

L-Lysine Fermentation

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Abstract: Amino acids are the basic bioelements of proteins, which are the most important macromolecules for the functions of humans and animals. Out of the 20 L-amino acids, ecumenically found in most of living organisms, L-lysine is one of the 9 amino acids which are essential for human and animal nutrition. L-lysine is useful as medicament, chemical agent, food material (food industry) and feed additive (animal food). Its demand has been steadily increasing in recent years and several hundred thousands tones of L-lysine (about 800,000 tones/year) are annually produced worldwide almost by microbial fermentation.

The stereospecificity of amino acids (the L isomer) makes the fermentation advantageous compared with synthetic processes. Mutant auxotrophic or resistant to certain chemicals strains of so-called gram positive coryneform bacteria are generally used, including the genera *Brevibacterium* and *Corynebacterium*, united to the genus.

The significance of Research and Development increased rapidly since the discovery of fermentative amino acid production in the fifties (S. Kinoshita *et al.*, Proceedings of the International Symposium on Enzyme Chemistry 2:464-468 (1957)), leading to innovative fermentation processes which replaced the classical manufacturing methods of L-lysine like acid hydrolysis. L-Lysine is separated and purified by suitable downstream processes involving classical separation or extraction methods (ultrafiltration or centrifugation, separation or ion exchange extraction, crystallization, drying) and is sold as a powder. Alternatively, spray dried pellets or liquid fermentation broth can be used as animal feed supplement. On behalf of today's strong competition in amino acid industry, Biotechnology companies are continuously aiming in innovative research developments and use complex management concepts and business strategies, towards gaining market leadership in the field of amino acid production.

Keywords: L-Lysine production, amino acids, corynebacteria, strain development, mutagenesis, fermentation, downstream process, ion exchange chromatography, recombinant DNA, feed additive, regulation.

INTRODUCTION

Out of the twenty naturally occurring amino acids, L-Lysine ($C_6H_{14}N_2O_2$; MW 146.19) is one of the 9 essential (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) and commercially important amino acids, ecumenically found in naturally occurring proteins of all living organisms. Its major commercial form is L-Lysine-HCl (L-Lysine monohydrochloride) [1-7]. L-lysine is commonly produced in a stable and non-hygroscopic hydrochlorinated form ($H_2N(CH_2)_4CHNH_2CO_2H.HCl \cdot 2H_2O$) of a purity higher than 98.5% and moisture content less than 1% [8].

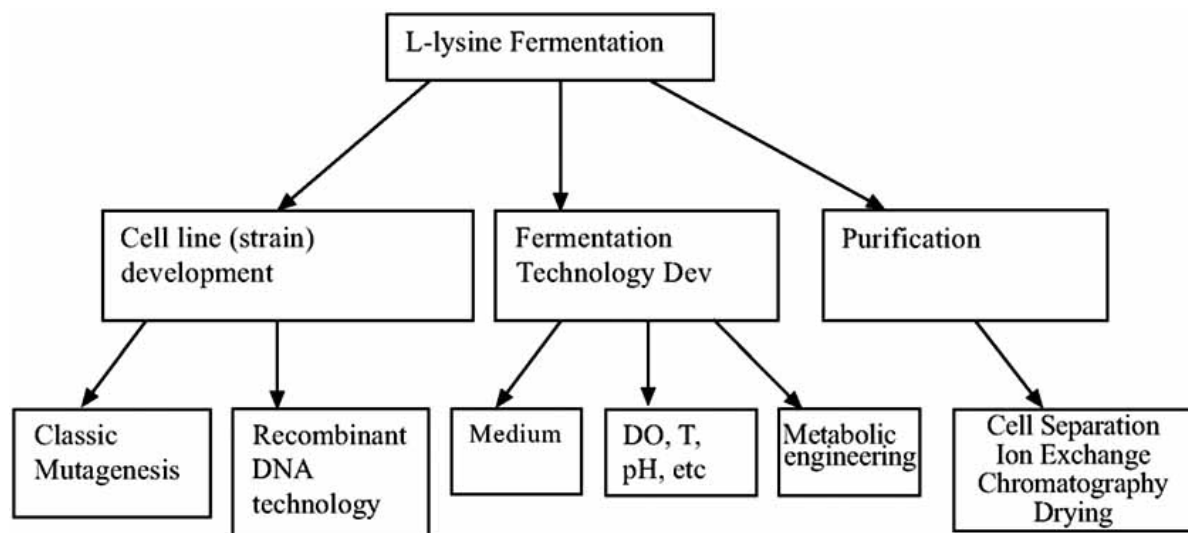
It is mainly used as a feed additive in the animal feed industry, mixed with various common livestock such as cereals which do not contain sufficient levels of L-lysine for the livestock's nutritional requirements, in especially for single-stomach (monogastric) animals like broilers, poultry and swine [6,7,9-12] and as a supplement for humans, improving the feed quality by increasing the absorption of other amino acids. US4327118 [13] describes a preparative method for long lasting solid lysine compositions, suitable for animal feed supplements, which don't agglomerate in the presence of moisture and can for time not necessitating the

use of expensive purified L-lysine. As a fine chemical, it is utilized in human medicine, in cosmetics and in the pharmaceutical industry, particularly as ingredients of infusion solutions for pharmaceutical applications [6,14]; and as precursor for industrial chemicals. Furthermore, a production method for industrially producing an optically active lysine derivative useful as a pharmaceutical intermediate is described in [15].

L-lysine can be produced either by a chemical or a biochemical method, which is more economic, even though relatively low yields are obtained during the extraction of L-lysine, requiring specific installations and the use of expensive products [13]. The stereospecificity of amino acids and the steadily increasing L-lysine demand necessitates indispensably their fermentative production (the L isomer) over synthetic processes [6,16]. Thus, L-lysine-producing strains of the gram positive corynebacteria, especially *Corynebacterium glutamicum*, *Brevibacterium flavum* and *Brevibacterium lactofermentum*, have been used for the last fifty years for the industrial production of amino acids.

Several hundred thousands tones of L-lysine (800,000 tones/year) are presumably produced annually worldwide, almost exclusively using bacterial fermentations. US 6984512 and WO 2005/059139 [6,16] refer to an annual L-lysine production of approximately 250,000 tons, instead. Thus, a variety of technologies for increasing the produc-

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tivity of amino acids are disclosed in the international patent bibliography.

L-LYSINE PRODUCTION

L-Lysine is mainly produced by fermentation using strains of corynebacteria, especially *Corynebacterium glutamicum*, which comprises a multi-step process including fermentation, cell separation by centrifugation or ultrafiltration, product separation and purification, evaporation and drying. Because of L-Lysine's great importance, efforts are constantly being made in order to improve the fermentation processes, comprising strain (e.g. increase of the microorganism's intrinsic productivity characteristics by classical mutagenesis and genetic engineering) and process development (e.g. effect of stirring and oxygen supply, temperature, pH and CO₂) as well as media optimization (e.g. influence of initial and operational sugar concentration, important nutrients and additives) and downstream processing (e.g. ion exchange chromatography) (General information; [17,18]. Batch, fed batch, repeated batch and repeated fed batch (feeding during cultivation) are used for the production of L-lysine and other L-amino acids, operating in mixing tank or air lift fermenters [11,14,19,20].

A continuous process may serve as an alternative application for the industrial production L-lysine. However, only a few successful attempts can be found in the international bibliography regarding continuous fermentation of L-lysine, because of technical difficulties and microbiological instability problems. The development of continuous fermentation processes for the production of citric acid and gluconic acid [21,22] as well as L-lysine (unpublished data received by Anastassiadis and Stephanopoulos, 1994-1996, MIT) have shown numerous advantages over the traditional discontinuous processes of the last 100 years. Thus, a continuous production of L-lysine also including the repeated fed batch mode seems to be very promising for future applications.

The development of a new multi-step biotechnological process requires three steps, comprising:

- Identification and characterization of a suitable biological system (microorganism, biocatalyst)
- Increase of bioreactor productivity by systematic media optimization and adaptation of fermentation technology to a developing process (process development and fermentation technology) (Fig. 1)
- Downstream process (cell separation by centrifugation or ultrafiltration, separation, evaporation and drying)

MICROORGANISMS AND STRAIN DEVELOPMENT

The fermentative production of amino acids started in the fifties (1956-57) with the discovery of glutamic acid producing bacteria by Japanese scientists. Since then, most of developmental work was devoted to the improvement of mutant strains involving classical and modern microbial genetics, while efficiency of commercial L-lysine production has steadily increased by the isolation of highly producing mutant strains.

Microorganisms employed in microbial amino acid production are divided into 4 classes, including the wild-type, auxotrophic mutant, regulatory mutant and auxotrophic regulatory mutant strains [6]. Mutant auxotrophic or resistant to certain chemicals strains of so called L-glutamic acid producing *Corynebacterium* or *Brevibacterium* sp. are employed for L-lysine production.

