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Research Article

Approaches for improving the production of L-Lysine in *Corynebacterium glutamicum*

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ABSTRACT

One of the most commonly used approaches for improving the production of L-Lysine in *Corynebacterium glutamicum* was classical mutagenesis which involves the repeated mutation and selection of the desired mutant. Single and/ or combined cloning and expression of the genes or disruption of certain genes in *Corynebacterium glutamicum* enabled the analysis of carbon flux control in response to elevation or removal of the respective enzyme activity. Based on these analyses, new strategies for the manipulation of this industrially important amino acid producer become possible. A quantitative description of how a pathway flux is controlled by individual pathway reactions and how this control changes in response to environmental and genetic changes will provide a rational basis for genetic manipulation. The key aspect of this approach is to enable a production strain to make full use of its intrinsic ability through eliminating all undesirable mutations accumulated in its genome. This review focuses on the approaches in the last 30 years in the field of industrial production of L-Lysine by *Corynebacterium glutamicum* from conventional methods like classical mutagenesis, metabolic flux analysis to the recent advancements like DNA microarray, genome based strain breeding and genome sequencing and functional genomics. This review also explains about the accomplishments by different approaches to the strain improvement in *Corynebacterium glutamicum* and also gives an insight for rationalizing production mechanism.

Keywords: Classical mutagenesis; *Corynebacterium glutamicum*; Genome Sequencing; L-Lysine; Metabolic Flux Analysis.

INTRODUCTION

L – Lysine is the third most frequently produced amino acid in a large industrial scale. Of that manufactured commercially, the largest amount 80% is produced by fermentation and 20% by chemical synthesis (N. Coello et. al., 2000). Industrially, L-Lysine is usually manufactured by a fermentation process using *Corynebacterium glutamicum* with production exceeding 750,000 tons a year (S. Georg and W.F. Volker 2007). Although other methods such as chemical synthesis, hydrolysis of proteins, enzymatic conversion, protoplast fusion technique, random mutation or repeated mutation and selection and metabolic flux analysis have extensively been used, the bulk of Lysine production throughout the world still depends solely on direct fermentative process. In recent years, advanced technologies like genome sequencing and DNA micro array based identification of novel target gene technology have improved the yield of L-Lysine production many folds and helped elucidate the detailed structural and functional insight of *Corynebacterium glutamicum*.

Since 1956, different strains of *Corynebacterium glutamicum* are being used for the production of L-Lysine. The biosynthesis of amino acids in micro organism is normally regulated to meet the organism's needs, but artificial distortion of metabolism can result in the overproduction of particular amino acids. The method of producing L-Lysine using the micro organism of *Corynebacterium* genus is very important and there have been many attempts made to improve the method. The accumulation of free amino acids in microbial cultures was originally studied from a physiological stand point under the topic, "Extra cellular nitrogen compound (ENC)''. In the early 1950`s, Dagleyand and his co-workers described the excretion of small amount of alanine, glutamic acid, aspartic acid and histidine in a culture of *E. coli.* They also found that addition of ammonium salt in excess of that required

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for growth resulted in increased amino acid production. The principles of the fermentative method quickly gained acceptance, and systematic work soon began on the production of other amino acids. This marked the birth of the amino acid fermentation industry (Aida, 1972).

DIFFERENT APPROACHES ARE DISCUSSED HERE UNDER

Classical Mutagenesis

L-Lysine was the first amino acid to be produced on an industrial scale with the aid of auxotroph when homoserine requiring auxotrophic mutants of *Corynebacterium glutamicum* were derived as L-Lysine producers (Kinoshita S. et. al., 1958, Nakayama K. et. al., 1961, Nakayama K. et. al., 1966).

The biosynthetic sequence of L-Lysine via the *diaminopimelate dehydrogenase* reaction, starting from the central metabolite, phosphoenol pyruvate involves the following enzymes; *phosphoenol Pyruvate carboxylase (ppc)*, *aspartate amino transferase (aspB), aspartokinase (lysC), aspartate semialdehyde dehydrogenase (asd), dihydropicolinate synthase (dap A), dihydropicolinate reductase (dapB), diaminopimelate dehydrogenase (ddh), diaminopimelate decarboxylase (lysA)* (L. Eggeling et al., 1991).

The over expression of each gene including *lysC (aspartate kinase), dap A (dihydropicolinate synthase), dapB (dihydropicolinate reductase) and asd (aspartate semialdehyde dehydrogenase)* revealed that sole over expression of wild type *dihydropicolinate synthase* resulted in lysine formation but in a lower amount (11m M), and the other enzymes had no effect on lysine secretion in *C. glutamicum* (L. Eggeling et al., 1991).

Phosphoenolpyruvate (PEP) carboxylase is an anaplerotic enzyme in *C. glutamicum* for growth and Lysine production. It had been found that PEP negative strains of *C. glutamicum* had an alternative enzyme *PEP carboxykinase* (GTP dependent) and it alone can fulfil the anaplerotic function required for growth on glucose and lysine production (B. J. E Ikmanns et al., 1993).

The Classical mutagenesis had been greatly used but most of the useful genetic properties have lain idle within individual mutant and have not so far been actively exploited.

