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The L-Lysine Story: From Metabolic Pathways to Industrial Production

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Abstract L-lysine is an essential amino acid required for nutrition of animals and humans. It has to be present in food and feed, which, in many cases, is realized by supplementation of the feed-stuffs with pure lysine. The high importance of lysine in nutrition has stimulated intensive research on the lysine biosynthetic pathways and their regulation and the search for microorganisms capable of over-producing this amino acid. As an important milestone, the glutamate producing soil bacterium *Corynebacterium glutamicum* was isolated in 1956 and soon received interest to be used for production of another amino acid stemming from the TCA cycle: lysine. Within a few years the first lysine producing strains were obtained. The past 50 years following the discovery of *C. glutamicum* were characterized by a huge progress towards understanding the physiology of this organism and developing and optimizing industrial production strains. This has resulted in effective biotechnological processes currently used for producing about 750 000 tons of lysine per year. Today, systems-oriented approaches aiming at investigating the link between the different components of cellular physiology such as transcriptome, fluxome and metabolome, provide a novel powerful platform that will surely drive future research towards holistic understanding of lysine over-producing microorganisms as well as the creation of superior production strains.

1 Introduction

The essential amino acid L-lysine is one of the most important amino acids applied as supplement in animal feed. Animal feed, which is typically based on corn, wheat or barley, is poor in lysine. The supplementation of such feed materials with a lysine rich source leads to optimized growth of e.g. pigs or chicken. The direct addition of lysine hereby has proven especially valuable. It does not cause an extra uptake and metabolization of other amino acids beyond their need so that superfluous formation of ammonia and environmental burden by increased nitrogen loads in the manure is avoided. The continuing development of an increased consumption of white meat in various countries of the western as well as the eastern world has led to an enormous market growth for lysine during the past decades (Fig. 1). Currently, the world-wide production of lysine is about 750 000 tons with a predicted market growth over the next years of about 8%. Since only the L-isomer of lysine is effective as feed supplement, all manufacturing processes utilize fermentative production (Leuchtenberger 1996). The cradle of biotechnological lysine production stands in Japan. Stimulated by the increased demand for amino acids, especially glutamate, a large screening program was initiated in Japan about 50 years ago which, in 1956, led to the discovery of the glutamate excreting microorganism *Corynebacterium glutamicum* (Kinoshita et al. 1957; Udaka 1960). Within a few years, the first lysine excreting mutants of *C. glutamicum* were available and applied for production in large scale (Kelle et al. 2005; Kinoshita et al. 1961). Since then a continuous optimization of producing strains and processes has led to efficient manufacturing of lysine from renewable resources covering the high demand for this compound required today (de Graaf et al. 2001; Eggeling and Sahm 1999; Hermann 2003; Pfefferle et al. 2003).

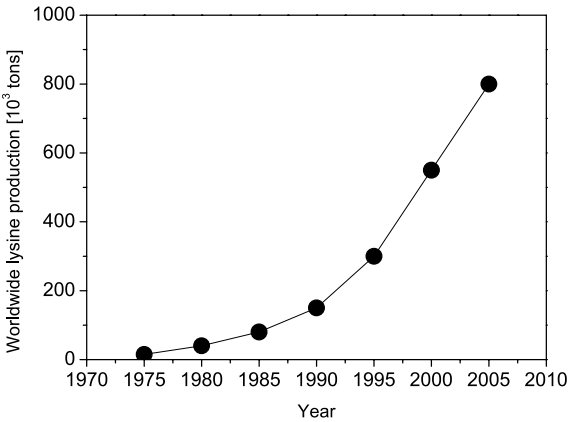


Fig. 1 Development of the world-wide annual biotechnological production of L-lysine

2 Lysine Biosynthetic Pathways

In microorganisms, lysine can be synthesized by two completely different routes: either from 2-oxoglutarate and acetyl-CoA via the α-aminoadipate route or from aspartate via the diaminopimelate route. Two variants of the α-aminoadipate route (for review see Velasco et al. 2002 and references therein) occur in higher fungi and in archaea like *Thermoproteus neutrophilus* on the one hand and in the bacterium *Thermus thermophilus* on the other hand. In five reactions that are catalyzed by homoisocitrate synthase (EC 4.1.3.21), homoaconitate hydratase/cis-homoaconitase (EC 4.2.1.36) and homoisocitrate dehydrogenase (EC 1.1.1.155), 2-oxoglutarate and acetyl-CoA are converted to α-aminoadipate. In one variant of the pathway, α-aminoadipate is converted to lysine by α-aminoadipate reductase (EC 1.2.1.32), saccharopine reductase (EC 1.5.1.10) and saccharopine dehydrogenase (EC 1.5.1.7 or 1.5.1.8). In another variant of the pathway, first described in *Thermus thermophilus*, the conversion of α-aminoadipate to lysine occurs via the acetylated intermediates N^2-acetyl-L-α-aminoadipate, N^2-acetyl-L-aminoadipyl-δ-phosphate, N^2-acetyl-L-α-aminoadipate semialdehyde and N^2-acetyl-L-lysine catalyzed by the gene products of *lysX*, *lysZ*, *lysY* and either *lysJ* or *argD* and either *lysK* or *argE*.

In bacteria and plants, lysine may be synthesized from aspartate by one or several of four variants of the diaminopimelate route. These pathway variants diverge at the common intermediate tetrahydrodipicolinate (Born and Blanchard 1999; Schrumpf et al. 1991; McCoy et al. 2006). As shown in Fig. 2, one of these pathways involves succinylated intermediates, while the acetylase pathway comprises acetylated intermediates and the so-called dehydrogenase pathway directly forms DL-diaminopimelate from tetrahydrodipicolinate (Schrumpf et al. 1991; Wehrmann et al. 1998). The aminotransferase pathway, recently described to operate in *Chlamydia*, converts tetrahydrodipicolinate to LL-diaminopimelate, which can then be epimerized to DL-diaminopimelate (McCoy et al. 2006). Most bacteria only comprise one of these pathways (Bartlett and White 1985; White 1983). Whereas the succinylase pathway is present both in gram negative and gram positive bacteria, the acetylase variant seems to be exclusively used by some *Bacillus* species (Bartlett and White 1985; Born and Blanchard 1999; Weinberger and Gilvarg 1970). Only in a few organisms like different species of the genera *Corynebacterium* and in *Bacillus macerans* two lysine biosynthetic pathways operate together (Bartlett and White 1985; Malumbres and Martin 1996; Schrumpf et al. 1991). A common feature involved in the different pathways concerns the regulation of pathway flux by feedback inhibition of aspartate kinase. In *C. glutamicum* only one isoenzyme of aspartate kinase (or aspartokinase) exists, which is encoded by two genes, *lysC*α and *lysC*β, representing the coding sequences for the two subunits of the enzyme (Kalinowski et al. 1990). Its activity is bio-

