions or the entirety of the constituents of the fermentation liquor and/or of the biomass remain in the end product.

The strains used preferably produce L-amino acids, especially L-lysine, even before attenuation of the mqo gene.

Preferred embodiments are to be found in the claims.

The term "attenuation" or "attenuate" in this context describes the diminution or exclusion of the intracellular activity of one or more enzymes (proteins) in a microorganism that are coded for by the corresponding DNA, by, for example, using a weak promoter or using a gene or allele that codes for a corresponding enzyme having a low level of activity, or by inactivating the corresponding gene or enzyme (protein), and optionally by combining those measures.

The microorganisms provided by the present invention are able to produce amino acids from glucose, saccharose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They may be representatives of coryneform bacteria, especially of the genus

Corynebacterium. In the case of the genus Corynebacterium, special mention may be made of the species Corynebacterium glutamicum, which is known to those skilled in the art for its ability to produce L-amino acids.

Suitable strains of the genus Corynebacterium, especially of the species Corynebacterium glutamicum, are especially the known wild-type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium melassecola ATCC17965
Corynebacterium thermoaminogenes FERM BP-1539
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869 and
Brevibacterium divaricatum ATCC14020

and L-amino acid-producing mutants or strains prepared therefrom

such as, for example, the L-lysine-producing strains

Corynebacterium glutamicum FERM-P 1709
Brevibacterium flavum FERM-P 1708
Brevibacterium lactofermentum FERM-P 1712
Corynebacterium glutamicum FERM-P 6463
Corynebacterium glutamicum FERM-P 6464
Corynebacterium glutamicum ATCC 21513
Corynebacterium glutamicum ATCC 21544
Corynebacterium glutamicum ATCC 21543
Corynebacterium glutamicum DSM 4697 and
Corynebacterium glutamicum DSM 5715.

Contrary to the prior art (EP-A-1038969) it has been found that coryneform bacteria produce L-amino acids in an improved manner after attenuation of the mqo gene.

The nucleotide sequence of the mqo gene of Corynebacterium glutamicum has been published by Molenar *et al.* (European Journal of Biochemistry 254, 395-403 (1998)) and can also be taken from the gene library under Accession Number AJ 22 4946. The nucleotide sequence is also shown in SEQ ID No. 1 and the amino acid sequence of the protein is shown in SEQ ID No. 2.

The sequences described in the mentioned references coding for malate quinone oxidoreductase can be used according to the invention. It is also possible to use alleles of malate quinone oxidoreductase, which are formed from the degeneracy of the genetic code or by sense mutations that are neutral in terms of function.

In order to achieve attenuation, either the expression of the mqo gene or the catalytic properties of the gene products can be diminished or excluded. The two measures are optionally combined.

Gene expression can be diminished by carrying out the culturing in a suitable manner or by genetic alteration (mutation) of the signal structures of gene expression. Signal structures of gene expression are, for example, repressor genes, activator genes, operators, promoters, attenuators, ribosome-binding sites, the start codon and terminators. The person skilled in the art will find information thereon, for example, in patent application WO 96/15246, in Boyd and Murphy (Journal of Bacteriology 170: 5949 (1988)), in Voskuil and Chambliss (Nucleic Acids Research 26: 3548 (1998), in Jensen and Hammer (Biotechnology and Bioengineering 58: 191 (1998)), in Pátek *et al.* (Microbiology 142: 1297 (1996)), and in known textbooks of genetics and molecular biology, such as, for example, the textbook of Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or that of Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990).

Mutations that lead to a change in or diminution of the catalytic properties of enzyme proteins are known from the prior art; examples which may be mentioned are the works of Qiu and Goodman (Journal of Biological Chemistry 272: 8611-8617 (1997)), Sugimoto *et al.* (Bioscience Biotechnology and Biochemistry 61: 1760-1762 (1997)) and Möckel ("Die Threonindehydratase aus Corynebacterium glutamicum: Aufhebung der allosterischen Regulation und Struktur des Enzyms", Berichte des Forschungszentrums Jülichs,Jül-2906, ISSN09442952, Jülich, Germany, 1994). Summaries are to be found in known textbooks of genetics and molecular biology, such as, for example, that of Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

There come into consideration as mutations transitions, transversions, insertions and deletions. In dependence on the effect of the amino acid substitution on the enzyme activity, missense mutations or nonsense mutations are referred to. Insertions or deletions of at least one base pair in a gene lead to frame shift mutations, as a result of which incorrect amino acids are incorporated or the translation breaks off prematurely. If a stop codon forms in the coding region as the result of a mutation, that likewise generally leads to premature breaking off of the translation.