Metabolic Flux Analysis

So far the development of highly productive strains was based on random mutagenesis, selection and screening procedures and thus has been an empirical and undefined pathways and their regulation and the availability of the genes involved now permit directed metabolic design, that is improvement of enzymatic and/ or regulatory functions of *C. glutamicum* with the application of recombinant DNA technology (H. Sahm et al., 1995). Nuclear magnetic resonance (NMR) method has become a powerful tool for analysing metabolite concentrations in cell extracts but also in whole cells. NMR technique is applied to study the actual physiological flux distribution between the two variants of lysine biosynthesis in the wild type strain and in several lysine producing strains. Hermann Sahm (1995) had demonstrated that an increase of the flux of L-aspartate semialdehyde to L-Lysine could be obtained in strains with increased *dehydrodipicolinate synthase* activity. Amplification of the genes directly associated with the synthesis of amino acids does not necessarily result in high product yields since carbon flux distribution at key branch points (nodes) in primary metabolism (such as those occurring in glycolysis, tri carboxylic acid [TCA] cycle and pentose phosphate pathway) must often be redirected from the flux distributions that are normally associated with balanced growth. Due to network rigidity (Stephanopoulos G. and Vallino, J.J., 1999) of intermediary metabolism, significant flux alterations are required in order to optimize amino acid biosynthesis. The metabolic rates in a given organism are not constant, they respond to environmental factors and stress conditions. By combined overexpression of deregulated *aspartate kinase* and *dihydropicolinate synthase*, the L-Lysine secretion could be increased (10-20%). This result shows that of the six enzymes that convert aspartate to lysine, *aspartate kinase* and *dihydrodipicolinate synthase* are responsible for metabolic flux control within this biosynthetic pathway. For construction of strong lysine-producing strains the activities of these enzymes must be increased (FEMS microbiology review, 1995).

Molecular and genetic data on amino acid biosynthesis have accumulated which now can be compared with the information obtained by classical methods. About 30 different genes have been sequenced and characterized in *C. glutamicum*, mostly concerned with intermediary metabolism or amino acid biosynthetic pathways (Malumbres M. et al., 1993). A quantitative understanding of the pathway flux control may be realised by metabolic control analysis (MCA) (Kacser H. And Burns J., 1973, Fell D.A. and Sauro M., 1985, Reder C., 1988 and Fell D.A., 1992). The application of MCA to the engineering of cellular activity has revitalized interest in the kinetic modelling of important pathways. The development of mathematical models offers a quantitative way of evaluating the effects of intracellular conditions on the synthesis of cellular metabolites (Nielson J. and Jorgensen H.S., 1995, Pissara P. N. et al., 1996, Malmberg L. H. and Hu w. S., 1992 and Leaf T. A. and Srienc F., 1998). A mathematical model describing intracellular lysine biosynthesis by *C. glutamicum* in batch fermentation was developed. It was mainly based on the mechanism of individual enzymatic reactions and could be applied in the identification of the rate limiting steps in whole cell metabolic systems when enzymatic reactions are available, although it becomes very complicated. Kazuyuki Shimizu and his co-workers performed the detailed analysis of the re-

sponse of the overall flux through the lysine synthetic pathway to changes in pathway enzyme activities and participating metabolite concentrations using the kinetic models and identified that the aspartate phosphorylation and lysine export are the rate limiting reactions for lysine synthesis (Kazuyuki Shimizu et al., 1999 and Kazuyuki Shimizu et al., 2000). Various studies had been performed to understand the physiological activity of *C. glutamicum* under fermentation conditions and its effect over the production of amino acids. The Total Reducing Activity (TRA) of cells was used to estimate the physiological activity of *C. glutamicum* under conditions of L-Lysine synthesis. A decrease in TRA of growing cells was related to an increase in bacterial lysine synthesis activity linearly correlated with the intracellular concentrations of RNA and the bacterial growth rate (Maija Ruklisha and Longina Paegle, 2001).

The main weakness of the extracellular metabolite balancing method was its inability to provide flux estimates in cases of metabolic network structural singularities (i.e. where the metabolic network is structured such that simple metabolite balances cannot provide flux estimation for separate reactions that lead to the same product from different substrates). For example, it was not possible to estimate carbon fluxes through the *PEP carboxylase* and *pyruvate carboxylase* anaplerotic reactions. In additions, the rates of extracellular metabolite excretion and consumption could only provide net fluxes, while no information about the extent of reversibility of a reaction could be obtained. Finally, assumptions about the cellular energy balance had to be included, thus raising further uncertainties (Mattheos Kofas and Gregory Stephanoulos, 2005).

To overcome these complications a novel Sensor Reactor approach was developed (Massaoudi et al.). A successful combination of this technology with NMR analysis, metabolite balancing methods and a mathematical description of ^{13}C- isotope labelling resulting in powerful tool for quantitative pathway analysis during batch fermentation. Its first application to a 160L, Lysine fermentation of *C. glutamicum* revealed for the first time that increased lysine formation correlated with substantial changes in the metabolic flux distribution at the anaplerotic node in this organism (H. Sahm et al., 2003). Metabolic imbalances often lead to unpredictable physiological responses and suboptimal metabolite productivity. This deficiency can be overcome by the coordinated overexpression of more than one flux controlling genes in a production pathway selected by considering their individual contributions on the cell physiology. Attempts have been made by simultaneous overexpression of *pyruvate carboxylase* and *aspartate kinase*, the two key enzymes in central carbon metabolism and the lysine production pathway in *C. glutamicum*.