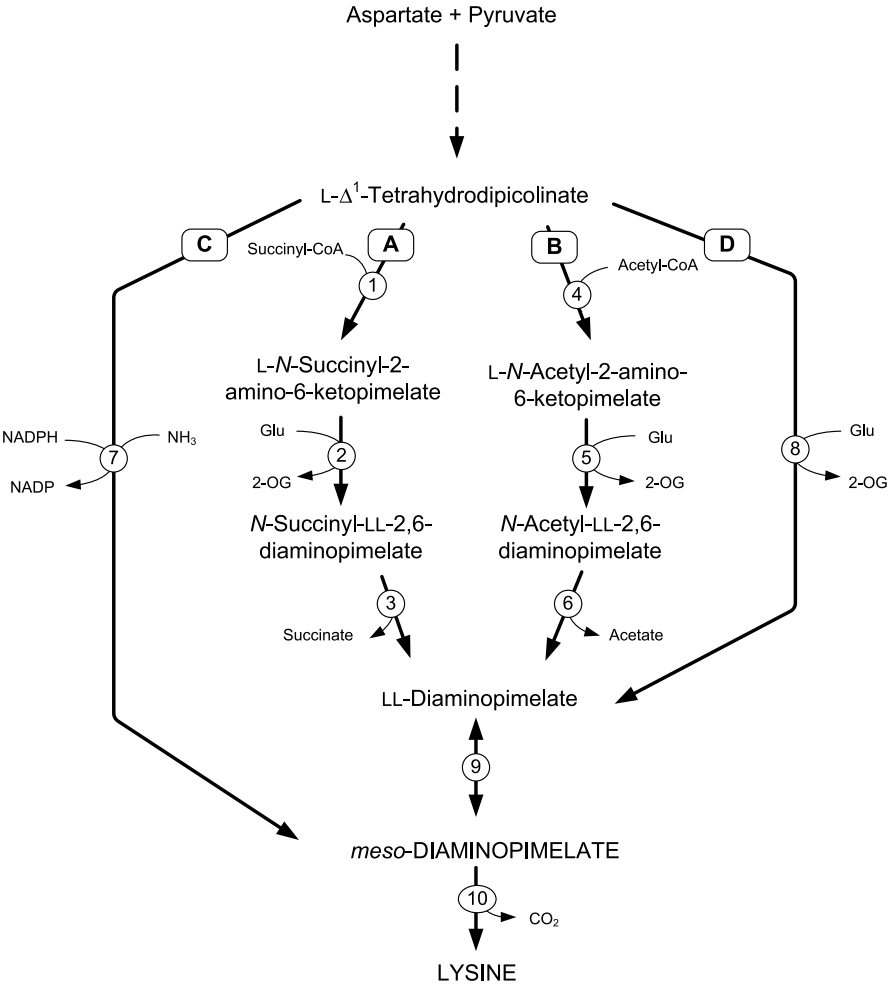


Fig. 2 The four pathways of D,L-diaminopimelate and lysine synthesis from aspartate in prokaryotes: succinylase pathway (**A**); acetylase pathway (**B**), dehydrogenase pathway (**C**), and aminotransferase pathway (**D**). Enzymes involved in the succinylase pathway are: (1) tetrahydrodipicolinate succinylase (DapD); (2) *N*-succinyl-aminoketopimelate aminotransferase (DapC); and (3) *N*-succinyl-diaminopimelate desuccinylase (DapE). Enzymes of the acetylase pathway are: (4) tetrahydrodipicolinate acetylase; (5) N-acetyl-aminoketopimelate aminotransferase; and (6) *N*-acetyl-diaminopimelate deacetylase. Via the aminotransferase pathway, L,L-diaminopimelate is formed by (8) tetrahydrodipicolinate aminotransferase. (9) Diaminopimelate epimerase (DapF) is common for these three pathways. The dehydrogenase pathway directly forms D,L-diaminopimelate via (7) diaminopimelate dehydrogenase (Ddh). Lysine is built from D,L-diaminopimelate by (10) diaminopimelate decarboxylase

chemically regulated through concerted feedback inhibition by lysine and threonine that bind at the regulatory β-subunits (Kalinowski et al. 1991; Malumbres and Martin 1996). A similar regulation has been described for lysine synthesis in *B. megaterium* which, however, has two different isoenzymes. One of the present isoenzymes is also inhibited by a concerted action of two amino acids, which are lysine and methionine, whereas the other isoenzyme is only subject to inhibition by threonine (Chatterjee and White 1982). Additionally to the modulation of enzyme activity by metabolites, some bacteria e.g. *E. coli* and *B. megaterium* also show a transcriptional regulation for enzymes involved in lysine biosynthesis (Chatterjee and White 1982; Cremer et al. 1988).

3 *Corynebacterium glutamicum* as a Production Organism

Corynebacterium glutamicum, including its subspecies *Brevibacterium flavum*, *Brevibacterium lactofermentum*, *Corynebacterium lilium*, *Corynebacterium efficiens* and *Brevibacterium divaricatum* (Liebl 2005; Liebl et al. 1991) is the most important organism for industrial lysine production. The only other species used for lysine production are recombinant *E. coli* strains (Imaizumi et al. 2005, 2006). The capability of *C. glutamicum* to secrete amino acids was discovered in the 1950s (Kinoshita et al. 1957; Udaka 1960). It is a gram-positive, rod-like, non-motile, aerobic bacterium (Fig. 3). Fur-

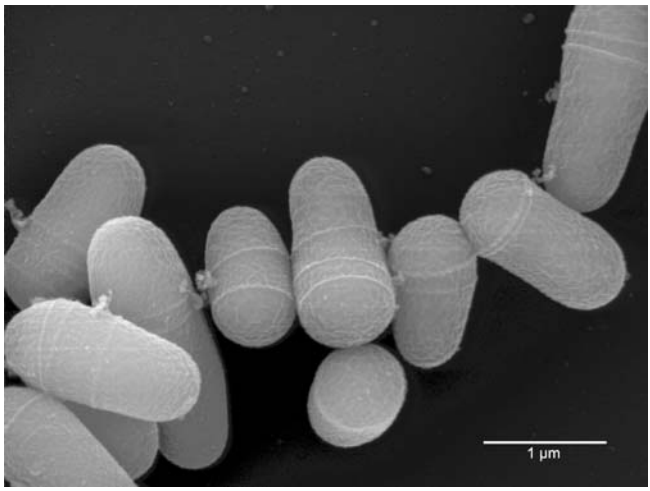
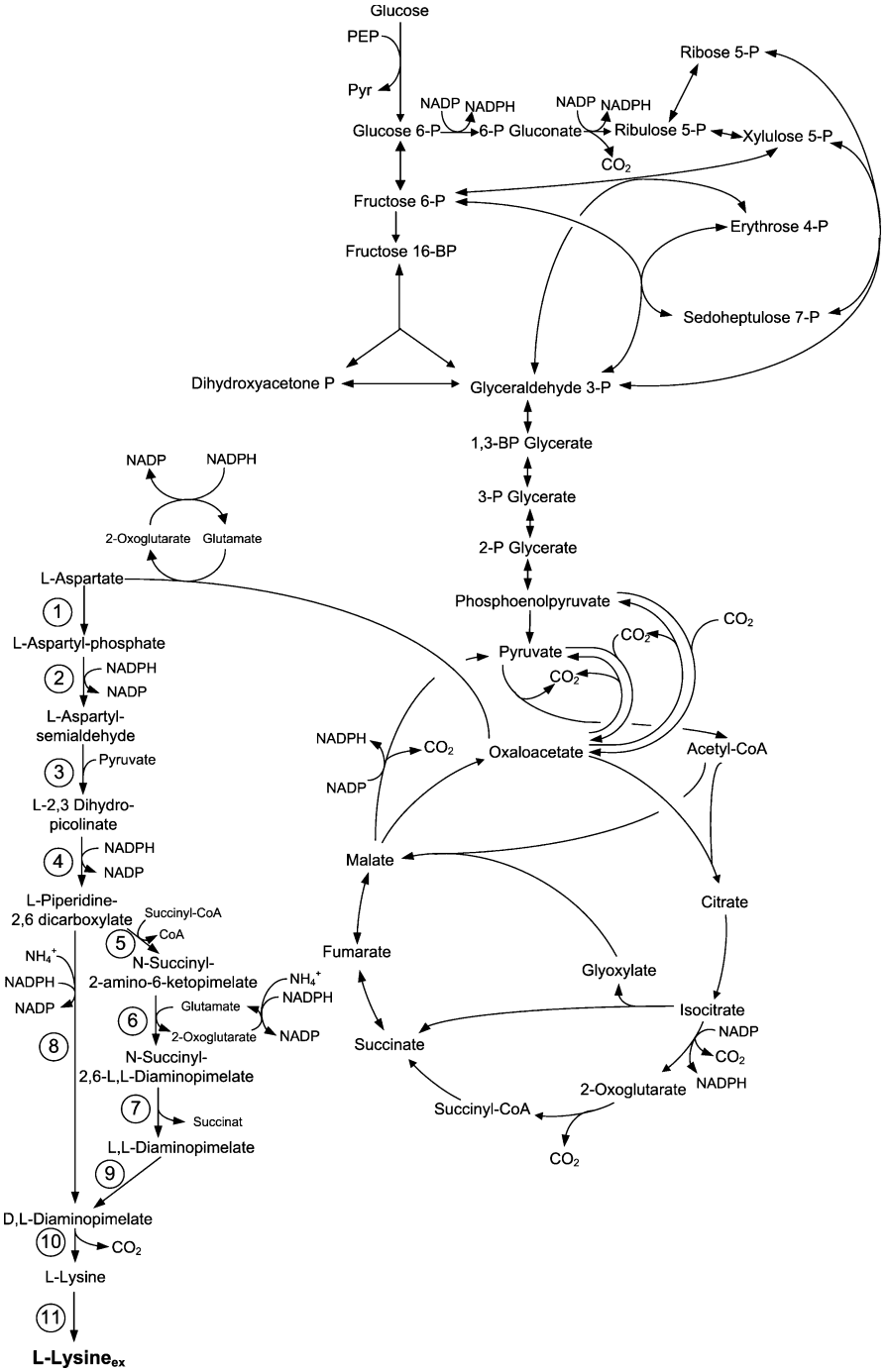