Process improvement for producing larger amounts of L-lysine using microorganisms remains a continual attempt [23], whereas the continuous development of classical and modern genetics resulted in the development of superior strains imparted with properties advantageous for the commercial production of L-lysine. Selected mutant strains with improved productivity characteristics are used today in industry for the production of L-lysine, which are obtained by various methods including classical mutagenesis, plasma fusion, genetic engineering and any other techniques commonly used for mutation of microorganisms. L-lysine yields (g product/g added carbon source x 100) between 40 and 50% are disclosed [9,10,24].

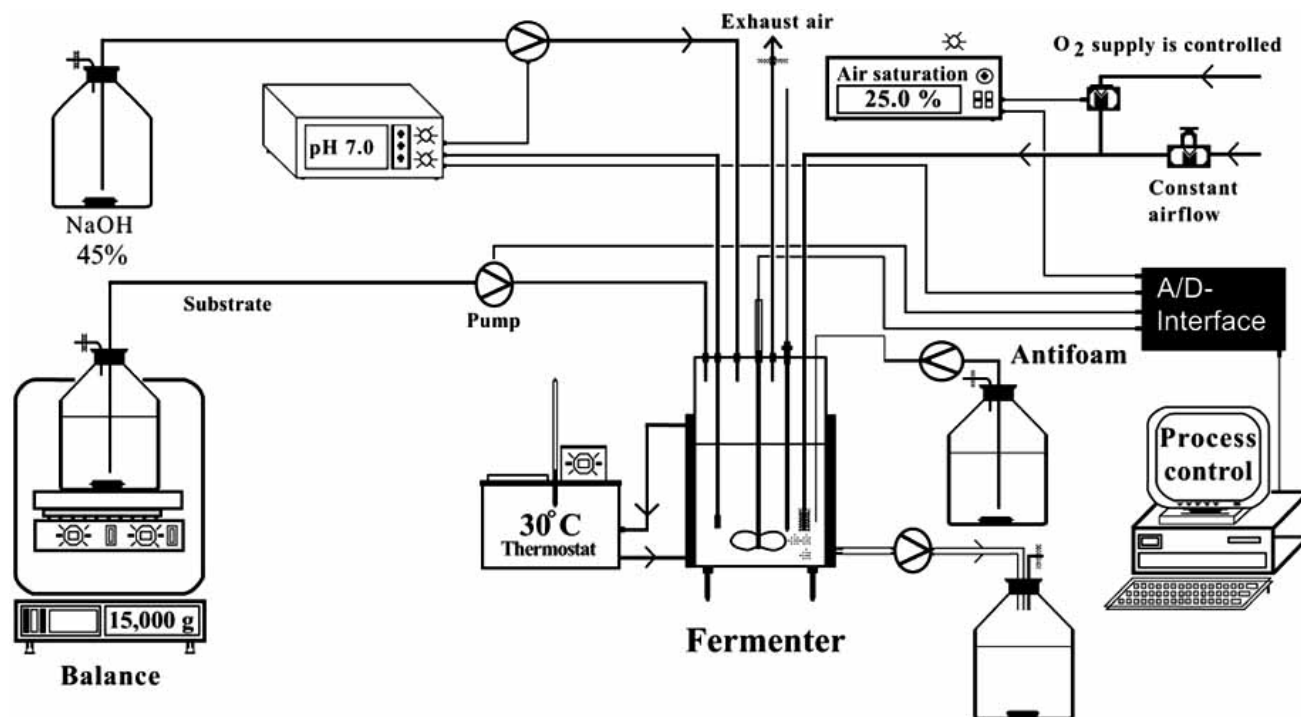


Fig. (1). Bioreactor for the continuous and discontinuous (batch, fed batch) L-lysine fermentation (A/D interface = Analog/digital converter).

Microorganisms employed in microbial processes for the production of amino acids are divided into 4 major classes: wild-type, auxotrophic mutant (auxotrophy), regulatory mutant and auxotrophic regulatory mutant strain (sensitivity and analog resistance) [25], pp. 649-661; cited in [7, 16]. Mutants of *Corynebacterium* and related organisms enable the inexpensive production of amino acids by direct fermentation of cheap carbon sources, e.g. molasses, acetic acid and ethanol.

CLASSICAL MUTAGENESIS

Classical mutagenesis of microorganisms comprises exposing to ultraviolet light irradiation (UV), X-ray irradiation, radiation irradiation (e.g. exposing to UV radiation at 30°C for about 180 sec) and chemical mutagen treatment (e.g. 250 or 500 µg/l N-methyl-N'-nitro-N-nitrosoguanidine (NTG) at room temperature, 30 or 32°C for 10-30 min (e.g. in tris/maleic buffer of pH 6.0; cells are washed with 0.1M tris buffer of pH 7.2) followed by mutant selection conducting replication on selective minimal agar plate media or LB agar plates [2,5,7,12,14,26-28,43].

Alternatively, genes relevant to a resistance (resistant mutants) or genes controlling the synthesis of selected amino acids (regulatory negative mutants) can be isolated from L-glutamic acid producing coryneform bacteria, then be subjected to a mutation treatment *in vitro* in order to obtain a resistant, regulatory or recombinant mutant gene type which will substitute the corresponding wild type gene on the bacterial chromosome by established homologous recombination techniques [2, 24, 29].

Several L-lysine fermentation processes utilizing various strains isolated for auxotrophic or resistance properties are

described in patent literature. Auxotrophic (e.g. for leucine or isoleucine, homoserine, threonine or threonine with methionine) or to certain chemicals and antimetabolites resistant (e.g. raffinate, L-lysine-analogues such as S-(2-aminoethyl)-L-cysteine (AEC; 0.4 g/l); acyl-lysine, methylated acyl-lysine, oxo-lysine, lysine-hydroxamate, -methyl-lysine and -chloro-caprolactam; arginine analogues and other analogues) and regulatory negative mutant strains of so-called coryneform bacteria with increased productivity are used as biocatalysts for the excretion of amino acids, including the genera [1-3,9,10,14,16,24,26,27,29-33]. Raffinate refers to a waste stream product from an ion-exchange operation for lysine recovery, containing a large amount of ammonia sulfate, L-lysine, other amino acids, salts, and carbohydrates such as isomaltose. Furthermore, sterilization of a medium using heat treatment produces amino acid derivatives and other metabolic antagonists which cause the inhibition of culture growth [16].

US2979439 [34] discloses mutants requiring homoserine or methionine and threonine [33] mutants having a nutritional requirement for threonine, methionine, arginine, histidine, leucine, isoleucine, phenylalanine, cystine, or cysteine. US3707441 [35] discloses a mutant having a resistance to a lysine analog [36] a mutant having both characteristics, an ability to produce L-lysine and a resistance to bacitracin, penicillin G or polymyxin. US3707441 [37] discloses mutants with nutritional requirements for homoserine, threonine, threonine and methionine, leucine, isoleucine or their mixtures and a resistance to lysine, threonine, isoleucine or their analogs, whereas [38] discloses a resistance mutant to a lysine analog and [39] L-lysine producing mutants of *Corynebacterium*, which are resistant to at least one of aspartic analogs and sulfa drugs.

Furthermore, [2] discloses mutant strains able to produce L-glutamic acid and L-lysine in the absence of any biotin action-suppressing agent and [7] 4 a strain of the genus *Corynebacterium* or *Brevibacterium*, resistant to 4-N-(D-alanyl)-2,4-diamino-2,4-dideoxy-L-arabinose 2,4-dideoxy-L-arabinose or its derivatives. Additionally, [26] describes a process for the production of L-lysine by mutant strains of *Brevibacterium* (*B. lactofermentum* AJ 12220 (FERM BP-996)) or *Corynebacterium*, which are resistant to superoxide production accelerators, to superoxide dismutase induction inhibitors, to superoxide radical reaction accelerators and to oxidizing agents that supply oxygen for the formation of superoxide.

Superior mutant strains of *Nocardia* sensitive to threonine or methionine and amino acid analogs such as alpha-amino-beta-hydroxy-valeric acid, alpha-amino-butyric acid or alpha-ketobutyric acid are described in [27], which don't require the expensive compounds threonine, methionine, leucine or iso-leucine.

US4066501 [5] describes a method of developing mutant strains of *Brevibacterium lactofermentum* or *Corynebacterium* resistant to γ -aminolauryllactam, γ -methyl-lysine or N γ -carbobenzoxy lysine. Additionally, further mutants are resistant to S-(2-aminoethyl)-L-cysteine or require L-alanine for cell growth. US20067008786 [40] discloses a Coryneform bacterium having resistance to the antibiotic monensin, whereas overproduction of L-lysine by resistant mutant strains of the genus *Corynebacterium* or *Brevibacterium* to purine and pyrimidine analogs or ethylene glycol is described in [41, 42], respectively.