There was only marginal change in the overall lysine productivity due to either reduced cell growth or reduced lysine specific productivity. In contrast, the simultaneous amplification of the activities of the two enzymes yielded more than 250% increase of the lysine specific productivity in lactate minimal medium without affecting the growth rate or final cell density of the culture (Gregory Stephanopoulos et al., 2002). The application of acetate as a substrate for lysine synthesis could be more attractive in comparison with glucose during the lysine synthesis phase when the bacterial growth rate is below its maximum. A decrease in the growth rate of cells of other *C. glutamicum* strains resulting from the application of acetate as glucose co-substrate also has been reported (Wendisch et al., 2000). Acetate low concentrations can be applied in as a glucose co-substrate to increase lysine biosynthesis productivity and lysine yields from carbon substrates by *C. glutamicum* RC 115. Pyruvate supplementation can be used as a method to improve these parameters under conditions of bacterial growth in glucose-acetate media with increased concentrations of acetate (Longina Paegle and Maija Ruklisha, 2003).

Genome Based Strain Breeding

Recent DNA technologies give the opportunity for the rational strain improvement of *C. glutamicum* by the targeted modification of genes (Sahm H., et al., 2000). The large potential of this approach was illustrated by a recent study in which a tremendous increase in lysine production was obtained by the mutation of only 3 genes in the wild type *C. glutamicum* strain ATCC 13032 (Ohnishi j. et al., 2002). One of the key tasks in targeted strain optimization is the identification of genetic modifications that lead to improved strain characteristics. The experience of past clearly shows that a detailed quantitative knowledge of metabolic physiology is required for the rational design of superior production strains. Extensive research has been used to sequence the whole genome of *C. glutamicum* and to investigate its genetic repertoire (Bathe B. et al., 1996, Haber G.H. et al., 2001, Mockel B. et al., 1999, Nakagawa S. et al., 2001, Tauch A. et al., 2002).

Metabolic reconstruction via functional gene annotation revealed fascinating insights into this organism, including functional predictions for >60% of the identified genes (Ikeda M. and S. Nakagawa, 2003). Gene expression (transcriptome) analysis with *C. glutamicum* has recently been realized by the development of specific DNA microarrays (Ikeda M. and S. Nakagawa, 2003) and was used to investigate gene expression during the growth of *C. glutamicum* on glucose and acetate (Hayashi M. et al., 2002, Muffler A. et al., 2002) and during the production of valine (Lange C. et al., 2003). Expression profiles of selected genes of central metabolism (Loos a. et al., 2001) and amino acid production (Glanemann C. et al., 1992) of *C. glutamicum* were determined. For proteome analysis of *C. glutamicum*, two dimensional gel electrophoresis was recently used to identify different proteins (Hermann T. et al., 1998, Hermann T. et al., 2000, Hermann T. et al., 2001, Schaffer S. et al., 2001) and to study the in-

fluence of nitrogen starvation on the proteome (Schmid R. et al., 2000). For the quantification of metabolic fluxes (the fluxome), comprehensive approaches combining ^{13}C tracer experiments, metabolite balancing, and isotopome remodelling have been developed (Kelleher J. K., 2001, Wiechert W. et al., 2001 ,Wiechert W. et al., 2002, Wittmann C. et al., 2002) and applied to *C. glutamicum*, involving, e.g., comparative fluxome analysis during growth, glutamate, and lysine production (Mockel B. et al., 1999, Sonntag K. et al., 1995), during lysine production in batch cultures (Wittmann C. et al., 2001) of different mutants of a lysine-producing strain genealogy (Wittmann C. et al., 2002), during growth on acetate and/or glucose (Wendisch V. F. et al., 2000), and during lysine production on different carbon sources (Kiefer P. et al., 2004).For a full description of the physiological state of a biological system, not one, but all, components (the genome, transcriptome, proteome, intracellular metabolite concentrations [metabolome], and fluxome) have to be analyzed.