Fig. 3 Raster electron micrograph of *Corynebacterium glutamicum* ATCC 13032 cultivated on minimal glucose medium. Samples were taken during exponential growth (Krömer, Heinzle and Wittmann 2006, unpublished results)


Glucose
PEP
Pyr
Glucose 6-P
NADP
NADPH
6-P Gluconate
Ribose 5-P
Ribulose 5-P
Xylulose 5-P
CO2
Fructose 6-P
Erythrose 4-P
Fructose 16-BP
Sedoheptulose 7-P
Dihydroxyacetone P
Glyceraldehyde 3-P
1,3-BP Glycerate
3-P Glycerate
2-P Glycerate
Phosphoenolpyruvate
Pyruvate
2-Oxoglutarate
Glutamate
L-Aspartate
L-Aspartyl-phosphate
L-Aspartyl-semialdehyde
L-2,3 Dihydro-picolinate
L-Piperidine-2,6 dicarboxylate
Succinyl-CoA
CoA
N-Succinyl-2-amino-6-ketopimelate
NH4+
Glutamate
N-Succinyl-2,6-L,L-Diaminopimelate
Succinat
L,L-Diaminopimelate
D,L-Diaminopimelate
L-Lysine
L-Lysine_ex
Oxaloacetate
Acetyl-CoA
Malate
Citrate
Fumarate
Glyoxylate
Isocitrate
Succinate
2-Oxoglutarate
Succinyl-CoA

◀ **Fig. 4** Central metabolic pathways and lysine biosynthesis in *Corynebacterium glutamicum*. The numbers given for the reactions in the lysine biosynthesis pathway relate to Table 1, where further details, for example on the corresponding enzymes, the encoding genes, and the patents claimed by major industrial players in the field are given

ther characteristics comprise a cell wall with arabino-galactan and mycolic acids with 26 to 36 carbon atoms and a murein sacculus with peptido-glycan cross linked via meso-diaminopimelic acid (Goodfellow et al. 1976). The GC-content of its genome is 53.8% (Kalinowski et al. 2003). Extensive biochemical studies in the last decades as reviewed in a recent handbook on *Corynebacterium glutamicum* (Eggeling and Bott 2005) and the recent unravelling of the genome sequence (Haberhauer et al. 2001; Ikeda and Nakagawa 2003; Tauch et al. 2002; Kalinowski et al. 2003) have contributed to a detailed knowledge of the reactions of lysine biosynthesis and central metabolism in this micro-organism (Fig. 4).

3.1 Lysine Biosynthesis

Lysine belongs to the aspartate amino acid family and in *C. glutamicum* is produced from pyruvate, oxaloacetate and two ammonia molecules involving the additional supply of four NADPH as reducing power (Michal 1999). Interestingly, the organism has a split pathway for the biosynthesis of lysine (Schrumpf et al. 1991; Sonntag et al. 1993). The two alternative branches give *C. glutamicum* an increased flexibility in response to changing environmental conditions, involving e.g. different ammonia levels (Sahm et al. 2000). DL-diaminopimelate as intermediate of the lysine pathway additionally is an essential building block for the synthesis of the murein sacculus (Wehrmann et al. 1998). Concerning regulation of lysine biosynthesis, aspartokinase (EC 2.7.2.4), catalyzing the formation of aspartyl phosphate from aspartate is the key enzyme. It is feedback regulated by concerted action of lysine and threonine (Kalinowski et al. 1991). Via the requirement for building blocks and cofactors lysine formation is closely linked to the central metabolism. The enzymes involved in lysine biosynthesis are summarized in Table 1.

3.2 Central Carbon Metabolism

The most relevant substrates for industrial lysine production are starch and molasses. They are based on glucose, fructose, and sucrose as major carbon sources. These compounds are taken up via phosphoenolpyruvate-dependent phosphotransferase systems (Dominguez and Lindley 1996; Dominguez et al. 1998; Malin and Bourd 1991; Moon et al. 2005). The carbon source has an in-

Table 1 Genes and enzymes involved in L-lysine biosynthesis in *C. glutamicum*. The information on patents and patent applications by the lysine producing companies Archer Daniels Midland (■), Ajinomoto (▲), BASF(●), Degussa (♦) and Kyowa Hakko Kogyo (▼) claiming an improvement of lysine production through modification of the lysine biosynthetic pathway in *C. glutamicum* is taken from Kelle et al. (2005). The reaction numbers relate to the corresponding metabolic reactions of lysine biosynthesis in the metabolic network shown in Fig. 4

Gene	Enzyme	EC number	Inhibitory ligands	Transcription unit	Transcriptional regulation	Reference	Reaction number	Patents
lysC	aspartate kinase	2.7.2.4	lysine threonine	lysC		(Kalinowski et al. 1991)	1	■ ▲ ● ♦ ▼
asd	aspartate semialdehyde dehydrogenase	1.2.1.11	lysine threonine	asd		(Cremer et al. 1988)	2	■ ● ▼
dapA	dihydrodipicolinate synthase	4.2.1.52		dapB-orf2-dapA-orf4		(Patek et al. 1997)	3	■ ▲ ● ♦ ▼
dapB	dihydrodipicolinate reductase	1.3.1.26		dapB-orf2-dapA-orf4		(Patek et al. 1997)	4	■ ▲ ● ♦ ▼
dapD	tetrahydrodipicolinate succinylase	2.3.1.117		dapD		(Wehrmann et al. 1998)	5	● ▼
dapC	succinyl-amino-keto-pimelate transaminase	2.6.1.17		dapC		(Hartmann et al. 2003)	6	● ♦ ▼
dapE	succinyl-diamino-pimelate desuccinylase	3.5.1.18		dapE		(Wehrmann et al. 1998)	7	● ▼
ddh	meso-diaminopimelate dehydrogenase	1.4.1.16		ddh		(Cremer et al. 1988)	8	■ ▲ ● ♦ ▼
dapF	diaminopimelate epimerase	5.1.1.7		dapF		(Hartmann et al. 2003)	9	● ♦ ▼
lysA	diaminopimelate decarboxylase	4.1.1.20		lysA	inhibited by lysine	(Cremer et al. 1988)	10	■ ▲ ● ▼
lysE	lysine permease			lysE	induced by lysine	(Vrljic et al. 1996)	11	● ♦ ▼