L-Lysine producing strains of *Corynebacterium* or *Brevibacterium* sensitive to fluoropyruvic acid, in addition to homoserine auxotrophy and resistance to S-(2-aminoethyl)-L-cysteine (AEC), are described in [43] and to aspartic acid analogs and sulfa drugs in [2, 39] describes coryneform L-glutamic acid and L-lysine producing mutants with a temperature-sensitive mutation (37°C) with respect to a biotin action-suppressing agent. The mutants produce L-glutamic acid and L-lysine in the absence of any biotin action-suppressing agents (they are normally added at an initial or intermediate stage of cultivation) such as surfactants (e.g. saturated fatty acids, fatty acid esters) or antibiotics (e.g. lactam antibiotics such as penicillin and cephalolysine) under excessive amounts of biotin higher than 10 $\mu\text{g/l}$, which is for example excessively present in molasses media [44].

WO04013340A2 [12] provides a method for the fermentative production of L-lysine using coryneform bacteria that are sensitive to diaminopimelic acid analogues such as 4-hydroxydiaminopimelic acid, 4-fluorodiamino-pimelic acid and 2,4,6-triaminopimelic acid (3-30 g/l). US5770412 [28] describes a method for the selection of high L-lysine producing mutants of the genera *Corynebacterium* and *Brevibacterium*, being resistant to feedback inhibition by 2-azido-epsilon-caprolactam (40 mM), which has considerably higher efficiency in the selection of mutants than fluoro- or chlorocaprolactam which are described in [28].

MUTAGENESIS BY PROTOPLAST FUSION

A process for the production of L-lysine is described in [45], which comprises culturing a protoplast fusion mutant strain derived by protoplast fusion between strains of *Corynebacterium glutamicum* and *Brevibacterium lactofermentum*.

MUTAGENESIS BY RECOMBINANT DNA TECHNOLOGY

More recent developments of recombinant DNA technology and molecular biology have also been used in order to improve L-lysine producing strains e.g. of *Corynebacterium glutamicum* or *Brevibacterium*, by amplifying individual biosynthesis genes, integrating genes into chromosomal DNA or inserting genes coding for enzymes being highly rate determining for the biosynthesis of L-amino acid (e.g. phosphoenol pyruvate carboxylase; [46] into one or at least two plasmid vectors, which have compatible replicating origins different from each other.

For example, the subunits carrying the biotin-carboxyl carrier protein domain and the biotin-carboxylase domain in the nucleotide sequence (accBC gene) encoding the enzyme acetyl-CoA carboxylase [18], an *lysC* allele coding for a feedback-resistant aspartate kinase [47,48], a recombinant DNA fragment conferring resistance to aminoethylcysteine [49], the *dapA* gene coding for dihydrodipicolinate synthase (EP-B-0 197 335), the *asd* gene coding for aspartate semi-aldehyde dehydrogenase (EP-A-0 219 027) and a nucleotide sequence coding for the *accDA* gene [50] are amplified and in particularly overexpressed [9,10,46,50-52].

Intracellular activity of one or more important enzymes (Table 1) can increase by increasing the copy number of the corresponding L-lysine biosynthetic pathway gene(s) (gene amplification) in a host cell chromosome and the expression (over expression), by using a stronger promoter or a gene encoding for a corresponding enzyme with higher activity and optionally combining these measures, or mutating the promoter and regulatory region or the ribosome binding site located upstream from the structural gene and increasing the promoter strength. Expression cassettes which are incorporated upstream from the structural gene work in the same way. Inducible promoters additionally can increase the expression during the course of L-lysine fermentation, whereas measures for prolonging the life of m-RNA also improve the expression. The enzyme activity is also enhanced by prolonging the life of an enzyme protein, coded by genes or gene constructs either being located in plasmids (shuttle vectors) of variable copy number or being integrated and amplified in the chromosome [9,53]. By means of enhancement, in particular overexpression measures, the activity or concentration of the corresponding protein is generally raised by at least 10% and at most up to 1000% or 2000%, referred to the activity or concentration of the wild type protein and/or the activity or concentration of the protein in the starting microorganism [12].

Alternatively, it may be advantageous for the production of L-lysine simultaneously to attenuate, in particular to reduce the expression of one or more genes. WO0401

Table 1. Enhancement or Overexpression of Important Genes for L-lysine Formation [12]

Gene	Gene description
LysC	coding for feedback-resistant aspartate kinase
DapA	coding for dihydrodipicolinate synthase
Gap	coding for glyceraldehyde-3-phosphate dehydrogenase
Pyc	coding for pyruvate carboxylase
Zwf	coding for glucose-6-phosphate dehydrogenase
simultaneously lysE	coding for the lysine export protein
Zwa1	coding for the Zwa1 protein
LysA	coding for diaminopimelic acid decarboxylase
the gene sigC	coding for the sigma factor C
the gene tpi	coding for triose phosphate isomerase
the gene pgk	coding for 3-hosphoglycerate kinase

3340A2 [12] claims a process, characterized in that bacteria are used in which the metabolic pathways that reduce the formation of L-lysine are at least partially switched off or attenuated (Table 2). The term "attenuation" describes in this connection the reduction (0-75%) or switching off of the intracellular activity of one or more enzymes (proteins) in a microorganism that are coded by the corresponding DNA, by using for example a weak promoter or a gene or allele that codes for a corresponding enzyme with a low activity or inactivating the corresponding gene or enzyme (protein), and optionally combining these measures [12].

ES2247987T, EP1067193 [17,18] Offenlegungsschrift DE-A-198 31 609 and [54] reported about increasing L-lysine productivity after the amplification of pyc gene coding for pyruvate carboxylase in mutant strains, in which additional genes from the group of dapA-Gen, lysC-Gen, lysE-Gen und dapB-Gen are overexpressed. EP1619252 [20] is related to mutant strains of coryneform bacteria with an increased pyc gene as well as enhanced dapA gene (dihydrodipicolinate gene) with a mutated dapA promoter (MC20 or MA16 mutation), in which occasionally further genes of the group lysC-Gen (Aspartate Kinase gene), lysE-Gen (Lysine-Export-Carrier-Gen) und dapB-Gen (Dihydrodipicolinate-Reduktase-Gen) are overexpressed, alone or in mixture. Offenlegungsschrift DE-A-195 48 222 cited in [9, 55] disclose enhanced activity of L-lysine export carrier coded by the lysE gene.

In addition to these attempts of amplifying individual single genes, attempts have also been approached to simultaneously amplify two or more genes in order to improve L-lysine production in corynebacteria [9]. Offenlegungsschrift [56] discloses the increase of L-lysine productivity by simultaneously amplifying the asd and the dapA gene from *Escherichia coli*, whereas Offenlegungsschrift [57] after the simultaneous amplification of a lysC allele coding for a

Table 2. Attenuation or Switch off of Genes Reducing L-Lysine Formation [12]

Gene	Gene description
pck	Gene coding for phosphoenol pyruvate carboxykinase
pgi	Gene coding for glucose-6-phosphate isomerase,
deaD	Gene coding for DNA helicase
citE	Gene coding for citrate lysase
menE	Gene coding for 0-succinylbenzoic acid CoA-ligase
mikE17	Gene coding for the transcription regulator Mike17j
poxB	Gene coding for pyruvate oxidase
zwa2	Gene coding for the Zwa2 protein

feedback-resistance and of the dapA gene by means of plasmid pJC50 cited in [9]. EP-A-0 841 395 particularly reports about increasing productivity after the simultaneous amplification of an lysC allele coding for a feedback-resistant (sic) and of dapB gene, whereas further improvements could be achieved by additional amplification of the dapB, lysA and ddh genes. EP-A-0 854 189 describes the increase in productivity after simultaneously amplifying a lysC allele coding for a feedback-resistance and of dapA, dapB, lysA and aspc genes. EP-A-0 857 784 particularly reports the increase in productivity after simultaneously amplifying an lysC allele coding for a feedback-resistance and of the lysA gene, whereas a further improvement could be achieved by additional amplification of the ppc gene.

Homoserine auxotroph or resistant strains (e.g. to homoserine dehydrogenase or S-(2-aminoethyl)-cysteine) of Coryneform bacteria (e.g. *Brevibacterium saccharolyticum* ATCC 14066, *B. immariophilum* ATCC 14068, *B. lactofermentum* ATCC 13869, *B. lactofermentum* ATCC 39134, *B. roseum* ATCC 13825, *B. flavum* ATCC 13826, and *Corynebacterium acetoacidophilum* ATCC 13870), incorporating an expression vehicle (plasmid) which was obtained from *Corynebacterium* or *E. coli* strains, carrying a genetic sequence coding for the synthesis of diaminopimelic acid decarboxylase with various restriction endonuclease cleavage sites in its DNA (flanked by two Pst I sites) and a molecular weight of 2.9 ± 0.05 megadaltons (Md), produce higher amount of L-lysine by fermentation under identical conditions than the host strain. US4346170 & US4954441 [23,58] discloses a process for producing L-lysine by *Coryne-bacterium* or *Brevibacterium* strains, transformed by a recombinant vector with a DNA fragment containing a gene involved in the synthesis of dihydrodipicolinic acid synthetase.