The different profiling tools have, however, mainly been applied separately to *C. glutamicum*. Very few studies have used the combined application of different profiling techniques (Glanemann C. et al., 2003). Therefore, our knowledge about metabolic control in *C. glutamicum* involving the understanding of the links between its different components, e.g., between the transcriptome (expression level of a certain gene) and the fluxome (flux catalyzed by the corresponding enzyme), is still limited. An in-depth analysis of the intracellular metabolite concentrations, metabolic fluxes, and gene expression (metabolome, fluxome, and transcriptome, respectively) of lysine-producing *C. glutamicum* ATCC 13287 was performed at different stages of batch culture and revealed distinct phases of growth and lysine production. For this purpose, ^{13}C flux analysis with gas chromatography-mass spectrometry-labelling measurement of free intracellular amino acids, metabolite balancing, and isotopome remodelling were combined with expression profiling via DNA microarrays and with intracellular metabolite quantification. The integrated approach was valuable for the identification of correlations between gene expression and in vivo activity for numerous enzymes. The glucose uptake flux closely corresponded to the expression of glucose *phospho transferase* genes. A correlation between flux and expression was also observed for *glucose-6-phosphate dehydrogenase*, *transaldolase*, and *transketolase* and for most TCA cycle genes. In contrast, cytoplasmic malate dehydrogenase expression increased despite a reduction of the TCA cycle flux, probably related to its contribution to NADH regeneration under conditions of reduced growth. Most genes for lysine biosynthesis showed a constant expression level, despite a marked change of the metabolic flux, indicating that they are strongly regulated at the metabolic level. Glyoxylate cycle genes were continuously expressed, but the pathway exhibited in vivo activity only in the later stage. The most pronounced changes in gene expression during cultivation were found for enzymes at entry points into glycolysis, the pentose phosphate pathway, the TCA cycle, and lysine biosynthesis, indicating that these might be of special importance for transcriptional control in *C. glutamicum* (Jens Olaf Kromer et al., 2004). Additionally, systems oriented analysis involving, e.g. fluxome, metabolome or transcriptome analysis has proven useful to gain understanding of the metabolism and to identify promising targets (Becker et al., 2005, Kromer et al., 2004, Wendisch et al., 2006). As example, previous metabolic flux studies of different *C. glutamicum* mutants revealed a correlation between lysine production and carbon flux through the pentose phosphate pathway (PPP) (Kiefer et al., 2004, Wittmann and Heinzle, 2002). Hereby, the importance of the PPP arises from the fact that it supplies NADPH required as cofactor in high amounts for the biosynthesis of lysine. The flux studies suggested an amplification of the PPP flux as promising target to improve lysine formation through increased availability of NADPH (Wittmann and Heinzle, 2002). The potential of this strategy was recently shown by over expression of the *fbp* gene, encoding fructose 1,6-bisphosphatase, in *C. glutamicum* which resulted in a significant increase of PPP flux and NADPH formation (Becker et al., 2005). As a consequence, an increase of the lysine yield of up to 40% could be achieved. Metabolic flux engineering of *C. glutamicum* was carried out to increase lysine production by focusing on engineering of the pentose phosphate pathway (PPP) flux by different genetic modifications. Over expression of the *zwf* gene, encoding *G6P dehydrogenase*, in the feedback-deregulated lysine-producing strain *C. glutamicum* ATCC 13032 *lysC fbr* resulted in increased lysine production on different carbon sources including the two major industrial sugars, glucose and sucrose. The strategy The additional introduction of the A243T mutation into the *zwf* gene and the over expression of *fructose 1,6-bisphosphatase* resulted in a further successive improvement of lysine production. Hereby the point mutation resulted in higher affinity of *G6P dehydrogenase* towards NADP and reduced sensitivity against inhibition by ATP, PEP and FBP. Overall, the lysine yield increased up to 70% through the combination of the different genetic modifications. Through strain engineering formation of trehalose was reduced by up to 70% due to reduced availability of its precursor G6P. Metabolic flux analysis revealed a 15% increase of PPP flux in response to over expression of the *zwf* gene. Overall a strong apparent NADPH excess resulted. Redox balancing indicated that this excess is completely oxidized by malic enzyme (Wittmann C., et al., 2007).Presently, the combined annual production of L-glutamate and L-Lysine using large scale batch fermentations with *C. glutamicum* strains amounts to more than 1,500,000 t (Kelle et al., 2005, Kimura, 2005 and Leuchtenberger, 1996). In the complex media used for the fermentations, a significant amount of phos-

phorus is often naturally present in the form of phytate *(myo-inositol-1, 2, 3, 4, 5, 6-hexakiphophate)*. The use of this phosphate by *C. glutamicum* either as phosphorus or as carbon source is prevented by the lack of an enzyme outfit. Neither the uptake nor the intra- or extracellular hydrolysis of phytate is known to occur naturally in *C. glutamicum* (Wendisch and Bott, 2005). *C. glutamicum* strains can be genetically engineered to utilize plant derived phytate as the sole source of phosphorus by heterologous expression of a beta- propeller phytase gene from *Bacillus sp*. For the lysine production strain investigated, *C. glutamicum* ATCC 21252 (*pWLQ2::phyC),* the lysine productivity as well as the bacterial growth rate supported by phytate utilization did not differ dramatically from results obtained under fermentation conditions additionally containing inorganic phosphate. This route of engineering *C. glutamicum* to utilize phytate as an alternative phosphorous source may be useful for the improvement of strains used in industrial amino acid production (Wolfgang Liebel and Mladen V. Tzvetkov, 2008).

Genome Sequencing and Functional Genomics

The reconstruction of classically derived production strains based on genomic information can be an effective approach for innovation of fermentation processes in the post genomic era. The availability of genomic data from industrial organisms allows PCR-based cloning and sequencing of any desirable genes of production strains derived from the organisms. By comparing sequences from wild type and classically derived production strains, it should be possible to decipher the results obtained from mutation-selection and define the genetic background that is required to achieve high-level production.

