Bioprocess Technology Development and Convalescing Production of L-Lysine in *Corynebacterium glutamicum*

Ramesh Malothu*, Dowlathabad MR and Balanarasaiah.T

***Abstract*—** L-lysine ($C_6H_{14}N_2O_2$), one of the essential and commercially important amino acids, is found in naturally-occurring proteins of all living organisms. 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 in 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.

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

I. 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 [28]. Industrially, L-lysine is usually manufactured by a fermentation process using *Corynebacterium glutamicum* with production exceeding 750,000 tons a year[15] . Although other methods such as chemical synthesis, hydrolysis of proteins, enzymatic conversion, protoplast fusion technique, random mutation or a 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 microarray based identification of novel target gene technology have improved the yield of L-lysine production by many folds and helped elucidate the detailed structural and functional insight of *Corynebacterium glutamicum*.

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II. CLASSICAL MUTAGENESIS

The accumulation of free amino acids in microbial cultures was originally studied from a physiological stand point under the topic, "Extracellular 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 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 [1]. 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 [7], [9],[11],[12]. The biosynthetic sequence of L-Lysine via the *diaminopimelate dehydrogenase* reaction, starting from the central metabolite, phosphoenolpyruvate involves the following enzymes; *phosphoenolpyruvate 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)* [6]. One of the most commonly used approaches was classical or random mutagenesis which involves the repeated mutation and selection of the desired mutant. The overexpression of each genes including *lysC (aspartate kinase), dap A (dihydropicolinate synthase),dapB ihydropicolinatereductase) and asd (aspartate semialdehyde dehydrogenase)* revealed that sole overexpression 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 [14]*. *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 [18]. 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.

III. Metabolic Flux Analysis

Single and/ or combined cloning and expression of the genes or disruption of certain genes in *C. 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. 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[16]. 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 and Sahm had demonstrated that an increase of the flux of L-aspartate semialdehyde to L-Lysine could be obtained in strains with increased dihydrodipicolinate 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.. By combined overexpression of deregulated *aspartate kinase* and *dihydropicolinate synthase*, the L-Lysine secretion could be increased (10-20%). For construction of strong lysine-producing strains the activities of these enzymes must be increased [16] . 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 [10] . 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. Such a quantitative understanding of the pathway may be realised by metabolic control analysis (MCA)[3] [5], [13]. 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 [20]. 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 response 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 [21]. 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 [22]. 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.To overcome these complications a novel Sensor Reactor approach was developed [23]. A successful combination of this technology with NMR analysis, metabolite balancing methods and a mathematical description of ^{13}C- isotope labelings resulting in powerful tool for quantitative pathway analysis during batch fermentation. Its first application to a 160 L 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 [18]. 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 changes 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 [24]. 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 [15]. Acetate low concentrations can be applied in as a glucose co-substrate to increase lysine biosynthesis productivity and lysine yiels from carbon substrates by *C. glutamicum* RC 115.

IV. Genome Based Strain Breeding

Recent DNA technologies give the opportunity for the rational strain improvement of *C. glutamicum* by the targeted modification of genes [16]. 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 [23]. 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 [27]. Metabolic reconstruction via functionalgene annotation revealed fascinating insights into this organism, including functional predictions for >60% of the identified genes [23]. Gene expression (transcriptome) analysis with*C. Glutamicum* has recently been realized by the development of specific DNA microarrays [23] and was used to investigate gene expression during the growth of *C. glutamicum* on glucose and acetate [17]. For proteome analysis of *C. glutamicum*, two dimensional gel electrophoresis was recently used to identify different proteins [4]. For the quantification of metabolic fluxes (the fluxome), comprehensive approaches combining ^{13}C tracer experiments, metabolite balancing, and isotope remodelling have been developed [8] and applied to *C. glutamicum*, involving, e.g., comparative fluxome analysis during growth, glutamate, and lysine production [27], during lysine production in batch cultures [26] of different mutants of a lysine-producing strain genealogy [26], during growth on acetate and/or glucose [15], andduring lysine production on different carbon sources [27]. For a full description of the physiological state of a biologicalsystem, not one, but all, components (the genome, transcriptome,proteome, intracellular metabolite concentrations[metabolome], and fluxome) have to be analyzed. The differentprofiling tools have, however, mainly been applied separatelyto *C. glutamicum*.Therefore, ourknowledge about metabolic control in *C. glutamicum*involvingthe understanding of the links between its different components,e.g., between the transcriptome (expression level of acertain gene) and the fluxome (flux catalyzed by the correspondingenzyme), 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 *Corynebacteriumglutamicum*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-labeling measurement of free intracellular amino acids, metabolite balancing, and isotopomermodeling 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 phosphotransferase 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 [26]. 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) [26]. 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 [26]. The potential of this strategywas 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 [26]. As a consequence, an increase of the lysine yield of up to 40% could be achieved. Metabolic flux engineering of *Corynebacterium 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*ATCC13032 *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 [26].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 L. Euchtenberger, 1996). In the complex media used for the these fermentations, a significant amount of phosphorus 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* [15]. *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 ATCC21252 (*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.

V. 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 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. 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 [25]. Interestingly, in one study by comparative genomic analysis revealed no mutations in either *lysE* or *lysG* 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 characteristics of L-Lysine excretion may be the reason why this bacterium has been widely used for the industrial fermentation of L-Lysine [25]. As the pentose phosphate pathway serves as primary route for NADPH generation in glucose-grown *C. glutamicum*[18], its flux and subsequently lysine production was increased either by deletion of the *phosphogluco isomerase gene pgi*, precluding utilization of *glucose-6-phosphate* in glycolysis [18] or by expression of mutant alleles of the *glucose-6-phosphate dehydrogenase gene zwf* or the *6-phosphogluconate dehydrogenase gene gnd* [19], [25]. After the genome sequence of the *C. glutamicum* wild type strain, ATCC13032, was determined [2] and the DNAs microarray technology established [15]. 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 improvement of lysine production could be identified in a discovery-driven approach based on global gene expression analysis [15].

VI. Conclusion

A lot of studies have been conducted on how each gene involved in amino acid biosynthesis affects L-Lysine production by amplifying/deletion of each of the genes and develop a recombinant *Corynebacterium*. 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 information in understanding 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 studies related to 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 bacteria. This review explains about the accomplishments by different approaches to strain improvement in C. glutamicum and also gives an insight for rationalizing production mechanism.

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