Moreover, [59] applies an autonomously replicable recombinant DNA in cells of coryneform bacteria comprising DNA sequences coding for an aspartokinase with substantially desensitized feed back inhibition by L-lysine and L-threonine and for diaminopimelate decarboxylase. [60] claims a method locating insertion elements (IS elements) or transposons in coryneform bacteria, a suitable positive

selection system, the IS elements and their use. US5804414 [60] describes a method of amplifying a desired gene in a chromosome of a coryneform bacterium, forming an artificial transposon in which a drug resistance gene and the desired gene are inserted into an insertion sequence of coryneform bacterium, and introducing the artificial transposon into the coryneform bacterium. The desired genes can be amplified by these ways in the chromosome of coryneform bacteria which are used in the industrial production of amino acids or nucleic acids.

Furthermore, recombinant strains of *Escherichia coli* are reported as high L-lysine producers [24]. Those strains are obtained by the incorporation into a host strain of the genus *Escherichia* of a hybrid plasmid having inserted therein a DNA fragment with genetic information controlling L-lysine production. The DNA fragment is derived from a donor strain which is resistant to an L-lysine analogue. US4278765 & US5932453 [29,16] describes L-lysine production by genetically modified strains of *Escherichia* (e.g. AK deficient wild-type *E. coli*), encoding a recombinant aspartokinase III (AKIII) with increased AKIII activity, insensitive to feedback inhibition with L-lysine. The recombinant strain can grow on a culture medium free from L-lysine, L-threonine, L-methionine and diaminopimelic acid or free from homoserine and diaminopimelic acid. Additionally, [31] discloses a process for expressing at least a foreign gene (e.g. from *E. coli*) to an L-lysine producing host microorganism (e.g. *Corynebacterium* or *Brevibacterium*) by insertion to a vector DNA (e.g. plasmid pAec5).

US4346170 [24] describes an alternative process for the production of L-lysine from carbohydrates using recombinant strains of *Escherichia*, usually constructed by gene recombination techniques starting with L-lysine auxotrophs or high producing strains as a recipient. Genetic chromosomal information controlling L-lysine production derived from induced artificial donor strains e.g. *Brevibacterium* or *Corynebacterium*, resistant to an L-lysine analogue such as *S*-(2-aminoethyl)-cysteine (AEC) or homoserine auxotroph, is inserted using a hybrid plasmid [24, 27, 62] describes an aerobic L-lysine fermentation process by mutant strains derived from *Nocardia alkanoglutinosa* No. 223, which are resistant to AEC (0.4 g/liter). Plasmids stability is maintained by optionally adding suitable selectively acting substances to the medium, e.g. antibiotics [9].

Deregulation and subsequently increasing expression or increasing activity of fructose-1,6-bisphosphatase and increasing metabolic flux through the pentose phosphate pathway, has been disclosed to result in increasing lysine production in *C. glutamicum* using fructose or sucrose as carbon source. Additionally, one or more overexpressed or underexpressed deregulated feedback resistant key enzyme-encoding genes can increase L-lysine production [6]. Furthermore, harboring of recombinant DNA coding for -ketoglutarate dehydrogenase (amplification of -KGDH activity) has been described to increase L-lysine production in corynebacteria as well. Deficient -KGDH gene activity favors L-glutamic acid productivity, instead [63]. L-lysine productivity is enhanced by amplifying a novel gene (*dtsR* gene encoding for the protein referred as DTSR protein) participating in L-glutamic acid production, which is derived

from a Coryneform bacterium as well, while L-glutamic acid productivity is enhanced by suppressing the function of the gene [64].

In addition to biochemical pathways of L-lysine synthesis, L-lysine secretion into the media by a specific transport carrier, stimulated by membrane potential, has also been shown to be another important factor to be considered in the development of L-lysine overproducing strains of *C. glutamicum* (S. Bröer and R. Krämer, *Eur. J. Biochem.* 202: 137-143 (1991), cited in [16, 66] and corresponding [9, 10, 65] relate to L-lysine-producing strains of corynebacteria with amplified *lysE* gene (lysine export carrier gene), in which additional genes, individually or together, are amplified and particularly overexpressed, comprising the group of *dapA* gene (dihydrodipicolinate synthase), the *lysC* gene (aspartate kinase), the *dapB* gene (dihydrodipicolinate reductase) and the *pyc* gene, in especially the *dapA* gene and the *lysC* gene.

FERMENTATION MEDIUM

In addition to physical parameters like pH, agitation and aeration rate, air saturation, temperature, dissolved CO₂ and foaming, medium composition is a very important factor strongly influencing fermentation processes, often being object of extensive process development and optimization studies. The culture medium must satisfy in a suitable manner the requirements of microbial growth and production.

Defined media acquiring pure growth requiring nutrients and essential additives or alternatively undefined media containing natural organic substances such as soybean-hydrolyzate, corn steep liquor, yeast extract or peptone are used for L-lysine fermentation. Common fermentation media for L-lysine production contain various carbon and nitrogen sources, inorganic ions and trace elements (Fe⁺⁺, Mn⁺⁺), amino acids, vitamins (biotin, thiamine-HCl, Nicotinamide) and numerous complex organic compounds (General bibliography; [12,43]. An overexpression of genes is also achieved by optimizing the composition of the media and the culture technique in addition to physiological and genetic parameters [9].

E. coli culture medium for L-lysine production contains one or more types of inorganic salts, including potassium monohydrogen phosphate (K₂HPO₄), potassium dihydrogen phosphate (KH₂PO₄), magnesium sulfate (MgSO₄), sodium chloride (NaCl), magnesium chloride (MgCl₂), ferrous chloride (FeCl₂), ferrous sulfate (FeSO₄) and manganese sulfate (MnSO₄), one or more organic compounds such as yeast extract, peptone, meat extract, corn steep liquor and a soybean or wheat effluent. Furthermore they require suitable amounts of a saccharide starting material, vitamins and the like as required [61]. Ethylene glycol resistant mutants has been reported to be insensitive to concentrated aqueous solutions of sodium chloride, potassium chloride, ammonium chloride, ammonium sulfate, potassium sulfate, sodium sulfate, glucose, fructose, sucrose, and maltose which would inhibit the growth of the parent strains [42]. Additionally, calcium carbonate (CaCO₃) is added to shake flask cultures acting as buffer [43].

CARBON SOURCE

Mutants of *Corynebacterium* and related microorganisms enable the inexpensive production of amino acids from cheap renewable carbon sources by direct fermentation. Various carbohydrates are utilized individually or as a mixture for the production of L-lysine such as glucose, fructose, sucrose, molasses (sucrose, glucose, fructose etc.), maltose, blackstrap molasses, starch hydrolyzate (glucose, oligosaccharides), lactose, maltose, starch and starch hydrolysates, cellulose, cellulose hydrolysate, organic acids such as acetic acid, propionic acid, benzoic acid, formic acid, malic acid, citric acid and fumaric acid, alcohols such as ethanol, propanol, inositol and glycerol and certainly hydrocarbons, oils and fats such as soy bean oil, sunflower oil, groundnut oil and coconut oil as well as fatty acids such as e.g. palmitic acid, stearic acid and linoleic acid. Those substances may be used individually or as mixtures [5, 6,12, 16, 19, 20, 39, 42, 43, 60, 62,].

Alternatively, US Patent 3,595,751 cited in [27] utilizes olefin and US Patent 3,440,141 ethyl alcohol as carbon source, yielding low quantities of L-Lysine employing bacteria of the genus *Nocardia*. US4123329A [27] describes improved L-lysine production by aerobically culturing of mutant strains (e.g. ATCC 31220, ATCC 31221) of *Nocardia alkanoglutinosa* No. 223 which was isolated from soil, utilizing various carbon sources like *n*-alkanes containing 10 to 30 carbon atoms, kerosene containing *n*-alkane, crude oil, animal and vegetable oil, animal and vegetable fat and fatty acids. Additionally, the new process applies ethyl alcohol, glucose, fructose, a hydrolysis product of molasses and starch, and organic acids such as acetic acid and citric acid.

Auxotrophic mutant strains require definite substance for their growth, which should be added to the culture medium. Alternatively, protein hydrolyzate, corn steep liquor, meat extract or yeast extract containing those substances can be added, instead.

NITROGEN SOURCE

Various sources of nitrogen are utilized individually or as mixtures for the commercial and pilot scale production of L-lysine, including inorganic compounds such as gaseous and aqueous ammonia, ammonium salts of inorganic or organic acids such as ammonium sulfate, ammonium nitrate, ammonium phosphate, ammonium chloride, ammonium acetate and ammonium carbonate. Alternatively, natural nitrogen containing organic materials like soybean-hydrolyzate, soyprotein HCl-hydrolyzate (total nitrogen of about 7%), soy bean meal, soybean cake hydrolysate, corn steep liquor, casein hydrolysate, yeast extract, meat extract, malt extract, urea, peptones and amino acids may also be utilized [5, 19, 20, 43, 67, 68,]. Interestingly, Inuzuka and Hamada (1976) claimed enhanced L-lysine yields by enriching L-lysine fermentation medium by the culture liquor (2-150 ml/l) of an L-leucine-producing microorganism [12, 69].

Nocardia alkanoglutinosa utilizes various nitrogen sources including ammonium acetate, ammonium sulfate, ammonium chloride, ammonium nitrate, ammonia water, amino acids, amino acid mixtures, yeast extract, peptone and meat extract [29].