fluence on lysine production by *C. glutamicum*, which is related to the resulting different entry points into the central metabolism (Kiefer et al. 2002). The central catabolic network previously identified in *C. glutamicum* comprises the pathways of glycolysis, pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle and glyoxylate cycle, while the Entner–Doudoroff pathway has not been detected (Eikmanns 2005; Kalinowski et al. 2003; Yokota and Lindley 2005). The oxidative part of the PPP comprises the NADPH dependent enzymes glucose 6-phosphate dehydrogenase (EC 1.1.1.49) (Ihnen and Demain 1969; Moritz et al. 2000) and 6-phophogluconate dehydrogenase (1.1.1.44) (Moritz et al. 2000; Ohnishi et al. 2005). Both enzymes were intensively studied regarding their kinetic properties (Moritz et al. 2000). *C. glutamicum* exhibits an extensive set of different enzymes for the interconversion of C_4 metabolites of the TCA cycle and C_3 metabolites of glycolysis (Fig. 4). For anaplerotic replenishment of the TCA cycle *C. glutamicum* possesses pyruvate carboxylase (EC 6.4.1.1) (Peters-Wendisch et al. 1998) and phosphoenolpyruvate carboxylase (EC 4.1.1.31). Malic enzyme (EC 1.1.1.40) (Gourdon et al. 2000), PEP carboxykinase (EC 4.1.1.32) (Jetten and Sinskey 1993; Riedel et al. 2001), oxaloacetate decarboxylase (EC 4.1.1.3) (Jetten and Sinskey 1995) and probably phosphoenolpyruvate synthetase (Jetten et al. 1994), of which the genes of oxaloacetate decarboxylase and phosphoenolpyruvate synthetase have not been found yet, catalyze decarboxylation reactions from the TCA cycle towards glycolysis. In selected cases, NADPH supply by malic enzyme supports growth and lysine production of *C. glutamicum* (Dominguez et al. 1998; Kim et al. 2006). However, deletion of *malE* does not affect growth of *C. glutamicum* on glucose or acetate, suggesting that the metabolic pathways involved in NADPH-production are highly flexible (Gourdon et al. 2000). It has been proposed that cyclic cooperation of the enzymes between pyruvate/phosphoenolpyruvate and malate/oxaloacetate is involved in the regeneration of excess ATP (Marx et al. 1996; Petersen et al. 2000; Riedel et al. 2001).

In addition to the central catabolic routes, also anabolic pathways have been studied in detail providing detailed information on the cellular composition and the precursor demand for growth (Wittmann and de Graaf 2005). Overall about (16.4 mmol NADPH) g^{-1} is required for biomass synthesis. It is obvious that the anabolic NADPH requirement competes with lysine production. Considering a biomass yield of 0.5 (g dry biomass) (g glucose)$^{-1}$, which is achieved by *C. glutamicum* under aerobic conditions this results in 1.7 mol NADPH (mol glucose)$^{-1}$ that have to be generated by the NADPH forming reactions in the PPP and the TCA cycle. It should be noted that the knowledge about the metabolic network is continuously being updated with novel findings such as the detection of pyruvate carboxylase as anaplerotic enzyme (Peters-Wendisch et al. 1998) or the consideration of new reactions for growth on specific substrates such as sucrose (Dominguez and Lindley 1996).

3.3 Maximal Lysine Production Capacity

The maximal capacity, i.e. the theoretical maximum yield, of a *C. glutamicum* cell for lysine production is an important characteristic, since it provides an estimate of the remaining optimization potential of a running industrial process and gives advice for process or genetic engineers. Previous stoichiometric calculation considering only the major pathways involved in lysine production have yielded a molar lysine yield on glucose of 75% (Hollander 1994). A more detailed insight can be gained via stoichiometric network analysis considering the full set of available pathways in the central metabolism with information on reversibility or irreversibility of the different reactions and additional assumptions and restrictions posed on the metabolic network. This approach, named elementary flux mode analysis (Schilling et al. 1999; Schuster et al. 2002), has been recently applied to elucidate the potential of *C. glutamicum* for methionine production (Krömer et al. 2006). The theoretical maximum molar yield of *C. glutamicum* for lysine production obtained by such an analysis is 82% (Fig. 5). The in silico pathway analysis also provides information on the reactions contributing to this theoretical optimum, i.e. the corresponding theoretical metabolic flux distribution. Shown for one of the optimal elementary flux modes, i.e. optimal lysine production, PPP and malic enzyme supply the required NADPH (Fig. 5). Hereby the concerted action of pyruvate carboxylase, malate dehydrogenase (EC 1.1.1.37) and malic enzyme form a transhydrogenase-like cycle converting NADH into NADPH and thus providing an additional amount of this important cofactor. This metabolic cycle is different from a previously described cyclic pathway involving PCx, PCK, and MDH as shown to operate in vivo (Petersen et al. 2000; Riedel et al. 2001). One should further notice that, in addition to the soluble malate dehydrogenase *C. glutamicum* possesses another enzyme for interconversion of oxaloacetate and malate, the membrane-bound malate:quinone oxidoreductase (MQO, EC 1.1.99.16). Both enzymes probably catalyze a cyclic reaction in vivo leading to a net oxidation of NADH (Molenaar et al. 2000). The dehydrogenase branch of lysine biosynthesis is the sole lysine producing route. Admittedly, the given scenario implies zero flux through the TCA cycle and no biomass formation, which can hardly be realized. The achievable optimum can be expected to be somewhat lower than the value calculated here, but should still be significantly higher than the yield achieved in practice, which is in the range of about 55% (Ikeda 2003; Kawahara et al. 1990).

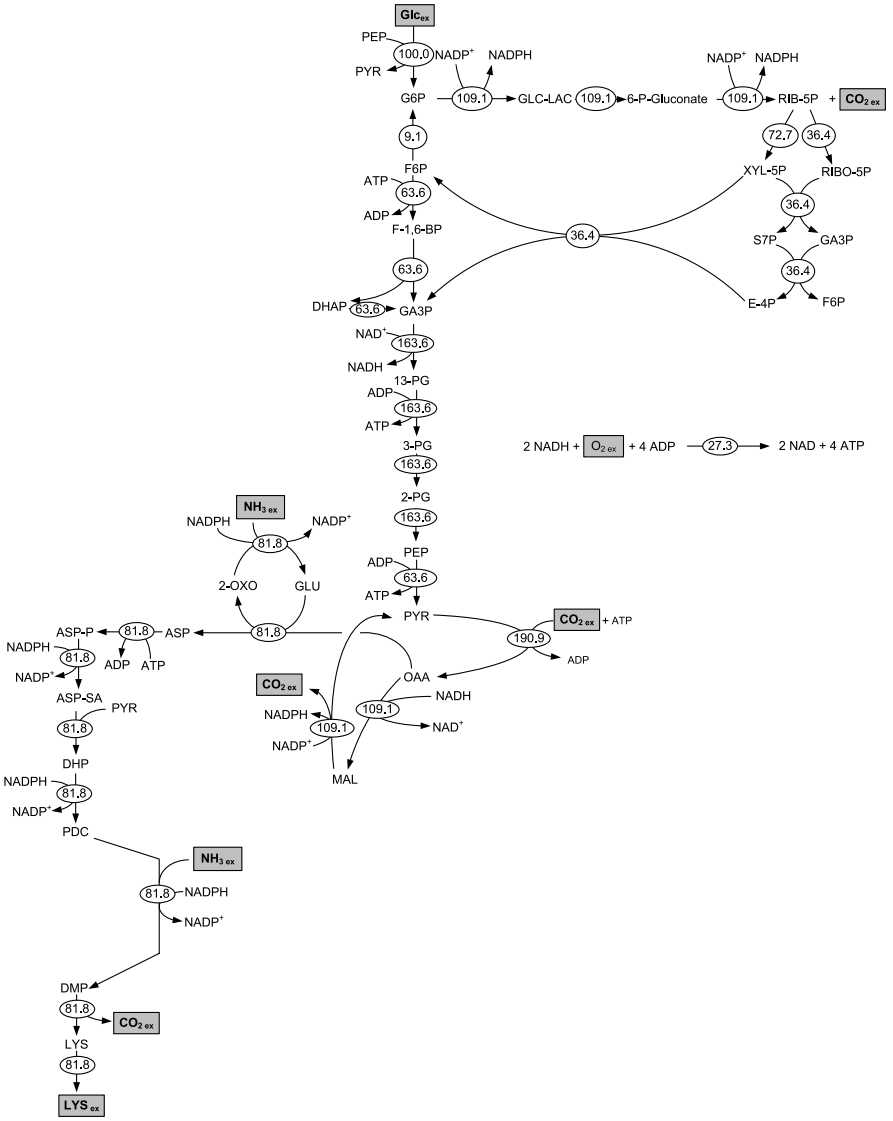


Fig. 5 Metabolic flux distribution of *Corynebacterium glutamicum* with maximal theoretical lysine carbon yield. All fluxes are given as relative molar fluxes to the glucose uptake in mol $(\text{mol})^{-1} \times 100$ (Krömer, Heinzle and Wittmann 2006, unpublished). One should note that additionally another elementary mode with optimal production exists which, however, only slightly differs in reaction steps involved in cofactor metabolism

4
Strain Engineering

After the discovery of its ability to produce and excrete amino acids (Kinoshita et al. 1957), *C. glutamicum* was used to establish a biotechnological production process for several amino acids. Through the years various methods for strain engineering have been developed to create more efficient production strains.