Deletions of several codons typically lead to complete loss of enzyme activity. Instructions for the production of such mutations are part of the prior art and can be found in known textbooks of genetics and molecular biology, such as, for example, the textbook of Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that of Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that of Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

The invention provides the allele 672, shown in SEQ ID No. 3, of the mqo gene, which allele carries the nucleotide adenine instead of the nucleotide guanine at position 672 of the DNA sequence (see SEQ ID No. 1), which leads to substitution of the TGG codon coding for the amino acid tryptophan-224 (see SEQ ID No. 2) by an opal (TGA) stop codon.

The invention also provides the allele 1230, shown in SEQ ID No. 4, of the mqo gene, which allele carries the nucleotide adenine instead of the nucleotide guanine at position 672 of the DNA sequence (see SEQ ID No. 1), which leads to substitution of the tgg codon coding for the amino acid tryptophan-224 (see SEQ ID No. 2) by an opal stop codon and which additionally carries a nucleotide substitution at position 1230 of cytosine to thymine.

A common method of mutating genes of C. glutamicum is the method of gene disruption and of gene replacement described by Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)).

In the method of gene disruption, a central portion of the coding region of the gene in question is cloned into a plasmid vector which is able to replicate in a host (typically E. coli), but not in C. glutamicum. Suitable vectors are, for example, pSUP301 (Simon *et al.*, Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer *et al.*, Gene 145, 69-73 (1994)), pK18mobsacB or pK19mobsacB (Jäger *et al.*, Journal of Bacteriology 174: 5462-5465 (1992)), pGEM-T (Promega corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-32684; US Patent 5,487,993), pCR®Blunt (Invitrogen, Groningen, Netherlands; Bernard *et al.*, Journal of Molecular Biology, 234: 534-541 (1993)) or pEM1 (Schrumpf *et al.*, 1991, Journal of Bacteriology 173:4510-4516). The plasmid vector containing the central portion of the coding region of the gene is then transferred to the desired strain of C. glutamicum by

conjugation or transformation. The method of conjugation is described, for example, in Schäfer *et al.* (Applied and Environmental Microbiology 60, 756-759 (1994)). Methods of transformation are described, for example, in Thierbach *et al.* (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch *et al.* (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a cross-over occurrence, the coding region of the gene in question is disrupted by the vector sequence, and two incomplete alleles lacking the 3'- and the 5'-end, respectively, are obtained. That method has been used, for example, by Fitzpatrick *et al.* (Applied Microbiology and Biotechnology 42, 575-580 (1994)) to exclude the recA gene of C. glutamicum.

In the gene replacement method, a mutation, such as, for example, a deletion, insertion or base substitution, is produced *in vitro* in the gene in question. The allele that is produced is in turn cloned into a vector that is not replicative for C. glutamicum, and the latter is then transferred to the desired host of C. glutamicum by transformation or conjugation. After homologous recombination by means of a first cross-over occurrence effecting integration and by means of a suitable second cross-over occurrence effecting an excision in the target gene or in the target sequence, incorporation of the mutation or of the allele is achieved.

That method has been used, for example, by Peters-Wendisch *et al.* (Microbiology 144, 915-927 (1998)) to exclude the pyc gene of C. glutamicum by means of a deletion. That method has been used by Schäfer *et al.* (Gene 145: 69-73 (1994)), for example, in order to incorporate a deletion into the hom-thrB gene region. In the same way, a deletion has been introduced into the cgl gene region of C.

glutamicum by Schäfer *et al.* (Journal of Bacteriology 176: 7309-7319 (1994)).

A deletion, insertion or a base substitution can thus be incorporated into the mqo gene.

In addition, it may be advantageous for the production of L-amino acids, in addition to attenuating the mqo gene, to enhance, especially to overexpress, one or more enzymes of the biosynthesis pathway in question, of glycolysis, of the anaplerotic pathway, of the citric acid cycle, of the pentose phosphate cycle, of amino acid export, and optionally regulatory proteins.

The term "enhancement" or "enhance" in this context describes an increase in the intracellular activity of one or more enzymes or proteins in a microorganism that are coded for by the corresponding DNA, by, for example, increasing the number of copies of the gene or genes, using a strong promoter or a gene or allele that codes for a corresponding enzyme or protein having a high level of activity, and optionally by combining those measures.