INORGANIC SALTS, TRACE ELEMENTS AND GROWTH FACTORS

Further components are necessarily added to fermentation media at the initiation and/or intermittently during the course of L-lysine fermentation, such as inorganic salts of various metals, like magnesium (e.g. magnesium sulfate), calcium, potassium, sodium, iron (e.g. iron sulfate), manganese, and zinc or traces of other metals. Phosphoric acid, potassium dihydrogen phosphate (KH_2PO_4) or dipotassium hydrogen phosphate (K_2HPO_4) or the corresponding sodium salts are commonly used as source of phosphorus for the production of L-lysine [12,19,20,27]. Essential growth factors such as amino acids and vitamins (e.g. vitamin B₁) and suitable precursors are also added to the culture in addition to the substances mentioned above, one-off batch or intermittently during the cultivation [12,20]. Table 3 illustrates the composition of a typical production medium for *C. alkanoglutinosa* [27,63].

Table 3. Composition of L-Lysine Fermentation Medium of *C. Alkanoglutinosa*

Compound	Shake flask	Bioreactor	Unit
<i>n</i> -Alkane (C ₁₄ -C ₁₈)	50.0	100.0	g/l
Ammonium Sulfate	30.0	35.0	g/l
CaCO ₃	30.0	1.0	g/l
K ₂ HPO ₄	0.5	1.0	g/l
KH ₂ PO ₄	0.5	1.0	g/l
MgSO ₄ ·7H ₂ O	0.5	0.5	g/l
NaCl	1.0	1.0	g/l
FeSO ₄ ·7H ₂ O	20.0	100.0	mg/l
ZnSO ₄ ·7H ₂ O	10.0	20.0	mg/l
MnSO ₄ ·4H ₂ O	10.0	20.0	mg/l
Tap water	940.0	870	ml
PH	7.0	7.0	
Steaming	15	15	Min

US5268293 [14] applies a fermentation medium containing: 75 g/l glucose, 40 g/l ammonium sulfate, 40 g/l CaCO₃, 100 g/l Corn Steep Liquor, 1 g/KH₂PO₄, 0.4 g/l MgSO₄·7H₂O, 0.01 g/l FeSO₄·7H₂O, 6 mg/l MnSO₄·4H₂O, 300 µg/l biotin, 500 µg/l thiamine-HCl, 0.01 g/l pantothenic acid at pH 7.6-8.0.

Alternatively, EP0923878B1 applies a fermentation medium with following composition: 30 g/l glucose, 20.0 g/l Soy hydrolysate, 20.0 g/l ammonium sulfate, 3.0 g/l urea, 1.0 g/l KH₂PO₄, 0.5 g/l MgSO₄·7H₂O, 0.002 g/l MnSO₄, 0.0001 g/l biotin and 0.0001 g/l thiamine-HCl. The effect of various amino acids (1-3 g/l) on L-lysine production was investigated by Oh *et al.* (1993) [14]. The addition of certain amino acids such as arginine, aspartic acid, isoleucine or

valine enhanced L-lysine production, in contrary to leucine. Furthermore, the degree of inhibition on bacterial growth caused by AEC (S-2-aminoethyl-cysteine) was reduced by the addition of 1 g/l of arginine to the culture medium.

INFLUENCE OF OXYGEN

No significant information has been found in patent literature regarding the effect of air saturation and a little is known regarding the real effect of oxygen on L-lysine fermentation. L-lysine fermentation is an aerobic process [42,43,63] demanding large amounts of oxygen and strongly influenced by the air saturation in bioreactor. Lactic acid is formed as a byproduct under anaerobic conditions, which is reconsumed after the establishment of aerobic conditions (unpublished data received by Anastassiadis and Stephanopoulos, MIT).

Aerobic conditions are maintained by aseptically adding to the culture oxygen containing gaseous mixtures, e.g. atmospheric air or pure oxygen [9,12]. Cultivation of L-lysine producing microorganisms is carried out with shaking of shake flasks (250-300 rpm) or by the aeration (0.5-1.5 vvm) of stirring bioreactors. US5268293 and US6984512 [14,16] describes bench scale fermentations operating at 2.1 vvm aeration rate of atmospheric air and changing agitation speed during the fermentation between 600 (at 0 h) and 900 rpm (at 19 h).

Enormous effects of air saturation (100% air saturation corresponds to saturation at 1 vvm aeration rate at 30°C and 600 rpm agitation rate) on continuous L-lysine production by *B. lactofermentum* have been found in chemostat process development experiments (unpublished data received by Anastassiadis and Stephanopoulos, MIT).

INFLUENCE OF TEMPERATURE

A wide range of optimum and operational temperatures have been disclosed in the international patent bibliography for L-lysine fermentation, in order to protect the whole usable range. US4275157 [43] claims L-lysine production at temperatures between 24 and 37°C, preferably at 31-33°C and [42] from 24 to 37°C for 2 to 7 days. US20066984512 and US5133976 [16, 67] operate at about 32°C and [14] at 25-35°C. Operation temperatures in the range between 25 and 40°C have been claimed in [68, 69, 10] (preferably 30-40°C), from 30-40°C in [7, 26, 45, 62]. US4623623 [63] describes a fermentation process claiming L-lysine production by protoplast fusion mutant strains at temperatures between 20 and 40°C for 1-6 days.

Alternatively, [67] claims increased L-lysine production employing the thermophilic strain *Corynebacterium thermoaminogenes* at temperatures from 25 up to 50°C, preferably about 35-45°C, and [9, 12] as well as [20] from 20 to 45°C (preferably 25-40°C). US Patent 5,846,790 discloses the rising of temperature to 33-40°C, preferably higher than 34°C, at a certain intermediate stage of L-lysine and L-glutamic acid fermentation. *Nocardia alkanoglutinosa* grows at temperatures between 10 and 40°C producing L-lysine at 20-40°C, optimally at 27-37°C [64]. Incubation of *E. coli* during L-lysine fermentation is conducted in submerged-aerial stirring, shaking or stationary culture at temperatures between 30 to 42°C, preferably at about 37°C

for 4 to 24 hours [61] or aerobically from 16 to 72 h at temperatures between 25 and 45°C [61].

INFLUENCE OF pH

The pH is a very important factor strongly influencing microbial fermentations. Basic compounds such as sodium hydroxide, potassium hydroxide, ammonium hydroxide, calcium carbonate, urea, ammonia and gaseous ammonia, or inorganic acid compounds such as phosphoric or sulfuric acid and organic acids are utilized for controlling pH in L-lysine cultures at a pH ranging from 5 to 9 [7,9,11,12,20,43, 63, 67,70] discloses L-lysine production by mutant strains at a pH spectrum between 5 and 8.5, [69] between 6.0 and 9.0 (preferably from 6.0 to 8.0), [26,62] in the range from 6 to 8 and [11] a pH in the range of 5.8 to 8.5, preferably 6.5 to 7.5.

A suitable initial pH of culture medium between 6 and 8 is disclosed in [61], whereas L-lysine production described in [16, 67] operates at pH 7.2 using NH₃ or NH₄OH and at pH between 5.0 and 8.0 in [14]. pH between 5 and 8 is referred in [68] and between 5.0 and 9.0 in [7, 42] operates at pH 6.5 to 7.0 and US Patent 4,066,501 at pH between 7.2 and 8.0, by feeding acetic acid and ammonium acetate (30°C). *Nocardia alkanoglutinosa* grows at pH between 6 and 9 [27]. The *E. coli* L-lysine fermentation process operates at pH between 5 and 8 using inorganic, acidic or alkaline or ammonia gas [61].

ANTIFOAMING

Foaming occurring during fermentation is controlled by the addition of antifoams such as fatty acid polyglycol esters [9,12,20] or silicone and polypropylene.

PLASMID PROTECTION

Suitable selectively acting substances e.g. antibiotics are added into the medium in order to maintain the stability of plasmids [12,20].

L-LYSINE FERMENTATION TECHNOLOGY

Amino acids are commercially produced in batch or fed-batch processes. All of nutrients are added at the beginning of the fermentation in batch operations [16].

For shake flask experiments, the strains are first of all incubated on agar plates e.g. for 24 hours at 33°C. Using this agar plate culture a preculture is inoculated (e.g. 10 ml of medium in a 100 ml Erlenmeyer flask equipped with baffles). The medium MM is used as medium for the preculture. The preculture is incubated e.g. for 24 hours at 33°C at 240 rpm on a shaker. In following, the main culture is inoculated using this preculture so that the initial optical density (e.g. OD at 660 nm) of the main culture reaches a certain level e.g. OD of 0.1 [12]. A production medium (e.g. MM medium) is used for the main culture. The culturing is carried out for example at 33°C and 80% atmospheric humidity [12]. OD, L-lysine and carbon source are determined during and at the end of fermentation

In batch fermentation microorganism grows until one or more of essential nutrients is exhausted or until fermentation conditions become unfavorable (e.g. product inhibition, oxygen limitation, pH decrease in shake flasks and uncontrolled fermentations etc.).