4.1
Classical Engineering

The first production strains were created within a few years after the discovery of *C. glutamicum* using an iterative procedure of random mutagenesis with UV light or chemical mutagens and subsequent strain selection (Nakayama et al. 1978). The key to success in these days was the use of toxic lysine analogues, such as S-(2-aminoethyl) cysteine, to screen for feedback resistant strains (Nakayama and Araki 1973). These strains later all revealed point mutations in the aspartokinase gene, through which the encoded enzyme was released from feedback inhibition by lysine and threonine (Kalinowski et al. 1991; Thierbach et al. 1990). This modification displays one of the most important characteristics of lysine production strains at all. Consequently, also strains were developed which exhibited a weakened or even blocked biosynthesis of threonine, i.e. auxotrophy for threonine (Nakayama and Araki 1973). Through further cycles of mutagenesis and selection strains with different auxotrophies for other amino acids, vitamins and resistance to other anti-metabolites were obtained (Kelle et al. 2005). The subsequent mutants from such a strain genealogy exhibited a stepwise improvement of production (Schrumpf et al. 1992; Wittmann and Heinzle 2002). Remarkable production properties such as a conversion yield up to 50% and a lysine·HCl titre above 100 g L^{-1} were achieved with such classically derived strains (Ikeda 2003; Leuchtenberger 1996). The additional nutrient requirement and the weak stress tolerance, due to the large number of undesired mutations accumulated during strain development (Ohnishi et al. 2002), display, however, severe disadvantages of conventional production strains and stimulated targeted approaches for strain optimization. After the gene targets improving lysine production had been identified (see below), it became possible to introduce mutant alleles of these genes isolated from the classically obtained producer strains into the wild type to generate stable and stress-tolerant lysine producer strains without additional nutrient requirements (Georgi et al. 2005; Hayashi et al. 2006a; Ohnishi et al. 2002, 2005).

4.2 Metabolic Engineering of Lysine Biosynthesis

The possibility to perform targeted genetic modifications through developments of molecular biology and genetic engineering tools initiated a number of efforts towards rational optimization to *C. glutamicum* (Ohnishi et al. 2002, 2005; Sahm et al. 2000). Logically, many of these studies have focussed on the optimization of the flux through the lysine biosynthesis by directly modifying enzymes of the pathway (Fig. 5). The modification of three of the enzymes, i.e. aspartate kinase (LysC), dihydrodipicolinate synthase (DapA, EC 4.2.1.52) and the lysine exporter (LysE), was especially valuable with respect to improvement of lysine production (Fig. 6). Aspartate kinase is the key enzyme with regard to metabolic control of the lysine pathway as it is subject to a feedback inhibition by threonine and lysine (Kalinowski et al. 1991; Malumbres and Martin 1996). Different point mutations in the *lysC* gene, i.e. in the region coding for its regulatory β-subunit, have been shown to release the enzyme from feedback control and lead to enhanced lysine formation (Cremer et al. 1991; Follettie et al. 1993; Kalinowski et al. 1991; Sugimoto et al. 1997). Similarly, also overexpression of the aspartate kinase gene stimulated production (Jetten et al. 1995). Today, the release of aspartate kinase from feedback control is regarded as one of the most important features of industrial lysine producer strains. This is also underlined by the various patents claiming different amino acid exchanges in this enzyme (Table 1) (Kelle et al.

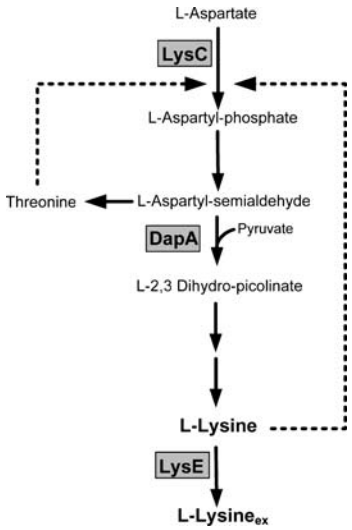


Fig. 6 Lysine biosynthetic pathway in *Corynebacterium glutamicum*. Metabolic regulation through feedback inhibition of asparate kinase by concerted action of lysine and threonine and important targets for optimization of lysine overproduction

2005). Plasmid encoded amplified expression of the *dapA* gene significantly increases lysine (Bonnassie et al. 1990; Cremer et al. 1991; Eggeling et al. 1998; Pisabarro et al. 1993). Amplification of *dapA* expression was further achieved through an extensive mutation of the promoter sequence (Vasicova et al. 1999), whereby a hot spot was discovered at the – 10 region (de Graaf et al. 2001). Also overproduction of diaminopimelate epimerase (DapF, EC 5.1.1.7) and succinyl-aminoketopimelate transaminase (DapC, EC 2.6.1.17), two enzymes of the succinylase branch, was beneficial for lysine formation (Kelle et al. 2005). A striking discovery with respect to lysine production was the discovery of the lysine exporter (LysE) and the subsequent overexpression of the *lysE* gene which resulted in an increased lysine secretion rate (Bellmann et al. 2001; Vrljic et al. 1996, 1999). The recently performed expression of *lysE* from *C. glutamicum* in a *Methylophilus methylotrophus* lysine producing strain was shown to also improve lysine production from methanol by this organism (Gunji and Yasueda 2006). Summarizing, the importance of engineering enzymes of the lysine pathway for efficient lysine production is underlined by the fact that today every single gene of the lysine biosynthetic pathway is covered with one or several patents by the major players in the field (Table 1).

4.3 Metabolic Engineering of NADPH Metabolism

NADPH is consumed by the lysine biosynthetic pathway in four steps either directly or indirectly through the assimilation of ammonium and its efficient supply appears crucial for lysine overproduction. A detailed insight into the NADPH metabolism of *C. glutamicum* has been obtained by ^{13}C metabolic flux studies under various physiological conditions (Becker et al. 2005; Kiefer et al. 2004; Marx et al. 1996, 1997; Sonntag et al. 1995; Wittmann and de Graaf 2005; Wittmann and Heinzle 2001a, 2002; Wittmann et al. 2004a). These allow establishing a NADPH balance. Glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and isocitrate dehydrogenase (EC 1.1.1.42), which uses NADP as cofactor in *C. glutamicum* (Chen and Yang 2000; Eikmanns et al. 1995), are hereby considered as NADPH-generating reactions, whereas NADPH-consuming reactions comprise lysine production and growth with a stoichiometric demand of 16.4 mmol NADPH (g biomass)$^{-1}$ (Wittmann and de Graaf 2005). Figure 7A illustrates that the NADPH metabolism of *C. glutamicum* is highly flexible adjusting to the given overall physiological needs of the cell. NADPH supply and consumption vary depending on the physiological growth state (Marx et al. 1997), the applied carbon source (Dominguez et al. 1998; Wendisch et al. 2000; Wittmann et al. 2004a), or the genetic background (Marx et al. 1999, 2003). In most cases this results in an apparent NADPH excess which seems to increase with diminishing NADPH oxidation. In selected cases even an apparent limitation for NADPH is observed. Examples are phases of maximal lysine production dur-