Accordingly, for the production of L-lysine, in addition to attenuating the mqo gene, one or more genes selected from the group

- the gene lysC coding for a feed-back resistant aspartate kinase (Accession No. P26512, EP-B-0387527; EP-A-0699759; WO 00/63388),
- the gene dapA coding for dihydrodipicolinate synthase (EP-B 0 197 335),
- the gene gap coding for glyceraldehyde-3-phosphate dehydrogenase (Eikmanns (1992). Journal of Bacteriology 174:6076-6086),

- at the same time the gene pyc coding for pyruvate carboxylase (DE-A-198 31 609),
- the gene zwf coding for glucose-6-phosphate dehydrogenase (JP-A-09224661),
- at the same time the gene lysE coding for lysine export (DE-A-195 48 222),
- the gene zwa1 coding for the Zwa1 protein (DE: 19959328.0, DSM 13115)
- the gene tpi coding for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086), and
- the gene pgk coding for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),

can be enhanced, especially overexpressed.

It may also be advantageous for the production of amino acids, especially L-lysine, in addition to attenuating the mqo gene, at the same time to attenuate, especially to diminish the expression of, one or more genes selected from the group

- the gene pck coding for phosphoenol pyruvate carboxy-kinase (DE 199 50 409.1, DSM 13047),
- the gene pgi coding for glucose-6-phosphate isomerase (US 09/396,478, DSM 12969),
- the gene poxB coding for pyruvate oxidase (DE:1995 1975.7, DSM 13114),
- the gene zwa2 coding for the Zwa2 protein (DE: 19959327.2, DSM 13113).

Finally, it may be advantageous for the production of amino acids, in addition to attenuating the mqo gene, to exclude

undesired secondary reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms produced according to the invention also form part of the invention and can be cultivated, for the purposes of the production of L-amino acids, continuously or discontinuously by the batch process or by the fed batch or repeated fed batch process. A summary of known cultivation methods is described in the textbook of Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook of Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/ Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the strains in question in a suitable manner. Descriptions of culture media for various microorganisms are to be found in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

There may be used as the carbon source sugars and carbohydrates, such as, for example, glucose, saccharose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as, for example, soybean oil, sunflower oil, groundnut oil and coconut oil, fatty acids, such as, for example, palmitic acid, stearic acid and linoleic acid, alcohols, such as, for example, glycerol and ethanol, and organic acids, such as, for example, acetic acid. Those substances may be used individually or in the form of a mixture.

There may be used as the nitrogen source organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soybean flour and

urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources may be used individually or in the form of a mixture.

There may be used as the phosphorus source phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts. The culture medium must also contain salts of metals, such as, for example, magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, may be used in addition to the above-mentioned substances. Suitable precursors may also be added to the culture medium. The mentioned substances may be added to the culture in the form of a single batch, or they may be fed in in a suitable manner during the cultivation.

In order to control the pH value of the culture, basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or ammonia water, or acid compounds, such as phosphoric acid or sulfuric acid, are expediently used. In order to control the development of foam, anti-foams, such as, for example, fatty acid polyglycol esters, may be used. In order to maintain the stability of plasmids, suitable substances having a selective action, such as, for example, antibiotics, may be added to the medium. In order to maintain aerobic conditions, oxygen or gas mixtures containing oxygen, such as, for example, air, are introduced into the culture. The temperature of the culture is normally from 20°C to 45°C and preferably from 25°C to 40°C. The culture is continued until the maximum amount of the desired product has formed. That aim is normally achieved within a period of from 10 hours to 160 hours.

Methods of determining L-amino acids are known from the prior art. The analysis may be carried out as described in Spackman *et al.* (Analytical Chemistry, 30, (1958), 1190) by

anion-exchange chromatography with subsequent ninhydrin derivatization, or it may be carried out by reversed phase HPLC, as described in Lindroth *et al.* (Analytical Chemistry (1979) 51: 1167-1174).

The present invention is explained in more detail in the following using working examples.

Example 1

Preparation of the expression vector pXK99Emobmqo for IPTG-induced expression of the mqo gene in C. glutamicum

1.1 Cloning of the mqo gene

From the strain ATCC 13032, chromosomal DNA was isolated by the method of Eikmanns et al. (Microbiology 140: 1817-1828 (1994)). On the basis of the sequence of the mqo gene known for C. glutamicum, the following oligonucleotides were chosen for the polymerase chain reaction (see SEQ ID No. 5 and SEQ ID No. 6):

mqo_oP1:
5`- GA GGA TCC GCA GAG AAC TCG CGG AGA TA-3`

mqo_hind:
5`- CT AAG CTT CGT AGC GAG CCT TGA TGT AT-3`

The primers were chosen here so that the amplified fragment contains the incomplete gene, starting with the native ribosome binding site without the promoter region, and the front region of the mqo gene. Furthermore, the primer mqo_oP1 contains the sequence for the cleavage site of the restriction endonuclease BamHI, and the primer mqo_hind the cleavage site of the restriction endonuclease HindIII, which are marked by underlining in the nucleotide sequence shown above.