The process of strain reconstruction avoids the complication of uncharacterized secondary mutations and contributes to rationalizing the mechanism of production through characterization of the genetic background for high level production. This approach allows the construction of a defined mutant without any foreign DNA, and thus is desirable for industrial use (J. Ohnishi et al., 2002). One intriguing finding involves L-Lysine export, which has been discussed in relation to L-Lysine overproduction (Vrljic et al., 1996, Hua et al., 2000). The export process is catalyzed by a recently discovered transport carrier, the product of *lys E* (Vrljic et al., 1996), the synthesis of which is controlled by the regulator protein encoded by *lys G*. Interestingly, in one study by comparative genomic analysis revealed no mutations in either *lys E* or *lys G* of strain B-6, indicating that *C. glutamicum* is naturally endowed with a high capability to excrete L-Lysine. This is also supported by the fact that introduction of *hom59, lysC311,* and *pyc458* into the wild-type strain achieved, to our knowledge, the highest rate of L-Lysine production to date. This prominent characteristic of L-Lysine excretion may be the reason why this bacterium has been widely used for the industrial fermentation of L-Lysine (J. Ohnishi et al., 2002). As the pentose phosphate pathway serves as primary route for NADPH generation in glucose-grown *C. glutamicum* (Marx et al., 1996, 1997, 1999), its flux and subsequently lysine production was increased either by deletion of the *phosphor gluco isomerase gene pgi,* precluding utilization of *glucose-6-phosphate* in glycolysis (Marx et al., 2003), or by expression of mutant alleles of the *glucose-6-phosphate dehydrogenase gene zwf* or the *6-phosphogluconate dehydrogenase gene gnd* (Ando et al., 2002, Ohnishi et al., 2005). Lysine production on sucrose (Georgi et al., 2005) and, in some strains, on fructose (Becker et al., 2005) could be improved by overexpression of *fbp*, which encodes *fructose-1,6-bisphosphate* (Rittmann et a., 2003), as a consequence of increased pentose phosphate pathway flux. After the genome sequence of the *C. glutamicum* wild type strain, ATCC 13032, was determined (Kalinowski et al., 2003) and the DNAs microarray technology established (Wendisch, 2003, Polen and Wendisch, 2004, Wendisch et. al., 2006). With these methods available, discovery-driven approaches to strain improvement of this amino-acid-producing bacterium became possible. Lysine production could be improved by about 40% through overexpression of NCg10855 or the *amtA-ocd- soxA* operon. Thus, novel target genes for improve- ment of lysine production could be identified in a dis- covery-driven approach based on global gene expres- sion analysis (Georg sindelar, Volker f. Wendisch, 2007).

CONCLUSION

Many studies on how each gene involved in amino acid biosynthesis affects L-Lysine production by amplifying the gene to develop microorganism of *Corynebacterium genes* have been conducted. Elimination of Non essential genes causes the reduced use of lysine which favours the unnecessary lysine consumption and also favours the lysine production under same condition. Metabolic flux analysis provided valuable data to understand the cellular response resulting from genetic engineering to visualize metabolic imbalance to guide further strain engineering. Especially for the optimization of lysine production in *C. glutamicum*, the consideration of the close connection between central metabolism and lysine biosynthetic pathway has turned out to be crucial. Novel approach to increase optimization of strain is a rational engineering of the cell. Genome based strain breeding involves identifying mutants by comparative genomic analysis, identifying mutations beneficial for production and assembling them in a single wild type background. Due to industrial interest in *C. glutamicum,* several studies have been performed to increase the industrial production of L-Lysine. By combining the knowledge of Genome based strain breeding, DNA microarray, metabolic flux analysis and overexpression of desired gene, we can achieve the desired strain with high productivity. The combinations of these advanced techniques enable us to meet

the increased production of L-Lysine by *C. glutamicum* at industrial scale.

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REFERENCES

Aida, K., Chibata, I., Nakayama, I., Takmami, K. and Yamada, H. "Biotechnology of amino acid production- Progress in Industrial Microbiology", Amsterdam, Vol. 24, 1986, 142-157.

Bathe, B., J. Kalinowski, and A. Puhler, "A physical and genetic map of the Corynebacterium glutamicum ATCC 13032 chromosome", Journal of Mol. Gen. Genet. 252, 1996, 255–265.

Becker, J., Klopprogge, C., Zelder, O., Heinzle, E., Wittmann, C., "Amplified expression of fructose 1,6-bisphosphatase in Corynebacterium glutamicum increases in vivo flux through the pentose phosphate pathway and lysine production on different carbon sources", Appl. Env. Microbiol. 71, 2005, 8587–8596.

Eikmarms, B.J., Eggeling, L. and Sahm, H., "Molecular aspects of lysine, threonine and isoleucine biosynthesis in Corynebacterium glutamicum. Antonie van Leeuwenhoek hit", J. Gen. M, 64, 1993, 145-163.

Fell, D. A. and Sauro, M., "Metabolic control and its analysis", Eur. J. Biochem., 148, 1985, 555-561.

Georg Sindelar & Volker F. Wendisch, "Improving lysine production by Corynebacterium glutamicum through DNA microarray-based identification of novel target genes", Appl Microbiol Biotechnol, 76, 2007, 677–689.

Georgi T, Rittmann D, Wendisch VF, "Lysine and glutamate production by Corynebacterium glutamicum on glucose, fructose and sucrose: Roles of malic enzyme and fructose-1, 6-bisphosphatase", Metab Eng 7, 2005, 291–301.

Glanemann, C., A. Loos, N. Gorret, L. B. Willis, X. M. O'Brien, P. A. Lessard, and A. J. Sinskey, "Disparity between changes in mRNA abundance and enzyme activity in Corynebacterium glutamicum: implications for DNA microarray analysis", Appl. Microbiol. Biotechnol. 61, 2003, 61–68.

Gregory Stephanopoulos, Mattheos A.G. Koffas and Gyoo Yeol Jung, "Engineering metabolism and product formation in Corynebacterium glutamicum by coordinated gene Over expression", Metabolic Engineering 5, 2003, 32–41.