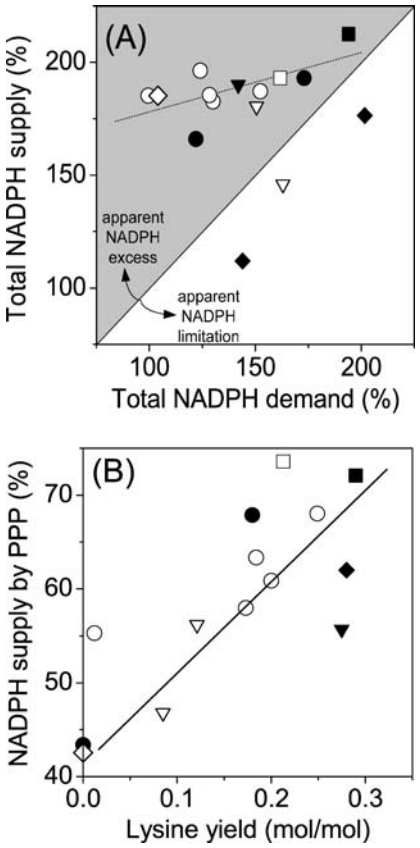


Fig. 7 NADPH metabolism of different *C. glutamicum* strains under various cultivation conditions as revealed by ^{13}C flux analysis: Overall flux through NADPH supply and demand (**A**), flux through the lysine pathway and the PPP (**B**). NADPH supply considers G6P dehydrogenase, 6PG dehydrogenase, and isocitrate dehydrogenase. Lysine production (4 NADPH/lysine) and anabolism are considered as NADPH-consuming reactions. Hereby, the NADPH demand for biomass formation, 16.4 mmol (g cell dry mass)$^{-1}$, was based on a detailed analysis of cellular composition (Wittmann and de Graaf 2005). Studies involved flux analysis of (i) non-producing *C. glutamicum* ATCC 13032 in batch culture on glucose (*open diamond*) (Sonntag et al. 1995), (ii) non-producing *C. glutamicum* ATCC 13032 and lysine producing *C. glutamicum* ATCC 13287, ATCC 21253, ATCC 21526, and ATCC 21543 in batch culture on glucose (*open circle*) (Wittmann and Heinzle 2002), (iii) lysine producing *C. glutamicum* ATCC 21253 during maximum production phase in batch culture on glucose (*open square*) (Wittmann and Heinzle 2001a), (iv) non-producing *C. glutamicum* LE4 and lysine producing *C. glutamicum* MH 20–22B in continuous culture on glucose (*closed circles*) (Marx et al. 1997), (v) lysine-producing *C. glutamicum* MH 20–22B in continuous culture on glucose (*closed square*) (Marx et al. 1996), lysine-producing *C. glutamicum* ATCC 21526 in batch culture on glucose, fructose (*closed diamond*) (Kiefer et al. 2004) and on sucrose (*closed triangle*) (Wittmann et al. 2004a), and lysine-producing *C. glutamicum lysC*^{fbr} and *C. glutamicum lysC*^{fbr} P_{EFTU}fbp during batch culture on glucose (*open triangle*) (Becker et al. 2005)

ing batch culture or production on fructose allowing only a small PPP flux (Kiefer et al. 2004).

The apparent imbalance between supply and demand for NADPH indicates that in *C. glutamicum* so far unassigned metabolic reactions are active in vivo, which either consume or supply NADPH. Possible candidates for NADPH consumption comprise superoxide-generating NADPH oxidase in the respiratory chain (Matsushita et al. 2001) or a metabolic cycle around the pyruvate node involving malic enzyme as hypothesized previously (de Graaf 2000; Petersen et al. 2000). Clear experimental evidence supporting the role of either one of these reactions has not been obtained to date. However, regarding the transhydrogenase-like cycle consisting of pyruvate carboxylase, malate dehydrogenase, and PEP carboxykinase, it has been shown that deletion of *pckA* improves lysine production and *pckA* overexpression reduces lysine production (Riedel et al. 2001) due to altered fluxes within this transhydrogenase-like cycle (Petersen et al. 2001). Concerning NADPH supply, the contribution of malic enzyme has been demonstrated for growth on fructose (Dominguez et al. 1998). Under these conditions cells carry an extremely low PPP flux due to an unfavorable entry point of the substrate into the central metabolism which probably activates malic enzyme (Kiefer et al. 2004). However, overexpression of the malic enzyme gene *malE* in a genetically defined lysine producing strain did neither improve lysine product yields on glucose nor on fructose or sucrose (Georgi et al. 2005).

Since the unassigned NADPH consumption flux becomes minimal with increasing lysine yield (dashed line in Fig. 7A) or NADPH even shows an apparent deficiency, a NADPH limitation of lysine production appears likely. This and the simple fact that four NADPH are required for synthesis of one lysine has stimulated metabolic engineering of the NADPH supply in *C. glutamicum* (Marx et al. 1999, 2003). As a prerequisite for successful modification of the NADPH metabolism, the key pathways supplying NADPH for lysine production in *C. glutamicum* were identified by different approaches. Stoichiometric investigation of the lysine network in the early 1990s already predicted that an increased lysine yield is linked to an increased flux through the PPP and a decreased flux through the TCA cycle (Kiss and Stephanopoulos 1992). The importance of the PPP for efficient lysine production was later shown by metabolic flux analysis (Marx et al. 1996) and by genetic experiments (Georgi et al. 2005; Marx et al. 2003; Ohnishi et al. 2005). As a combined outcome of various flux studies with different strains and under different conditions a close correlation of lysine production with the flux through the PPP could be observed (Fig. 7B). The importance of the PPP for efficient lysine production becomes also obvious from the theoretical flux distribution corresponding to optimal production (Fig. 5). In contrast to the PPP, the contribution of isocitrate dehydrogenase decreases with increasing lysine production (Wittmann and Heinzle 2002). In summary, it turns out that (i) lysine production is likely limited by NADPH availability and that (ii) the

PPP is the major pathway for supply of NADPH. In light of this, different approaches have aimed at redirecting the flux through the PPP in order to increase lysine production by *C. glutamicum*. As example, deletion of the phosphoglucose isomerase gene *pgi*, forcing the cell to metabolize the substrate glucose completely via the PPP, resulted indeed in increased lysine production on glucose (Marx et al. 2003). This strategy, however, appears useful only for glucose-based processes, since sucrose-based processes require an active phosphoglucose isomerase for recycling carbon into the PPP and full NADPH supply (Becker et al. 2005; Wittmann et al. 2004a). Amplified expression of the fructose 1,6-bisphosphatase gene *fbp* in a genetically defined strain of *C. glutamicum*, only carrying a point mutation in the *lysC* gene, was shown to increase lysine yield on glucose, fructose, and sucrose up to about 40% (Becker et al. 2005). Hereby, flux analysis of the mutant revealed that the overexpression of fructose 1,6-bisphosphatase gene indeed resulted in a 10% enhanced PPP flux. On the other hand, it was shown that *fbp* overexpression in a genetically defined strain carrying point mutations in *lysC*, *hom*, *pyc*, and *zwf* improved lysine yields due to reduced intracellular concentrations of fructose 1,6-bisphosphate, an inhibitor of glucose 6-phosphate DH and 6-phosphogluconate DH, in the engineered strain only on sucrose (Georgi et al. 2005). These differences are very likely to be caused by the different background of the strains, as the strain used by Georgi et al. for amplified expression of *fbp* already had an engineered PPP (Georgi et al. 2005). Further successful examples comprise the introduction of point mutations into PPP genes, previously identified by comparative sequencing of the *C. glutamicum* wild type and a classically derived production strain. The substitution A243T in the *zwf* gene encoding for glucose 6-phosphate dehydrogenase (Georgi et al. 2005; Zelder et al. 2005) and the substitution T1083C in the *gnd* gene encoding for 6-phosphogluconate dehydrogenase (Ohnishi et al. 2005) both led to a significantly increased lysine titre. The modification of 6-phosphogluconate dehydrogenase caused an 8% increase in the PPP flux (Ohnishi et al. 2005) which probably resulted from positively changed kinetics of the enzyme.