The primers shown were synthesized by MWG-Biotech AG (Ebersberg, Germany) and the PCR reaction was carried out

by the standard PCR method of Innis et al. (PCR protocols. A guide to methods and applications, 1990, Academic Press) with Pwo-Polymerase from Roche Diagnostics GmbH (Mannheim, Germany). With the aid of the polymerase chain reaction, the primers allow amplification of a DNA fragment 468 bp in size, which carries the incomplete mqo gene, including the native ribosome binding site.

The mqo fragment 468 bp in size was cleaved with the restriction endonucleases BamHI and HindIII and then isolated from the agarose gel with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

1.2 Construction of the expression vector pXK99Emob

The IPTG-inducible expression vector pXK99Emob was constructed according to the prior art. The vector is based on the Escherichia coli expression vector pTRC99A (Amann et al., Gene 69: 301-315 (1988)) and contains the trc promoter, which can be induced by addition of the lactose derivative IPTG (isopropyl $\beta$-D-thiogalactopyranoside), the termination regions T1 and T2, the replication origin ColE1 from E. Coli, the $lacI^q$ gene (repressor of the lac operon from E.coli), a multiple cloning site (mcs) (Norrander, J.M. et al. Gene 26, 101-106 (1983)), the kanamycin resistance gene aph(3´)-IIa from E. coli (Beck et al. (1982), Gene 19: 327-336) and the RP4-mobilization-site from the cloning vector pK18mobsacB (Schaefer et al, Gene 14: 69-73 (1994).

It has been found that the vector pXK99Emob is quite specifically suitable for regulating the expression of a gene, in particular effecting attenuated expression in coryneform bacteria. The vector pXK99Emob is an E. coli expression vector and can be employed in E. coli for enhanced expression of a gene.

Since the vector cannot replicate independently in coryneform bacteria, this is retained in the cell only if it is integrated into the chromosome. The peculiarity of this vector here is the use for regulated expression of a gene after cloning of a gene section from the front region of the corresponding gene in the vector containing the start codon and the native ribosome binding site, and subsequent integration of the vector into coryneform bacteria, in particular C. glutamicum. Gene expression is regulated by addition of metered amounts of IPTG to the nutrient medium. Amounts of 0.5 µM up to 10 µM IPTG have the effect of very weak expression of the corresponding gene, and amounts of 10 µM up to 100 µM have the effect of a slightly attenuated to normal expression of the corresponding gene.

The E. coli expression vector pXK99Emob constructed was transferred by means of electroporation (Tauch et al. 1994, FEMS Microbiol Letters, 123: 343-347) into E. coli DH5αmcr (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649). Selection of the transformants was carried out on LB Agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), which had been supplemented with 50 mg/l kanamycin.

Plasmid DNA was isolated from a transformant by conventional methods (Peters-Wendisch et al., 1998, Microbiology, 144, 915 - 927), cleaved with the restriction endonuclease NcoI, and the plasmid was checked by subsequent agarose gel electrophoresis.

The plasmid construct obtained in this way was called pXK99Emob (Figure 1). The strain obtained by electroporation of the plasmid pXK99Emob in the E. coli strain DH5αmcr was called E. coli DH5alphamcr/pXK99Emob.

1.3 Cloning of the mqo fragment in the E. coli expression vector pXK99Emob

The E. coli expression vector pXK99Emob described in Example 1.2 was used as the vector. DNA of this plasmid was cleaved completely with the restriction enzymes BamHI and HindIII and then dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250).

The mqo fragment approx. 458 bp in size described in 1.1, obtained by means of PCR and cleaved with the restriction endonucleases BamHI and HindIII was mixed with the prepared vector pXK99Emob and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation batch was transformed in the E. coli strain DH5αmcr (Hanahan, In: DNA cloning. A Practical Approach. Vol. I, IRL-Press, Oxford, Washington DC, USA). Selection of plasmid-carrying cells was made by plating out the transformation batch on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l kanamycin. After incubation overnight at 37°C, recombinant individual clones were selected. Plasmid DNA was isolated from a transformant with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and cleaved with the restriction enzymes BamHI and HindIII to check the plasmid by subsequent agarose gel electrophoresis. The resulting plasmid was called pXK99Emobmqo. It is shown in Figure 2.

The following microorganism was deposited as a pure culture on 15th February 2002 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty:

- Escherichia coli DH5alphamcr /pXK99Emobmqo (= DH5αmcr/

pXK99Emobmqo) as DSM 14815.