H. Sahm, A. Drysch, M. El Massaoudi, C. Mack, R. Takors, A.A. and De Graaf, "Production process monitoring by serial mapping of microbial carbon flux distributions using a novel Sensor Reactor approach: II—13Clabeling-based metabolic flux analysis and l-lysine production", Metabolic Engineering 5, 2003, 96–107.

Haberhauer, G., H. Schroder, M. Pompejus, O. Zelder, and B. Kroger, "Corynebacterium glutamicum genes encoding proteins involved in membrane synthesis and membrane transport" Patent WO, 2001. 01/00805.

Hayashi, M., H. Mizogushi, N. Shiraishi, M. Obayashi, S. Nakagawa, J. Imai, S. Watanabe, T. Ota, and M. Ikeda, Transcriptome analysis of acetate metabolism in Corynebacterium glutamicum using a newly developed metabolic array", Biosci. Biotechnol. Biochem. 66, 2002, 1337–1344.

Hermann Sahm, Lothar Eggeling, Bernd Eikmanns, Reinhard Krimer, "Metabolic design in amino acid producing bacterium - Corynebacterium glutamicum", FEMS Microbiology Reviews 16, 1995, 243-252.

Hermann, T., G. Wersch, E. M. Uhlemann, R. Schmid, and A. Burkovski, "Mapping and identification of Corynebacterium glutamicum proteins by two-dimensional gel electrophoresis and micro sequencing", Electrophoresis 19, 1998, 3217–3221.

Hermann, T., M. Finkemeier, W. Pfefferle, G. Wersch, R. Kra¨mer, and A. Burkovski, "Two-dimensional electrophoretic analysis of Corynebacterium glutamicum membrane fraction and surface proteins", Electrophoresis 21, 2000, 654–659.

Hermann, T., W. Pfefferle, C. Baumann, E. Busker, S. Schaffer, M. Bott, H. Sahm, N. Dusch, J. Kalinowski, A. Pu¨hler, A. K. Bendt, R. Kra¨mer, and A. Burkovski, "Proteome analysis of Corynebacterium glutamicum", Electrophoresis 22, 2001,1712–1723.

Hua, Q., Yang, C., Shimizu, K., "Metabolic control analysis for lysine synthesis using Corynebacterium glutamicum and experimental verifications", J. Biosci. Bioeng. 20, , 2000, 184–192.

Ikeda, M., and S. Nakagawa, "The Corynebacterium glutamicum genome: features and impacts on biotechnological processes", Appl. Microbiol. Biotechnol. 62, 2003, 99–109.

Jens Olaf Kro¨mer, Oliver Sorgenfrei, Kai Klopprogge, Elmar Heinzle and Christoph Wittmann, "In-Depth Profiling of Lysine-Producing Corynebacterium glutamicum by Combined Analysis of the Transcriptome, Metabolome, and Fluxome", Journal of bacteriology, Vol. 186, No. 6, Mar. 2004, 1769–1784.

Kacser, H., Burns, J.A., "Control of *enzyme+ flux", Symp. Soc. Exp. Biol. 27, 1973, 64–104.

Kalinowski J, "The complete Corynebacterium glutamicum ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins", J Biotechnology, 104, 2003, 5–25.

Kazuyuki Shimizu and Chen Yang, Qiang Hua, "Development of a Kinetic Model for L-Lysine Biosynthesis

in Corynebacterium glutamicum and Its Application to Metabolic Control Analysis", Journal of bioscience and bioengineering, Vol. 88, No. 4, 1999, 393-403.

Kazuyuki Shimizu and Qiang Hua, Chen Yang, "Metabolic Control Analysis for Lysine Synthesis Using Corynebacterium glutamicum and Experimental Verification", Journal of bioscience and bioengineering, Vol. 90, No. 2, 2000, 184-192.

Kelle, R., Hermann, T., Bathe, B, "L-Lysine production in handbook of Corynebacterium glutamicum, CRC Press, Taylor & Francis, Boca Raton, FL, USA, 2005, 465–488**.**

Kelleher JK., "Flux estimation using isotopic tracers: common ground for metabolic physiology and metabolic engineering", Metab Eng., Apr;3(2), 2001, 100-110.

Kiefer, P., E. Heinzle, O. Zelder, and C. Wittmann, "Comparative metabolic flux analysis of lysine-producing Corynebacterium glutamicum on glucose and fructose", Appl. Environ. Microbiol, 70, 2004, 229–239.

Kimura, E., In: Eggeling, L., Bott, M. (Eds.), "Handbook of Corynebacterium glutamicum", CRC Press, Taylor & Francis, Boca Raton, FL, USA, pp. 439–463, 2005.

Kinoshita, S., Nakayama, K. And Kitada, S. Gen. Appl. Microbiol. 4, 128-129, 1958.

Kromer J.O., Sorgenfrei O, Klopprogge K, Heinzle E, Wittmann C, "In-depth profiling of lysine-producing Corynebacterium glutamicum by combined analysis of the transcriptome, metabolome, and fluxome", J Bacteriol, 186:1769-1784, 2004.

L. Eggeling, Josef Cremer and Hermann Sahm, "Control of the Lysine biosynthesis sequence in Corynebacterium glutamicum as analyzed by Over expression of the individual corresponding genes", Applied and environmental microbiology, Vol. 57,6, June 1991, 1746-1752.