In fed-batch or extended fed-batch fermentations one or more nutrients are continuously or intermittently supplied to the culture medium, either from the beginning of fermentation or after the culture has reached a certain age, or when nutrient(s) are exhausted. The microorganism grows at a growth rate dictated by the rate or timing of nutrient feed. In generally a single nutrient, very often the carbon source (e.g. ethanol, glucose), is fed into fermenter in order to overcome substrate inhibition and high osmotic pressure. Furthermore, continuous feeding of complete medium simulates chemostat conditions acquiring combined advantages of both, batch as well as chemostat operation.

An interesting variant of extended batch or fed-batch fermentation is the repeated batch or fed-batch or fill-and-draw fermentation. A part of fermentation broth is removed at a certain time of operation, while feeding continues extending fermentation operation and increasing high product concentrations, or fermenter is filled with fresh medium at the end of fermentation. Favorable fermentation conditions are maintained by controlling pH, temperature, oxygen concentration, feeding essential nutrient(s) to the culture or alternatively, sophisticated feeding strategies are employed in fed batch mode (e.g. linear or logarithmic increasing mode) significantly enhancing L-lysine production and yields (MIT studies).

The continuous fermentation or chemostat (Fig. 1) uses continuous feeding of a complete medium, while culture fluid is continuously or semi-continuously withdrawn in such a way that the working fermenter volume remains constant. Generally, a single nutrient becomes the limiting factor for growth, e.g. nitrogen, phosphorus, an amino acid, a vitamin or the carbon source. A continuous fermentation can in principle run for an infinite time under steady state conditions. Continuous L-lysine fermentation offers many advantages over batch fermentations (unpublished data received by Anastassiadis and Stephanopoulos, MIT, 1994).

A new method is described in [11] maintaining total carbon source concentration at low concentration less than 5 g/l (e.g. sugar concentration) in fed-batch, continuous or cell-recycling continuous cultures, by monitoring increase of pH or dissolved oxygen (based on the carbon consumption) and intermittently adding medium into bioreactor, at a calculated feed rates using a computerized feed control device. Several purposes are accomplished so far, including overcome of

substrate inhibition, effective carbon source utilization, easy product isolation and prevention of environmental pollution, combining the advantages of conventional continuous and batch fermentation at once. Oxygen consumption, emission of carbon dioxide, pH, by-product production and addition of ammonia are further parameters, previously considered (predetermined proportional coefficient) for ineffectively controlling carbon source in bioreactor [11]. In contrary to customary fed batch processes (simultaneous feeding of carbon sources, trace elements and growth-limiting amino acids), [3] describes a novel process, feeding carbon source and limiting amino acid through two or more infeed currents.

Continuous fermentation or chemostat applies continuous feeding of complete medium to a constant fermentation volume aiming reaching of steady state conditions. A single nutrient, like carbon source, nitrogen, sulphur, phosphorous or alternatively oxygen, a vitamin or an amino acid (applies for auxotrophic microorganisms) will become growth limiting [16]. Continuous fermentation processes are described in [71] for the production of amino acids like L-glutamic acid, whereas [11] describes a cell-recycling continuous process for the production of L-lysine.

L-LYSINE FERMENTATION RESULTS

A little of the work described in the patent literature has been devoted to the fermentation process development and optimization, compared with the genetic improvement of L-lysine production strains. According to international patent bibliography, bacterial strain and process development has resulted in the continuous increase of L-lysine titers and yields the last 50 years of L-lysine fermentation [72-74]. Fermentation usually operates until concentration of L-lysine or of desired product reaches a maximum. This target is normally achieved within 10 hours to 160 hours for L-lysine production [12]. US6984512 [16] completes batch fermentation in 48 hrs.

L-lysine concentrations between 11.1 and 15.4 g/l were reached by various mutant strains in baffled shake flasks after 48 h [9, 20] Table 4 compares the results from shake flask experiments between wild and mutant strain [12].

Large-scale fermentation processes produce an impure 80-110 g/l L-lysine containing broth [75]. Fermentation times between 30 and 100 h have been reported reaching 50-100 g/l of L-lysine are produced by mutant strains within 30-

Table 4. Comparison of Results Obtained by Wild and Mutant Strain

Patent number	Mutant characteristics	L-lysine (g/l) Wild type	L-lysine (g/l) Mutant strain(s)
US7122369 B2 and US6984512 B1	Raffinate-resistant	9.4 g/l (SF) 88.3 g/l (BF)	14.1-24.6 g/l (SF) 122.3 g/l (BF), 6.6 l
US5770412	resistant to feedback inhibition by 2-azido-epsilon.-caprolactam		10% more than wild strain
WO 2004/013340 A2	sensitive to diaminopimelic acid analogues	18.9 g/l (SF)	19.6 (SF) DSM13994-Hdap-s

SF = Shake flask experiments
BF = Batch fermenter

100 hours based on molasses media [76], or alternatively, 70 g/l L-lysine, 44% yield and 15% solids (w/w) are reached after 50 h at 35°C [68].

L-Lysine concentrations of 41-96 g/l and yields of 17-32% have been reached after 72 h by aerobically culturing mutant strains at 30°C and pH 7.2-8.0 (acetic acid and ammonium acetate) utilizing ethylalcohol as single carbon source, 20-39 g/l L-lysine with beet molasses and 18-37 g/l with cane molasses [5]. L-lysine titers between 32 and 41 g/l and yields between 32 and 41% were obtained by fluoropyruvic acid sensitive mutant strains [43]. US2979439 72 or 84 g/l of L-lysine and a yield of 29-32% were obtained after 55 hours under batch cultivation by two mutants of *B. lactofermentum* from acetic acid and 68 or 80 g/l and 27 or 31% maintaining ethanol at about 0.3%. Furthermore, L-lysine-HCl concentrations between 10 and 21.3 g/l and yields between 21.7 and 29.7 were reached after 72 h by mutant strains of *Corynebacteria* at 31.5°C [7].

US6025169 [11] reports increased yields from 32% (batch culture of prior art) and 33% (feed culture of prior art) to 35% and volumetric productivities from 2.2 g/(l*h) (batch culture of prior art) and 2.3 g/(l*h) (feed culture of prior art) to 2.8 g/(l*h) maintaining sugar concentration lower than 5 g/l.

L-Lysine-HCl concentrations between 6.3 g/l and 28.7 g/l of L-lysine-HCl have been produced in shake flasks after 5 days by mutant strains of *N. alkanoglutinosa* (ATCC 31220 and ATCC 31221) at 33°C, depending on carbon source used and 52.5 g/l after 96 h in bioreactor at 33°C, pH 7.0 (ammonia water), 400 rpm and 0.5 vvm aeration rate [27]. 70 g/liter of lysine has been reached after 50 h by a *Corynebacterium* strain (United States Patent 5133976). 120 g/l of L-lysine-HCl, 45% yield and 1.6-2.5 g/l arginine were reached by mutant strain *C. glutamicum* CS-755 [14]. An increase from 12.5 g/l to 39 g/l L-lysine between host strain *B. lactofermentum* and a plasmid carrying transformant has been disclosed in [58] after 70 h cultivation on a synthetic medium with 100 g/l glucose at 30°C. Furthermore, [16] claimed production of more than 10 g/l L-lysine after 24 hours using a mutant strain of *Corynebacterium* grown on a medium containing at least 1% raffinose. Necessarily, L-lysine productivity, concentration and yield have still to be enhanced, whereas a productivity of lysine of 3.5-3.9 g/(l*h) and a yield of lysine of 31-43% has been reached by using various feeding strategies [11].

Usually, production companies don't reveal information regarding titers and yields of L-lysine, because of today's strong competition in industry. About 170 g/l of L-lysine are produced discontinuously after 2 days using superior recombinant strains of *C. glutamicum* (Research Center Jülich, Germany, 2006). About 100 g/l of L-lysine were continuously produced under optimized chemostat conditions at short residence times by a strain of *B. lactofermentum* (ATCC) without applying any genetic improvement, emphasizing the great still latent potential of process development (unpublished data received by Anastassiadis and Stephanopoulos, 2004-2006, MIT, USA).

BIOCHEMISTRY AND REGULATION OF L-LYSINE FERMENTATION

Because of the significance L-lysine economics, biochemical pathways of L-lysine synthesis have been intensively investigated and elucidated, aiming in increasing lysine production and reduction of production costs.

Upon cellular absorption, glucose is phosphorylated to glucose-6-phosphate with the consumption of phosphoenolpyruvate (phosphotransferase system), whereas sucrose is converted into fructose and glucose-6-phosphate by the phosphotransferase system and invertase reaction. Glucose is catalyzed through the Embden-Meyerhof-Parnas (glycolysis) and pentose phosphate pathways. During glucose catabolism, glucose-6-phosphate isomerase (EC 5.3. 1.9) and glucose-6-phosphate dehydrogenase (EC 1.1. 14.9) compete for the substrate glucose-6-phosphate, resulting into either fructose-6-phosphate or 6-phosphogluconolactone, respectively [6].