4.4 Metabolic Engineering of Precursor Supply

Oxaloacetate is a direct precursor of aspartate-derived amino acids, including lysine. In *C. glutamicum*, the anaplerotic enzymes phosphoenolpyruvate carboxylase (Eikmanns et al. 1989; O'Regan et al. 1989; Ozaki and Shiio 1969) and pyruvate carboxylase (Park et al. 1997; Peters-Wendisch et al. 1996, 1998) are involved in supplying oxaloacetate. The importance of these enzymes for lysine production becomes obvious from the correlation between the lumped anaplerotic net carboxylation flux and the flux into the lysine biosynthetic pathway under various conditions determined by ^{13}C metabolic flux analy-

sis (Fig. 8). Pyruvate carboxylase is today regarded as the major anaplerotic enzyme in *C. glutamicum* (Petersen et al. 2000) and overexpression of its gene has been shown to improve lysine production (Peters-Wendisch et al. 2001). This makes sense taking into account that, due to the presence of the phosphotransferase system for sugar uptake, high amounts of pyruvate are generally formed during growth of *C. glutamicum*. However, deletion of the pyruvate carboxylase gene *pyc* leads to the inability to grow on lactate as a sole carbon source (Peters-Wendisch et al. 1998), whereas overexpression of *pyc* in a lysine-producing strain strongly increased production (Peters-Wendisch et al. 2001). Knowing the importance of pyruvate carboxylase for lysine production, the point mutation C1372T, identified in a classically derived producer, was introduced into the *pyc* gene and resulted in a strong increase of lysine production (Ohnishi et al. 2002). It should be noted that, however, also overexpression of the phosphoenolpyruvate carboxylase gene *ppc* is beneficial for the formation of amino acids of the aspartate family (Sano et al. 1987).

The metabolism around the pyruvate node in *C. glutamicum*, was further unravelled by genetic, biochemical, and ^{13}C metabolic flux analyses,

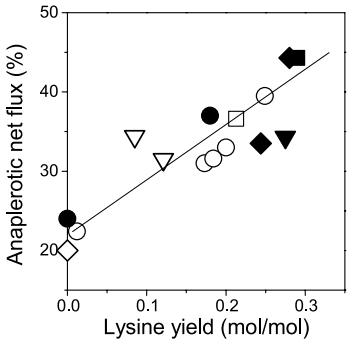


Fig. 8 Correlation between lysine yield and anaplerotic net flux in different *C. glutamicum* strains under various cultivation conditions as revealed by ^{13}C flux analysis. Studies involved flux analysis of (i) non-producing *C. glutamicum* ATCC 13032 in batch culture on glucose (*open diamond*) (Sonntag et al. 1995), (ii) non-producing *C. glutamicum* ATCC 13032 and lysine-producing *C. glutamicum* ATCC 13287, ATCC 21253, ATCC 21526, and ATCC 21543 in batch culture on glucose (*open circle*) (Wittmann and Heinzle 2002), (iii) lysine-producing *C. glutamicum* ATCC 21253 during maximum production phase in batch culture on glucose (*open square*) (Wittmann and Heinzle 2001a), (iv) non-producing *C. glutamicum* LE4 and lysine-producing *C. glutamicum* MH 20–22B in continuous culture on glucose (*closed circles*) (Marx et al. 1997), (v) lysine-producing *C. glutamicum* MH 20–22B in continuous culture on glucose (*closed square*) (Marx et al. 1996), lysine-producing *C. glutamicum* ATCC 21526 in batch culture on glucose, fructose (*closed diamond*) (Kiefer et al. 2004) and on sucrose (*closed triangle*) (Wittmann et al. 2004a), and lysine-producing *C. glutamicum lysC*^{fbr} and *C. glutamicum lysC*^{fbr} P_{EFTU}fbp during batch culture on glucose (*open triangle*) (Becker et al. 2005)

showing the presence of decarboxylating enzymes converting C_4 metabolites of the TCA cycle into C_3 metabolites of glycolysis. These enzymes, phosphoenolpyruvate carboxykinase and malic enzyme, operate in addition to the carboxylation enzymes and establish a highly flexible metabolic reaction cycle around the pyruvate node, which is also present in various other microorganisms (Sauer and Eikmanns 2005). It might function in wasting excess ATP under certain conditions (Sauer et al. 1997; Wittmann and Heinzle 2001b) or equilibrating the intracellular pool sizes of metabolites around the pyruvate node (Sauer and Eikmanns 2005). Additionally, a contribution to the NADPH metabolism has been hypothesized (Cocaign-Bousquet and Lindley 1995; de Graaf 2000). In light of these findings, deletion of the genes coding for the decarboxylating enzymes displayed a promising strategy to enhance the anaplerotic net flux. Indeed deletion of the phosphoenolpyruvate carboxykinase gene *pckA* resulted in a significant improvement of lysine production (Riedel et al. 2001). Neither the deletion nor the overexpression of the malic enzyme gene, however, influenced the metabolism of *C. glutamicum* on sugars markedly (Gourdon et al. 2000; Netzer et al. 2004). Moreover, overexpression of the malic enzyme gene did not change lysine production by *C. glutamicum* (Georgi et al. 2005). This might be due to the fact that typically phosphoenolpyruvate carboxykinase catalyzes the major decarboxylation flux in *C. glutamicum*, whereas malic enzyme obviously only plays a minor role (Gourdon et al. 2000; Petersen et al. 2000). It can, however, not be excluded that the situation might be different under certain physiological conditions.

4.5 Global Strain Engineering through Systems Biotechnology Approaches

The experience of the past clearly shows that detailed quantitative knowledge of metabolic physiology is required for rational design of superior production strains. Especially for the optimization of amino acid production by *C. glutamicum*, characterized by a close connection between central metabolism and product biosynthetic pathways, understanding of global metabolic regulation has turned out to be crucial. The powerful experimental and computational tools available today enable a detailed quantitative investigation of the metabolism of the industrial lysine producer *C. glutamicum*. A milestone in this research was the sequencing of the genome of *C. glutamicum* and the investigation of its genetic repertoire (Bathe et al. 1996; Haberhauer et al. 2001; Ikeda and Nakagawa 2003; Kalinowski et al. 2003; Tauch et al. 2002). Transcriptome analysis in *C. glutamicum* through DNA microarrays provided valuable insights into gene expression under various conditions (Wendisch 2003; Wendisch et al. 2006), such as growth on different carbon sources like glucose or acetate (Gerstmeir et al. 2003; Hayashi et al. 2002; Muffler et al. 2002) or production of lysine (Hayashi et al. 2006b; Krömer et al. 2004). Sim-

ilarly, also the analysis of the proteome, based on 2-D gel electrophoresis (Bendt et al. 2003; Hermann et al. 2001; Schaffer and Burkovski 2005; Schluesener et al. 2005) has proven valuable to understand important metabolic processes including, for example, nitrogen starvation (Schmid et al. 2000). The enormous contribution of the quantification of metabolic fluxes (fluxome) to our current understanding of the *C. glutamicum* metabolism has already been pointed out above. For this purpose comprehensive approaches combining ^{13}C tracer experiments, metabolite balancing, and isotopomer modelling have been developed and applied to *C. glutamicum* (de Graaf 2000; Wittmann and de Graaf 2005). Important studies to be mentioned are the comparative analysis of fluxes during growth, glutamate, and lysine production (Marx et al. 1997; Sonntag et al. 1995), in different mutants of a lysine-producing strain genealogy (Wittmann and Heinzle 2002), during co-utilization of acetate and glucose (Wendisch et al. 2000), and during lysine production on different industrially relevant carbon sources (Kiefer et al. 2004; Wittmann et al. 2004a). Further achievements comprise the miniaturization of flux analysis to the μL scale for screening purposes (Sauer 2004; Wittmann et al. 2004b), novel approaches towards the analysis of large-scale production processes (Drysch et al. 2003, 2004; El Massaoudi et al. 2003; Yang et al. 2003, 2006a,b) and current developments aiming at flux analysis under dynamic conditions (Nöh et al. 2006; Nöh and Wiechert 2006; Wiechert and Nöh 2005).