Lange C, Rittmann D, Wendisch VF, Bott M, Sahm H, "Global expression profiling and physiological characterization of Corynebacterium glutamicum grown in the presence of L-valine", Appl Environ Microbiol, 69, 2003, 2521–2532.

Leaf, T. A. and Srienc, F, "Metabolic modeling of polyhydroxy butyrate biosynthesis", Biotechnol. Bioeng., 57,1998, 557-570.

Leuchtenberger, W, "Amino acids—technical production and use in biotechnology, Germany, 1996, 466–502.

Longina Paegle and Maija Ruklisha, "Lysine synthesis control in Corynebacterium glutamicum RC 115 in mixed substrate (glucose-acetate) medium", Journal of Biotechnology 104, 2003, 123-128.

Loos, A., C. Glanemann, L. B. Willis, X. M. O_Brien, P. A. Lessard, R. Gerstmeier, S. Guillouet, and A. J. Sinskey, "Development and validation of Corynebacterium DNA microarrays", Appl. Environ. Microbiol. 67, 2001, 2310–2318.

Maija Ruklisha and Longina Paegle, "Metabolic fluxes and L-lysine synthesis by Corynebacterium glutamicum in relation to cellular total reducing activity", Process Biochemistry ,36, 2001, 1233–1240.

Malumbres, M., Gil, J.A. and Martin, J.F, "Codon preference in corynebacteria", Gene 134, 1993, 15-24.

Marx A, de Graaf AA, Wiechert W, Eggeling L, Sahm H , "Determination of the fluxes in the central metabolism of Corynebacterium glutamicum by nuclear magnetic resonance spectroscopy combined with metabolite balancing", Biotechnol. Bioeng., 49, 1996, 111–129.

Marx A, Eikmanns BJ, Sahm H, de Graaf AA, Eggeling L, "Response of the central metabolism in Corynebacterium glutamicum to the use of an NADH-dependent glutamate dehydrogenase", Metab. Eng. 1, 1999, 35–48.

Marx A, Hans S, Mockel B, Bathe B, de Graaf A, "Metabolic phenotype of phospho glucose isomerase mutants of Corynebacterium glutamicum", J Biotechnology: 104, March 1996, 185–197.

Marx A, Striegel K, de Graaf AA, Sahm H, Eggeling L, "Response of the central metabolism of Corynebacterium glutamicum to different flux burdens", Biotechnol. Bioeng. 56, 1997, 168–180.

Mattheos A.G. Koffas, Gyoo Yeol Jung, and Gregory Stephanopoulos, "Engineering metabolism and product formation in Corynebacterium glutamicum by coordinated gene Over expression", Metabolic Engineering 5, 2003, 32–41.

Mo¨ckel, B., A. Weissenborn, W. Pfefferle, J. Kalinowski, B. Bathe, and A. Puhler, "Genome sequencing of industrial microorganisms: the Corynebacterium glutamicum ATCC 13032 genome project", Microb. Comp. Genomics 4, 1999, 111.

Muffler, A., S. Bettermann, M. Haushalter, A. Horlein, U. Neveling, M. Schramm, and O. Sorgenfrei, "Genome-wide transcription profiling of Corynebacterium glutamicum after heat shock and during growth on acetate and glucose", J. Biotechnol. 98, 2002, 255–268.

Nakagawa, S., Mizoguchi, H., Ando, S., Hayashi, M., Ochiai, K., Yokoi, H., Tateishi, N., Senoh, A., Ikeda, M. and Ozaki, A, "Novel poly nucleotides", European patent, 1,108, 2001, 790.

Nakayama, K., Kitada, S. and Kinoshita, S., "J. Gen. Appl. Microbiol. 7, 1961, 145-154.

Nakayama, K., Tanaka, H., Hagino, H. and Kinoshita, S., "Agric. Biol. Chem. 30, 1966, 611-616.

Nereida Coello , Daniella Bianchi, Olivier Bertrand, Karsten Haupt, "Effect of gluconic acid as a secondary carbon source on non-growing L-lysine producers cells of Corynebacterium glutamicum. Purification and properties of 6-phosphogluconate dehydrogenase", Enzyme and Microbial Technology, 28, 2001, 754–759.

Ohnishi J, Katahira R, Mitsuhashi S, Kakita S, Ikeda M, "A novel gnd mutation leading to increased L-lysine production in Corynebacterium glutamicum", FEMS Microbiol. Lett. 242, 2005, 265–274.

Ohnishi, J., Mitsuhashi, S., Hayashi, M., Ando, S., Yokoi, H., Ochiai, K. and Ikeda, M, "A novel methodology employing Corynebacterium glutamicum genome information to generate a new L-lysine-producing mutant", Appl. Microbiol. Biotechnol., 58, 2002, 217–223.

Pissara, P. N., Nielsen, J., and Bazin, M. J, "Pathway kinetics and metabolic control analysis of a high-yielding strain of Penicillium chrysogenum during fed batch cultivations", Biotechnol. Bioeng., 51,1996, 168-176.

Polen T, Wendisch VF, "Genome wide expression analysis in amino acid-producing bacteria using DNA microarrays", Appl. Biochem. Biotechnology, 118, 2004, 215–232.

Reder, C, "Metabolic control analysis: A structural approach", J. Theor. Biol, 135, 1988, 175-201.

Rittmann D, Lange C, Wendisch VF, Bott M, Sahm H, "Global expression profiling and physiological characterization of Corynebacterium glutamicum grown in the presence of L-valine," Appl. Environ Microbiol., 69, 2003, 2521-2532.