Example 2

Integration of the vector pXK99Emobmqo into the genome of the C. glutamicum strain DSM5715

The vector pXK99Emobmqo mentioned in Example 1 was electroporated by the electroporation method of Tauch et al.,(1989 FEMS Microbiology Letters 123: 343-347) in the strain C. glutamicum DSM5715. The vector cannot replicate independently in DSM5715 and is retained in the cell only if it has integrated into the chromosome. Selection of clones with integrated pXK99Emobmqo was carried out by plating out the electroporation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which had been supplemented with 15 mg/l kanamycin and IPTG (1mM).

A selected kanamycin-resistant clone which has the Plasmid pXK99Emobmqo, mentioned in Example 1, inserted in the chromosomal mqo-gene of DSM5715, was called DSM5715::pXK99Emobmqo.

Example 3

Preparation of lysine

The C. glutamicum strain DSM5715::pXK99Emobmqo obtained in Example 2 was cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant was determined. By addition of IPTG, attenuated expression of the mqo gene occurs, regulated by the trc promoter.

For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with kanamycin (25 mg/l) and IPTG (10 µM)) for 24 hours at 33°C. Starting from this agar plate culture, a preculture was

seeded (10 ml medium in a 100 ml conical flask). The complete medium Cg III was used as the medium for the preculture.

Medium Cg III

| | |
|---|---|
| NaCl | 2.5 g/l |
| Bacto-Peptone | 10 g/l |
| Bacto-Yeast extract | 10 g/l |
| Glucose (autoclaved separately) | 2% (w/v) |

The pH was brought to pH 7.4

Kanamycin (25 mg/l) and IPTG (10 µM) were added to this. The preculture was incubated for 16 hours at 33°C at 240 rpm on a shaking machine. The OD (660 nm) of the preculture was 0.5. 500 µl of this preculture were transinoculated into a main culture. By transfer of IPTG-containing medium from the preculture, the IPTG concentration in the main culture was approx. 0.5 µM. Medium MM was used for the main culture.

Medium MM

| | |
|---|---|
| CSL (corn steep liquor) | 5 g/l |
| MOPS (morpholinopropanesulfonic acid) | 20 g/l |
| Glucose (autoclaved separately) | 50 g/l |
| Salts: | |
| $(NH_4)_2SO_4$ | 25 g/l |
| $KH_2PO_4$ | 0.1 g/l |
| $MgSO_4 * 7 H_2O$ | 1.0 g/l |
| $CaCl_2 * 2 H_2O$ | 10 mg/l |
| $FeSO_4 * 7 H_2O$ | 10 mg/l |
| $MnSO_4 * H_2O$ | 5.0 mg/l |
| Biotin (sterile-filtered) | 0.3 mg/l |
| Thiamine * HCl (sterile-filtered) | 0.2 mg/l |
| Leucine (sterile-filtered) | 0.1 g/l |
| $CaCO_3$ | 25 g/l |

The CSL, MOPS and the salt solution are brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions are then added, and the $CaCO_3$ autoclaved in the dry state is added.

Culturing was carried out in a 10 ml volume in a 100 ml conical flask with baffles. Kanamycin (25 mg/l) was added. Culturing was carried out at 33ºC and 80% atmospheric humidity.

After 72 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann

Instruments GmbH, Munich). The amount of lysine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivation with ninhydrin detection.

The result of the experiment is shown in Table 1.

Table 1

| Strain | OD (660 nm) | Lysine HCl g/l |
|---|---|---|
| DSM5715 | 6,8 | 12,82 |
| DSM5715::pXK99Emobmqo | 6,4 | 14,85 |

Brief Description of the Figures:

Figure 1: Map of the plasmid pXK99Emob,

Figure 2: Map of the plasmid pXK99Emobmqo.

The abbreviations and designations used have the following meaning.

Kan: Kanamycin resistance gene aph(3')-IIa from Escherichia coli

BamHI Cleavage site of the restriction enzyme BamHI

HindIII Cleavage site of the restriction enzyme HindIII

NcoI Cleavage site of the restriction enzyme NcoI

| | |
|---|---|
| Ptrc | trc promoter |
| T1 | Termination region T1 |
| T2 | Termination region T2 |
| lacIq | lacIq repressor of the lac operon of Escherichia coli |
| oriV | Replication origin ColE1 from E. coli |
| mob | RP4-mobilization site |
| mqo | Cloned region of the mqo gene |

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

INTERNATIONAL FORM

Degussa AG
Kantstr. 2
33790 Halle

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

| I. DEPOSITOR | II. IDENTIFICATION OF THE MICROORGANISM |
|---|---|
| Name: Degussa AG<br>Kantstr. 2<br>Address: 33790 Halle | Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:<br>DSM 14815<br>Date of the deposit or the transfer[1]:<br>2002-02-15 |

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 2002-02-15 [2].
On that date, the said microorganism was

( )[3] viable

( )[3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

| Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH<br>Address: Mascheroder Weg 1b<br>D-38124 Braunschweig | Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):<br>V. Weiß<br>Date: 2002-02-18 |
|---|---|

1 Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
2 In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
3 Mark with a cross the applicable box.
4 Fill in if the information has been requested and if the results of the test were negative.