The oxidative part of pentose phosphate cycle converts glucose-6-phosphate into ribulose-5-phosphate, supplying reduction equivalents in the form of NADPH. Proceeding pentose phosphate cycle, pentose, hexose and triose phosphates are interconverted. 5-phosphoribosyl-1-pyrophosphate (pentose phosphate) is required in the biosynthesis of nucleotides and as a precursor of aromatic amino acids and L-histidine. NADPH acts as a reduction equivalent in numerous anabolic biosyntheses, while four molecules of NADPH are consumed for the biosynthesis of one molecule of lysine from oxaloacetic acid [6]. Thus, carbon flux towards oxaloacetate (OAA) remains constant regardless of system perturbations [6, 77].

Pyruvate carboxylase (PEP carboxylase), phosphoenol pyruvate carboxylase (4.1.1.31 phosphoenol pyruvate carboxylase; PEPC) and pyruvate carboxylase have been identified as anaplerotic enzymes, which catalyze the reaction of adding 1 mole of carbon dioxide to pyruvate and phosphoenol pyruvate, fulfilling the TCA with oxaloacetic acid (OAA) and playing an important role in supplying aspartic acid and subsequently forming lysine, threonine, isoleucine (they are formed from aspartic acid) by metabolic processes and in the production of amino acids formed from TCA-cycle organic acids (e.g. glutamic acid, glutamine, proline, arginine, citrulline, ornithine, etc.) [46].

Entry into lysine pathway begins with L-aspartate, which is synthesized by the transamination of oxaloacetate. *C. glutamicum* has the ability of converting the L-lysine intermediate piperidine 2,6-dicarboxylate to diaminopimelate through two different routes, by reactions involving succinylated intermediates or by the single reaction of diaminopimelate dehydrogenase [16].

Overall, carbon flux into the pathway is regulated at two points: first, through feedback inhibition of aspartate kinase by the levels of both L-threonine and L-lysine; and second through the control of the level of dihydrodipicolinate synthase. Increased production of L-lysine may be therefore obtained in *Corynebacteria* by deregulating, desensitizing feedback inhibition by L-lysine and L-threonine [16, 51] and by increasing the activity of these two enzymes.

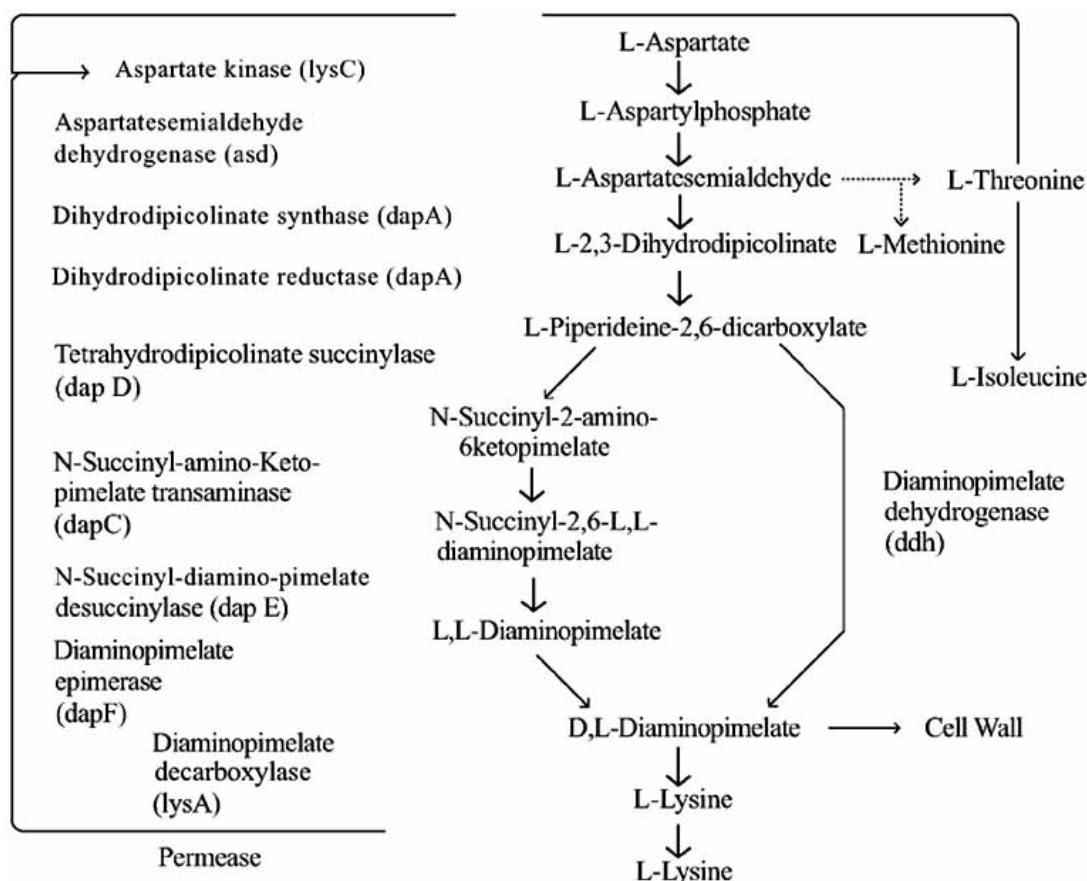


Fig. (2). Flow scheme of L-lysine formation pathway.

In addition to biochemical pathway of L-lysine synthesis, L-lysine export has also been shown to be another important factor influencing L-lysine production in *C. glutamicum*. It possesses following L-lysine properties: (1) the transporter possesses a rather high K_m value for lysine (20 mM); (2) the transporter is an OH^- symport system (uptake systems are H^+ antiport systems); and (3) the transporter is positively charged, and membrane potential stimulates secretion [16, 65].

Metabolic engineering has also been successfully used to redirect the flux of glucose derived carbons toward aromatic amino acid formation [6,16,78].

QUALITATIVE AND QUANTITATIVE L-LYSINE ANALYSIS

Qualitative and quantitative analysis of L-lysine formed is also an important point of consideration in international literature. After centrifugation and filtration of fermentation broth, L-lysine is determined by various means of analytical methods.

In US4275157 [43] a colorimetric determination method is used. The concentration of L-lysine (as monohydrochloride salt) accumulated in the culture broth is also analyzed by the acidic ninhydrin method or using High Pressure Liquid Chromatography (HPLC, reversed phase HPLC) with precolumn derivatization with ortho-phthalaldehyde and amino acid separation e.g. on a Hypersil AA

column (Agilent). Amino acid composition in the culture broth is analyzed with an amino acid autoanalyzer by means of ion exchange chromatography and postcolumn reaction with ninhydrin detection [6,7,9, 12, 14, 20]. Furthermore, L-lysine was measured by a bioassay method using a mutant strain of *Escherichia coli* or by using an amino acid analyzer, e.g. from Eppendorf-BioTronik (Hamburg, Germany) [12, 27]. The glucose content is determined with a glucose analyzer [9] or may be analyzed by HPLC applications.

DOWNSTREAM PROCESSES OF L-LYSINE

Product separation and purification is a very important factor enormously affecting fermentation process effectiveness and production costs, steadily requesting improvements in the recovery process of amino acids, especially L-lysine. L-lysine is recovered from fermentation broth in various ways. For many years, L-Lysine-HCl solid has been produced following various steps such as fermentation, separation, purification, crystallization and drying. L-lysine of resultant culture broth can be recovered by known conventional methods such as using ion exchange resins, or by directly crystallization of L-lysine from culture broths. After cell separation by cell filtration or centrifugation, L-Lysine may be recovered from fermentation broth by an ion exchange step and thereafter concentrated by evaporation and spray drying.

CELL SEPARATION

The fraction of L-Lysine fermentation broth is obtained by any suitable separating methods such as ultrafiltration or centrifugation [4, 14]. In order to overcome difficulties occurring in due of high viscosity of culture broth, filterability can be improved by adding a mineral acid (e.g. concentrated sulfuric acid) to adjust the pH to 1-3 or by adding an alkali to adjust the pH to at least 9 and then heating the culture broth to 80°C (100°C) for at least 5 minutes (e.g. 20 minutes) [27].

ION EXCHANGE CHROMATOGRAPHY

A variety of ion-exchange chromatographic methods are applied for the separation of L-lysine and other amino acids, either before or after biomass removal by filtration or centrifugation. They include primary cation exchange resins, based on the isoelectric point of desired amino acid, passing the solution at a pH of 1.5-4 and eluting L-lysine from resin with an eluent (e.g. ammonium hydroxide) with a pH>9.59, higher than the isoelectric point (Table 5), and ion-exchange chromatography using a fixed bed or simulated moving bed resins, adsorbing the desired amino acid onto the resin. Those purification methods are more economical compared with a costly crystallization step, also providing additional advantages and flexibility regarding the form of final product. It may be powder (spray drier), coarse powder or a fine granular product (fluid bed drier), flakes (drum drier) or intermediate to coarse granules (granulator drier) [8, 16].