Rittmann D, Schaffer S, Wendisch VF, Sahm H, "Fructose-1,6- bisphosphatase from Corynebacterium glutamicum: expression and deletion of the fbp gene and biochemical characterization of the enzyme", Arch Microbiol, 180, 2003, 285–292.

Sahm H, Eggeling L, de Graaf A, "Pathway analysis and metabolic engineering in Corynebacterium glutamicum", Biol. Chem. 381, 2000, 899–910.

Schaffer, S., B. Weil, V. D. Nguyen, G. Dongmann, K. Gunther, M. Nickolaus, T. Hermann, and M. Bott, "A high-resolution reference map for cytoplasmic and membrane-associated proteins of Corynebacterium glutamicum", Electrophoresis 22, 2001, 4404–4422.

Schmid, R., E.-M. Uhlemann, L. Nolden, G. Wersch, R. Hecker, T. Hermann, A. Marx, and A. Burkovski, "Response to nitrogen starvation in Corynebacterium glutamicum", FEMS Microbiol. Lett.,187, 2000, 83–88.

Sonntag, K., J. Schwinde, A. A. de Graaf, A. Marx, B. J. Eikmanns, W. Wiechert, and H. Sahm, "13C NMR studies of the fluxes in the central metabolism of Corynebacterium glutamicum during growth and overproduction of amino acids in batch cultures", Appl. Microbiol. Biotechnol, 44, 1995, 489–495.

Tauch, A., I. Homann, S. Mormann, S. Ru¨berg, A. Billault, B. Bathe, S. Brand, O. Brockmann-Gretza, C. Ru¨ckert, N. Schischka, C. Wrenger, J. Hoheisel, B. Mo¨ckel, K. Huthmacher, W. Pfefferle, A. Puhler, and J. Kalinowski., "Strategy to sequence the genome of Corynebacterium glutamicum ATCC 13032: use of a cosmid and a bacterial artificial chromosome library", J. Biotechnol. 95, 2002, 25–38.

Vallino, J.J., Stephanopoulos, G., "Metabolic flux distributions in Corynebacterium glutamicum during growth and lysine overproduction", Biotechnol. Bioeng., 67, 872–885 (Reprinted from Biotechnology and Bioengineering, vol. 41,1993, 633–646.

Vrljic, M., H. Sahm, and L. Eggeling, "A new type of transporter with a new type of cellular function: L-lysine export from Corynebacterium glutamicum", Mol. Microbiol., 22, 1996, 815–826.

Wendisch VF, "Genetic regulation of Corynebacterium glutamicum metabolism", J Microbiol. Biotechnol. 16, 2006, 999.

Wendisch VF, "Genome-wide expression analysis in Corynebacterium glutamicum using DNA microarrays", J Biotechnol. 104, 2003, 273–285.

Wendisch, V. F., A. A. de Graaf, H. Sahm, and B. J. Eikmanns, "Quantitative determination of metabolic fluxes during co utilization of two carbon sources: comparative analyses with Corynebacterium glutamicum during growth on acetate and/or glucose", J. Bacteriol. 182, 2000, 3088–3096.

Wendisch, V.F., Bott, M., "Phosphorus metabolism. In: Eggeling, L., Bott, M (Eds.), Handbook of Corynebacterium glutamicum", CRC Press, Taylor & Francis, Boca Raton, FL, USA, 2005, 377–396.

Wendisch, V.F., Bott, M., Kalinowski, J., Oldiges, M., Wiechert, W., "Emerging Corynebacterium glutamicum systems biology", J. Biotechnol. 124, 2006, 74–92.

Wiechert, W, "An introduction to 13C metabolic flux analysis", Genet. Eng. (New York) 24, 2002, 215–238.

Wiechert, W., M. Mollney, S. Petersen, and A. A. de Graaf. ,"A universal framework for 13C metabolic flux analysis", Metab. Eng. 3, 2001, 265–283.

Wittmann C, Becker J, "The L-lysine story: from metabolic pathways to industrial production. In: Wendisch VF, amino acid biosynthesis—pathways, regulation and metabolic engineering", Springer, Heidelberg, Germany. DOI 10.1007/7171, 2006 ,089.

Wittmann C, Heinzle E, "Genealogy profiling through strain improvement by using metabolic network analysis: metabolic flux genealogy of several generations of lysine-producing Corynebacteria", Appl. Environ Microbiol., 68, 2002, 5843-5859.

Wittmann, C, "Metabolic flux analysis using mass spectrometry", Adv. Biochem. Eng. Biotechnol., 74, 2002, 39–64.

Wittmann, C. and Heinzle, E., "Application of MALDI-TOF MS to lysine producing Corynebacterium glutamicum : A novel approach for metabolic flux analysis", Eur. J. Biochem. 268, 2001, 2441–2455.

Wittmann, C. and Heinzle, E., "Genealogy profiling through strain improvement by using metabolic network analysis: metabolic flux genealogy of several generations of lysine producing Corynebacteria", Appl. Environ. Microbiol, 68, 2002, 5843– 5859.

Wolfgang Liebl and Mladen V. Tzvetkov1, "Phytate utilization by genetically engineered lysine-producing Corynebacterium glutamicum", Journal of Biotechnology, 134, 2008, 211–217.