These tools have contributed significantly to our current understanding of the *C. glutamicum* metabolism. To fully describe the physiological state of a biological system, however, not one but all of its functional components (genome, transcriptome, proteome, metabolome, and fluxome) have to be analyzed. First examples of such systems-oriented studies already reveal a great potential (Krömer et al. 2004; Lange et al. 2003; Silberbach et al. 2005) and have stimulated the development of systems biotechnology approaches for future characterization and engineering of *C. glutamicum* (Wendisch et al. 2006). Such approaches are especially promising for the targeted multidimensional alteration of complex regulatory networks towards better tolerance of production strains to high temperature or salt levels, or extreme pH values (Kelle et al. 2005).

5
Industrial Production Processes

5.1
Large-Scale Manufacturing

Today large plants are in use for industrial lysine production and the feed-grade amino acid market is developing towards a few major suppliers (Ajinomoto, ADM, BASF, Cheil Jedang, Degussa, Global Biochem, Kyowa Hakko).

Fig. 9 Lysine production plant of the BASF AG located in Gunsan, South Korea with an annual capacity of about 100 000 tons. Copyright BASF AG—The chemical company (2003). Reproduced with permission

As an example Fig. 9 shows the lysine production facility of BASF AG in Gunsan, located at the west coast of South Korea. This plant has an annual production capacity of about 100 000 tons, which accounts for about 15% of the total world market. Industrial large-scale manufacturing of lysine can be separated into different steps involved in upstream processing, the fermentation process itself, and the downstream processing. Upstream processing comprises raw material testing, delivery and storage, the preparation of media from the raw materials, and the preparation of the inoculum for the production. The major industrial carbon sources for lysine production are cane molasses, beet molasses, sucrose and dextrose, whereby the latter is obtained from hydrolysis of starch (Ikeda 2003). Because of batch-to-batch variation of these complex nutrient sources, extensive media testing is carried out to ensure the suitability of a certain raw material with respect to product yield or titre. The carbon source is the major cost factor in industrial lysine production (Kelle et al. 2005). Related to this, sugar suppliers and lysine producers are subject to close alliances or companies cover even both, sugar supply and lysine production. Because of the strong impact of the sugar costs the conversion yield is of major importance for the economy of the production process. The preparation of the inoculum typically

involves successive cultivation of the production strain in increasing culture volumes, since *C. glutamicum* exhibits elevated lag phases when inoculated at a biomass concentration below 0.1 g L^{-1} (Kelle et al. 2005). Today, lysine-producing plants use large-scale fermenter vessels with 500 m^3 volume or even more to benefit from the economy of scale. The purification and formulation of the product in the downstream processing is a further important cost factor (Hermann 2003). One applied route comprises cell separation by vacuum filtration, evaporation, and spray drying for product formulation as shown in the flow chart in Fig. 9. Additionally, alternative strategies are used, mainly depending on the lysine preparation finally obtained (Kelle et al. 2005). During the past, lysine was mainly purified from the broth by ion exchange with separation of the biomass, followed by addition of HCl, evaporation, and drying (Hermann 2003). The crystalline lysine·HCl formed is much less hygroscopic than the corresponding sulfate salt (Kelle et al. 2005) and displayed the major product form through the past. In such processes the biomass of *C. glutamicum*, classified as GRAS organism and thus suitable for animal feed, is utilized separately for feed purposes. Today different developments allow a more economical downstream processing and have led to a number of different lysine preparations, such as liquid lysine (50% purity), granulated lysine sulfate (40–50% purity), or liquid lysine sulfate (20–30% purity), which are today well established on the market (Kelle et al. 2005). The granulated product contains the entire fermentation broth without separation of biomass reducing costs and adding additional nutritional value to the product.

5.2 Process Optimization

The optimization of the lysine production process can significantly contribute to reduction of the production costs. The improvement of the downstream processing leading to different lysine preparations as shown above is one illustrative example of the efficiency of such approaches. Other efforts aim at intensification of the process through a change of the operation mode. Repeated batch or fed-batch runs without preparation of the reactor or the inoculum display an interesting approach. After the production is finished a certain fraction of the broth is left in the reactor and is mixed with fresh medium, which significantly decreases the down-time and thus increases the volumetric productivity, i.e. the capacity of a given plant. Problems arise in cases where the production strain is genetically not stable, as in the case of lysine-producing mutants auxotrophic for other amino acids (Hermann 2003). Further improvement could be made by running the lysine production as a continuous process, but that has not been realized yet at a large scale (Ikeda 2003). Studies at a smaller scale, such as the optimization of lysine production in continuous culture under simultaneous phosphate and

carbon source limitation, however, show the potential of such approaches (Hirao et al. 1989). Instability of production strains is still a major problem in converting current batch or fed-batch processes into processes with extended runtime, but might be overcome in the near future through new insights into the underlying metabolic processes related to genetic instability or application of, for example, flow cytometry to quantify inoculum viability.

Process optimization or process design can be additionally supported by process modelling. Process models do not only consider process stoichiometry via the mass streams into the process and operational parameters of the different unit operations, but also take environmental aspects into consideration by weighing the environmental burden of utilized and released components (Heinzle et al. 2006). In this regard, process modelling allows a detailed insight into the process, the estimation of process efficiency, and the identification of conditions for optimal yield, product titre, selectivity, or minimized environmental burden. Amongst various examples, this has been recently demonstrated for the case study of lysine production (Knoll and Büchs 2006). In this example, a process model of lysine production, related to the flow chart shown in Fig. 10, was established and implemented

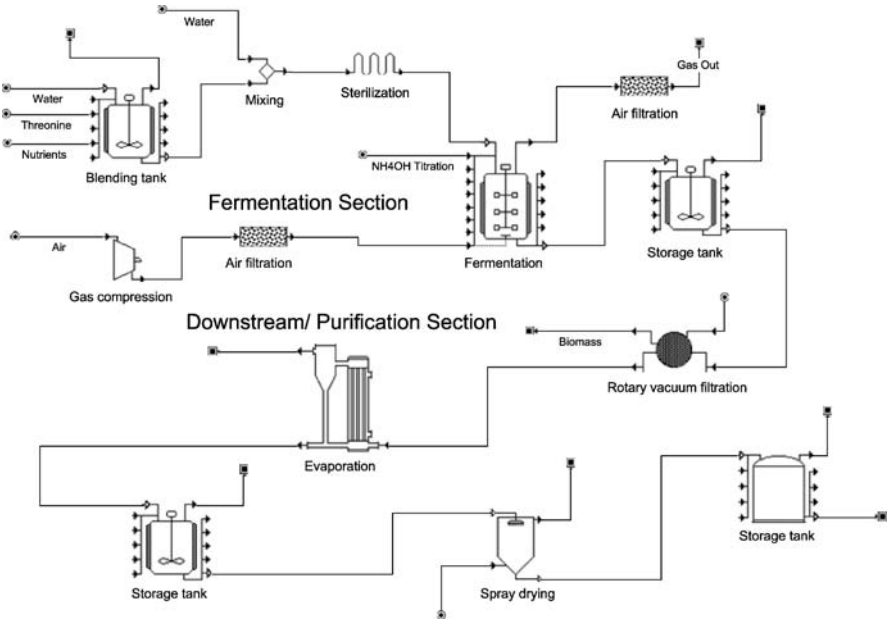


Fig. 10 Process flow diagram of a lysine production plant. Picture taken from: Knoll A, Büchs J (2006) L-lysine—coupling of bioreaction and process model. In: Heinzle, Biwer, Cooney, (eds) Development of Sustainable Bioprocesses—Modelling and Assessment. Copyright: Wiley. Reproduced with permission

into a process modelling software. For the given process setup, coupled to a simple biological model of lysine production, the authors could nicely show that the operational conditions allowing minimum production costs are different from those providing maximum space-time yield. Such modelling approaches are not only interesting for existing processes, but can also support optimal development of new plants. Through variation of the process setup, for example the comparison of alternative down-stream processing routes, optimal process configurations can be identified in an early phase of development, when the degree of freedom is still high.

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