Form DSMZ-BP/9 (sole page) 12/2001

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

INTERNATIONAL FORM

Degussa AG
Kantstr. 2
33790 Halle

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

| I. IDENTIFICATION OF THE MICROORGANISM | |
|---|---|
| Identification reference given by the DEPOSITOR:<br>DH5alphamcr/<br>pXK99Emobmqo | Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:<br>DSM 14815 |
| II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION | |
| The microorganism identified under I. above was accompanied by:<br>( x ) a scientific description<br>( x ) a proposed taxonomic designation<br>(Mark with a cross where applicable). | |
| III. RECEIPT AND ACCEPTANCE | |
| This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2002-02-15 (Date of the original deposit)[1]. | |
| IV. RECEIPT OF REQUEST FOR CONVERSION | |
| The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion). | |
| V. INTERNATIONAL DEPOSITARY AUTHORITY | |
| Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH<br>Address: Mascheroder Weg 1b<br>D-38124 Braunschweig | Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):<br>U. Weiss<br>Date: 2002-02-18 |

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

Form DSMZ-BP/4 (sole page) 12/2001

**What is claimed is:**

1. Process for the production of L-amino acids, especially L-lysine, by fermentation, which method comprises carrying out the following steps:

   a) fermentation of the coryneform bacteria producing the desired L-amino acid, in which at least the mqo gene is attenuated,

   b) concentration of the desired product in the medium or in the cells of the bacteria, and

   c) isolation of the desired L-amino acids, in which optionally portions or the entirety of the constituents of the fermentation liquor and/or of the biomass remain in the end product.

2. Process according to claim 1, wherein there are used bacteria in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally enhanced.

3. Process according to claim 1, wherein there are used bacteria in which at least some of the metabolic pathways reducing the formation of the desired L-amino acid are excluded.

4. Process according to claim 1, wherein expression of the polynucleotide(s) coding for the mqo gene is diminished.

5. Process according to claim 1, wherein the catalytic properties of the polypeptide (enzyme protein) for which the polynucleotide mqo codes are diminished.

6. Process according to claim 1, wherein, for the production of L-lysine, there are fermented coryneform microorganisms in which at the same time one or more genes selected from the group

6.1 the gene lysC coding for a feed-back resistant aspartate kinase,

6.2 the gene dapA coding for dihydrodipicolinate synthase,

6.3 the gene gap coding for glyceraldehyde-3-phosphate dehydrogenase,

6.4 the gene pyc coding for pyruvate carboxylase,

6.5 the gene zwf coding for glucose-6-phosphate dehydrogenase,

6.6 at the same time the gene lysE coding for lysine export,

6.7 the gene zwa1 coding for the Zwa1 protein,

6.8 the gene tpi coding for triose phosphate isomerase, and

6.9 the gene pgk coding for 3-phosphoglycerate kinase

are enhanced, especially overexpressed.

7. Process according to claim 1, wherein, for the production of L-amino acids, there are fermented coryneform microorganisms in which at the same time one or more genes selected from the group

7.1 the pck gene coding for phosphoenol pyruvate carboxykinase,

7.2 the pgi gene coding for glucose-6-phosphate isomerase,

7.3 the gene poxB coding for pyruvate oxidase, or

7.4 the gene zwa2 coding for the Zwa2 protein

are attenuated.

8. Process according to one or more of the preceding claims, wherein microorganisms of the species Corynebacterium glutamicum are used.

9. Coryneform bacteria in which at least the mqo gene is present in attenuated form.

10. Coryneform bacteria according to claim 9, wherein the attenuation is effected by means of a deletion, inversion, transition or transversion mutation.

11. Coryneform bacteria according to claim 9 or 10, wherein the mqo gene contains a stop codon.

12. Coryneform bacteria according to claim 11, wherein the stop codon, especially of the opal type, is located according to position 224 of the amino acid sequence of SEQ ID No. 2.

13. Coryneform bacteria according to one or more of claims 9 to 12, containing the mqo allele according to SEQ ID No. 3.

14. Coryneform bacteria according to one or more of claims 9 to 12, containing the mqo allele according to SEQ ID No. 4.

15. Escherichia coli strain DH5alphamcr /pXK99Emobmqo (= DH5αmcr/ pXK99Emobmqo) deposited as DSM 14815 at the German Collection for Microorganisms and Cell Cultures (DSMZ, Brunswick, Germany).