Table 5. Isoelectric Points of Certain Important Amino Acids (US Patent 5,684,190)

Amino acid	Isoelectric point
Lysine	9.59
Glycine	5.97
Threonine	5.64
Proline	6.3
Arginine	11.15
Methionine	5.74

Alternatively, calcium hydroxide is added to the culture broth to adjust its pH to 11.0. The culture broth is then heated at 100°C for 30 minutes, aerated at 1 vvm flow rate for 2 hours. Concentrated sulfuric acid is added to adjust the pH to 5.0, followed by filtration. Specifically, the filtrate is passed through an ion-exchange resin (IR-120, NH₄⁺ type) such as IR-120 or IRC-84 for the adsorption of L-lysine, which is washed with water and then eluted with an ammonia solution [27].

Eluate fractions, containing L-lysine and impurities such as ammonia, potassium, calcium, sodium and magnesium ions and other amino acids, are concentrated by evaporation. Ammonia is simultaneously stripped off using multiple effect evaporators. In following, they are neutralized or acidified with concentrated hydrochloric acid and crystal-

lized either by means of a batch cooling crystallizer or an evaporative crystallizer (crystallisation process) to obtain L-lysine-HCl of more than 98% purity.

Potassium (as K⁺) makes up approximately 80% of the non-ammonium cationic impurities in the L-lysine liquor after the primary ion-exchange operation. The wet crystals of L-lysine are separated from mother liquor using a centrifuge or filter and then dried by a typical fluid bed drier, which can be recycled and a portion of lysine is lost by purging a waste stream. Impurities that are not separated from L-lysine in the primary (conventional) ion exchange resin step are removed by passing the first solution over a secondary (also a third) cation exchange resin (12-30 m/h feeding rate), which is located in a bed, operating as a fixed bed, as a countercurrent rotating fixed bed or as a countercurrent moving bed, enormously reducing impurity levels, recovery losses and capital and operating costs and thus don't necessitating further purification by crystallization [8, 27].

Suitable primary and secondary cation exchange resins include the strong acid cation exchange resins DOWEX XUS 43518 and XUS 40406 and DOWEX C500ES (registered trade mark of Dow Chemical Company), which typically are sulphonated copolymers of styrene and divinyl benzene with a resin capacity of 1.9 equivalents/liter. The column is regenerated with 5-10% sulphuric or hydrochloric acid and then washed with several bed volumes of demineralised water. US5684190 and TW0521996B [8,80] pro-vides a process for separating basic amino acids (e.g. L-lysine) from an aqueous solution (pH 4 by adding sulfuric acid) using cation exchange resin towers in series (DIAION SK-1B, Amberlite IR-120 and Duolite C-20, all are trademarks), comprising repetitive adsorption and elution steps in sequence (also cited in [16]). Cation exchange resins may be employed in the form of ammonium-type, sodium-type, etc. [79].

According to [43], the supernatant is passed through a column of "Amberlite IR-120" OH type after the centrifugation of fermentation broth and removal of cells and other precipitates. L-Lysine which is absorbed on the resin is eluted with 3% ammonia water, the eluate is evaporated and HCl is added, so that L-lysine-HCl-2 H₂O crystallines are obtained upon cooling.

US5268293 [14] describes a purification procedure including adjustment of filtrate with sulfuric acid to pH to 5.0, decolorization by active carbon, concentration, crystallization and then separation into crystals and liquid fraction. L-Lysine is adsorbed on a strong acidic cation exchange resin Duolite C-20N by loading the eluent (10 ml) from bottom to the top of a column at a space velocity of 5. This upflow adsorption method makes the remove of microorganisms by centrifugation unnecessary, reducing therefore L-lysine losses.

After the removing of ammonia, the L-lysine rich fraction is mixed with the filtrate in the ultrafiltration step and subjected to pH adjusting, decolorizing and concentrating to obtain the pure crystal (recovery yield of 94% and purity of above 99%). After completing the adsorption, the resin column is sufficiently washed with water in order to remove microorganisms and particulate materials. The L-

lysine adsorbed is eluted with 2N-NH₄OH and the L-lysine fraction which is 0.6 fold of the resin volume is collected (129 g/l L-lysine.HCl, 97% recovery yield from culture broth). The fractions with low L-lysine concentration are recycled back to the next cycle. The crystals obtained by this method are dried in hot air to prepare 993 g of L-lysine hydrochloride (recovery yield of 94% and purity above 99.5%) [14].

L-Lysine-HCl solution is usually crystallized to produce L-Lysine-HCl dihydrate crystals (L-Lysine-HCl 2H₂O), which are thereafter dried to have less than 1% moisture. This product is still dusty and lumpy, resulting in the loss of valuable material, sometimes causing an incomplete formulation. The use of an ion exchange makes the process expensive. Extending the previous method, [4] describes a new method for the production of L-lysine feed supplement with a final L-lysine purity in the range between 35 and 80%.

DRYING STEP

The purification process also comprises a drying step which may involve any suitable drying means such as a spray granulator, spray dryer, tray dryer, drum dryer, rotary dryer, and tunnel dryer [4, 67, 80]. Concentrated L-lysine solutions are obtained by heating fermentation broths under reduced pressure by steam at 130°C using a multipurpose concentrator or thin film evaporator. The microorganism is deactivated, whereas concentrated broths are stable for long time and L-lysine is not degraded during storage for six months at 20°C [68, 76].

Direct spray drying of an L-Lysine fermentation broth avoids extensive purification steps associated with the L-lysine hydrochloride process, in particular the use of expensive ion-exchange. However, a consistent L-lysine concentration in the final dry product is difficult to achieve, because of considerably varying L-lysine concentration in a fermentation broths. Also, the dry product may be dusty and difficult to use [4]. EP0923878B1 and TW0521996B [67,80] describe a new process for making granular L-lysine feed supplement, substantially drying the enriched L-lysine broth, whereas L-lysine content isn't solely dependent on initial L-lysine concentration of fermentation broth [67].

SOLID FEED ADDITIVE OF L-LYSINE

TW0501996B [81] describes a process for the production of an amino acid feed supplement which still contains most of fermentation broth solids. [68] describes a fermentation process for the preparation of stable long lasting (6 months at 30°C) L-lysine sulphate (35 to 48% by weight of lysine) for animal nutrition, adding sulfuric acid or ammonium sulphate. Concentrated L-lysine fermentation solutions have the same nutritive properties as purified lysine and biomass, other medium components or their derived products do not have any detrimental effect on animal health [68]. US5622710 [82] describes a process for the preparation of non-dusty biomass containing (or not) granular animal feed particles by spray drying the fermentation broth, which are converted into pellets by means of costly high shear mixing equipment. European Application Number 91460051.5 describes a method of making a dust free, free-flowing

granulated L-lysine product from a liquid solution or slurry by a spray granulation process.

Care should be taken either to use or to properly dispose the cell rich L-lysine broth, which is frequently treated as a waste by-product. Occasionally, non-granular L-lysine feed supplement with an adjustable amount of L-lysine purity is desirable on economic grounds. International Publication Number WO9523129 and application Ser. No. 08991145 (filed on Dec. 16, 1997) describe the production of non-stoichiometric salts of L-lysine in granular form, wherein the amount of L-Lysine content in the final product is adjustable. The spray granulation step can be replaced with alternative methods of drying such as spray drying, drum drying, rotary drying, tray drying, and tunnel drying [4]. Additionally, an animal feed additive in form of stable pellets is produced by pelletizing from a fermentation broth. The biomass may be completely present, partly present or not present at all in the pellets [82]. US3089824 [83] Describes the use of a fluidized bed for the manufacturing of compressed tablets for medical use. Moreover, [12] provides L-lysine feedstuffs additives that contain no, only traces or 90-100% of biomass and/or constituents from the fermentation broth formed.

AQUEOUS L-LYSINE FOOD ADDITIVE

Alternatively, [84] describes various processes relating to the production of an aqueous cell-free feed supplement containing between 300 and 600 g/l L-lysine, thus fulfilling various specific needs of individual customers. L-lysine content does not solely depend on L-lysine concentration in bioreactor and cell free amino acid solution can additionally be purified by chromatography columns, e.g. by an ion exchange column. The above process may be applied to any amino acid which can be produced by fermentation, e.g. L-lysine, threonine and tryptophan [84].

CURRENT & FUTURE DEVELOPMENTS

L-lysine production by means of fermentation methods applying a variety of mutant strains of corynebacteria and other microorganisms obtained either by classical mutagenesis or recombinant technology still continues to remain a first runner in biotechnological production of bulk chemicals. It is assumed that no other microbial process has been undertaken to such extensive efforts of gaining better mutant strains. A little work has been devoted to the fermentation process development and optimization, still leaving large opportunities for further improvements in terms of improving productivity concentration and yield of L-lysine. The results (about 100 g/l L-lysine) obtained in continuous cultures (chemostat) at low residence times using a regular L-lysine producing ATCC strain (Anastassiadis and Stephanopoulos, 1994-1996, MIT, USA) confirmed those thoughts. In comparison, the legendary fermentative production of citric acid is reflecting the development of fermentation technology and process development occurring in the last 100 years of modern biotechnology.

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