Figure 1: pXK99Emob

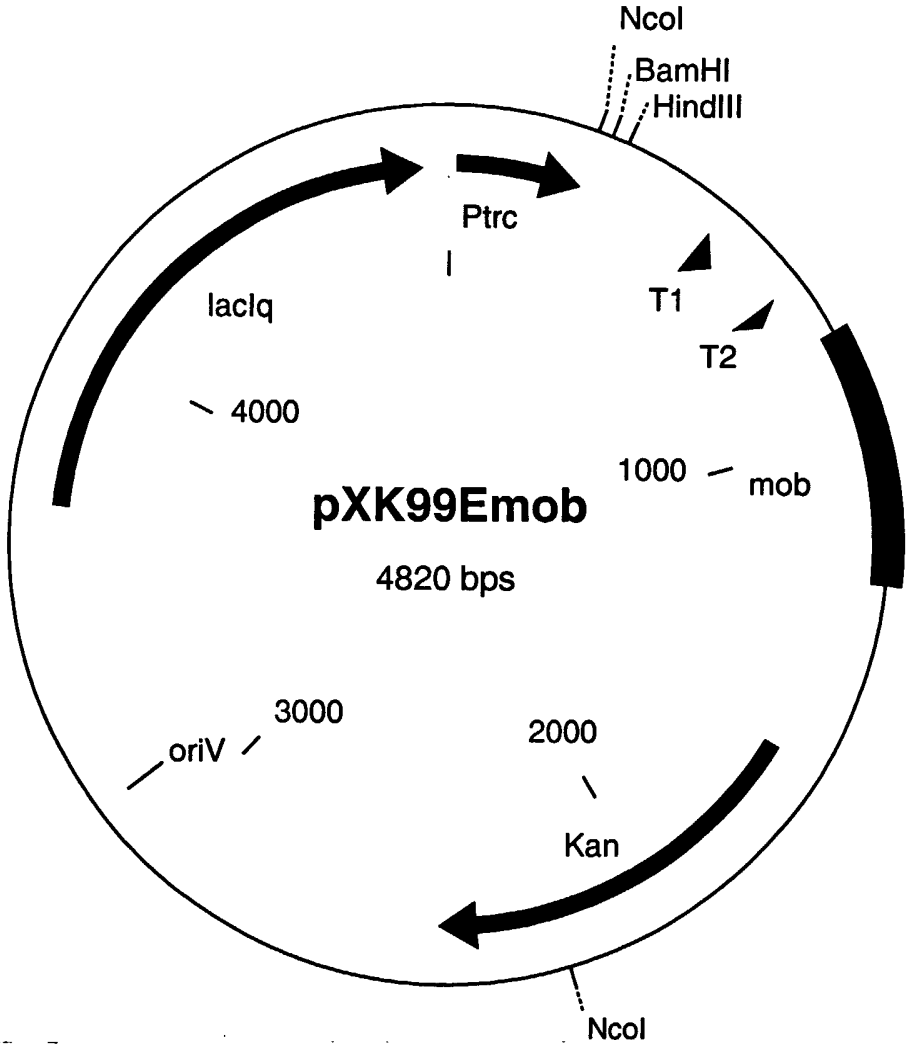
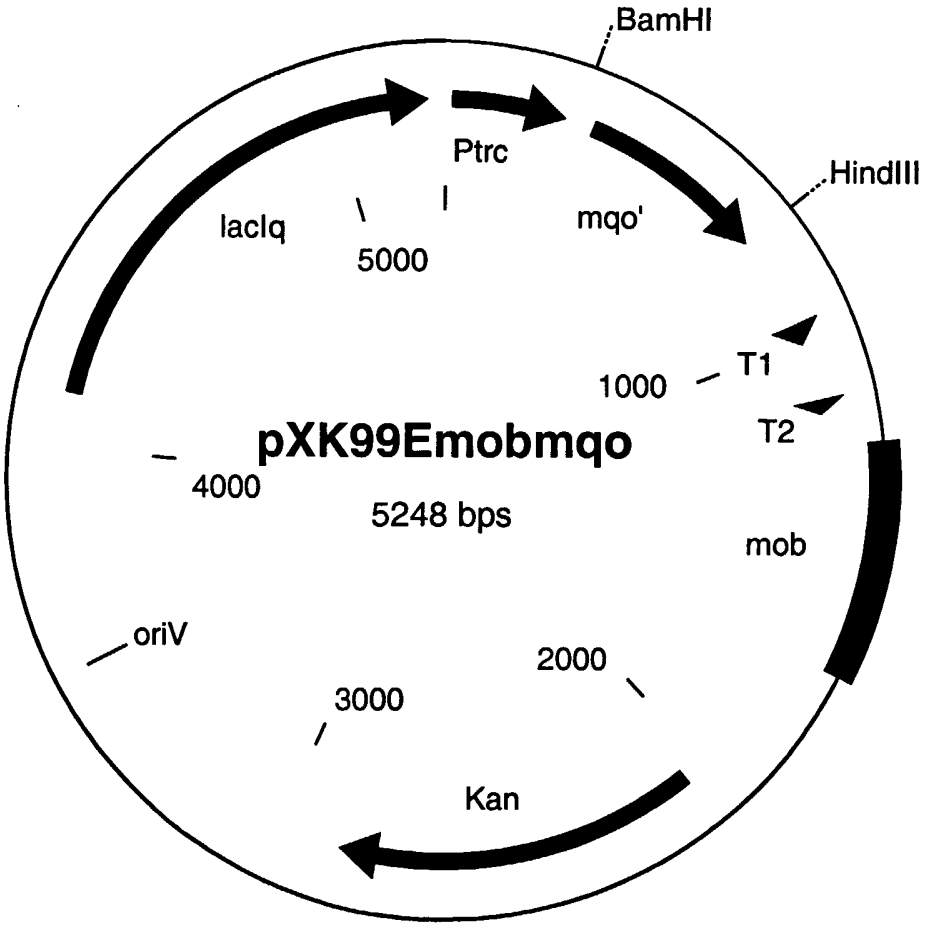


Figure 2: pXK99Emobmqo



SEQUENCE LISTING

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Val Val Leu Ile Gly Ala Gly Ile Met Ser Ser Thr Leu Gly Ala Met
            20                  25                  30

ctg cgt cag ctg gag cca agc tgg act cag atc gtc ttc gag cgt ttg      144
Leu Arg Gln Leu Glu Pro Ser Trp Thr Gln Ile Val Phe Glu Arg Leu
        35                  40                  45

gat gga ccg gca caa gag tcg tcc tcc ccg tgg aac aat gca gga acc      192
Asp Gly Pro Ala Gln Glu Ser Ser Ser Pro Trp Asn Asn Ala Gly Thr
    50                  55                  60

ggc cac tct gct cta tgc gag ctg aac tac acc cca gag gtt aag ggc      240
Gly His Ser Ala Leu Cys Glu Leu Asn Tyr Thr Pro Glu Val Lys Gly
65                  70                  75                  80

aag gtt gaa att gcc aag gct gta gga atc aac gag aag ttc cag gtt      288
Lys Val Glu Ile Ala Lys Ala Val Gly Ile Asn Glu Lys Phe Gln Val
                85                  90                  95

tcc cgt cag ttc tgg tct cac ctc gtt gaa gag gga gtg ctg tct gat      336
Ser Arg Gln Phe Trp Ser His Leu Val Glu Glu Gly Val Leu Ser Asp
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cct aag gaa ttc atc aac cct gtt cct cac gta tct ttc ggc cag ggc      384
Pro Lys Glu Phe Ile Asn Pro Val Pro His Val Ser Phe Gly Gln Gly
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gca gat cag gtt gca tac atc aag gct cgc tac gaa gct ttg aag gat      432
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cac cca ctc ttc cag ggc atg acc tac gct gac gat gaa gct acc ttc      480
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Val Ala Ile Ser Trp Ile Asp Glu Gly Thr Asp Ile Asn Tyr Gly Ala
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cag acc aag cag tac ctg gat gca gct gaa gtt gaa ggc act gaa atc      624
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Ile Val Thr Val Lys Asn Val His Thr Gly Asp Thr Lys Thr Ile Lys
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            260                 265                 270

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Val Pro His Leu Asp Thr Arg Val Ile Glu Gly Glu Lys Gly Leu Leu
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ttt gga cct tac ggt ggc tgg acc cct aag ttc ttg aag gaa ggc tcc     1008
Phe Gly Pro Tyr Gly Gly Trp Thr Pro Lys Phe Leu Lys Glu Gly Ser
                325                 330                 335

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Tyr Leu Asp Leu Phe Lys Ser Ile Arg Pro Asp Asn Ile Pro Ser Tyr
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gaa gtt ctc aag gac cag gac aag cgt atg gat gct ctt cgc gag tac     1152
Glu Val Leu Lys Asp Gln Asp Lys Arg Met Asp Ala Leu Arg Glu Tyr
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Met Pro Glu Ala Gln Asn Gly Asp Trp Glu Thr Ile Val Ala Gly Gln
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