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Review

Fermentative production of lysine by *Corynebacterium glutamicum*: transmembrane transport and metabolic flux analysis

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Abstract

Since the 1950s the production of amino acids by fermentative methods has become a very important aspect of industrial microbiology, leading to numerous studies to understand and improve the metabolic conditions driving to amino acid overproduction. In this review, in addition to a brief historic background of Coryneform bacteria, the various strategies used for strain improvement, such as and the use of auxotrophic strains and regulatory mutants, are discussed. Metabolic pathways involved in the production of L-lysine by *Corynebacterium glutamicum* and the mechanisms mediating its efflux and secretion are discussed. Metabolic flux analysis, which is considered to be a very powerful tool providing valuable information regarding bottlenecks in the production of desired metabolites, is also covered in relation to lysine secretion in conjunction with the significance of transport mechanisms.

Keywords: *Corynebacterium glutamicum*; L-Lysine; Transport mechanisms; Biosynthetic pathways; Metabolic flux analysis

1. Introduction

Amino acids have now been produced with the aid of microorganisms for nearly 50 years. The economic importance of these cellular building blocks is significant, hence, demand is continually growing and constant efforts to increase production performance are directed towards the microorganisms themselves, as well as towards technical improvements of the respective processes. The highest produced amino acid (approximately 900,000 tonnes per year) is L-glutamic acid, followed by L-lysine (420,000 tonnes per year) and DL-methionine (350,000 tonnes per year) while the other amino acids trail behind. The reason for the increased demand for amino acids stems from their utilization as food additives, feed supplements, therapeutic agents and precursors for the synthesis of peptides or agrochemicals. L-Lysine is required as a feed additive for poultry and pig breeding [1,2], for example, and hence *Corynebacterium glutamicum* has traditionally occupied a special position within the amino acid producing microorganisms.

Up until the 1950s no appropriate commercial process for production of natural L-amino acids existed except by isolation from natural proteins. For that reason, continuous efforts were made in order to improve the nutritional value of low cost vegetable proteins by enrichment with essential amino acids. In 1957, Kinoshita et al. discovered a potent amino acid-producing microorganism, *C. glutamicum* (initially named *Micrococcus glutamicus*), which provided a novel method for producing natural amino acids [3,4]. *C. glutamicum* is a Gram-positive, non-sporulating bacterium that may be isolated from soil. It is not motile, with pleomorphic short rods ($(0.7-1)\times(1-3)$ μm in size) producing yellowish colonies and having a DNA G + C content of 53–55%. It requires biotin in order to grow, cultivation temperatures of approximately 30 °C, with most strains able to utilize acetic acid, ethanol, glucose or sucrose for amino acid production [5].

Coryneform bacteria are central to the industrial production of amino acids. When compared to chemical methods, fermentative production has the advantage of yielding the optically active and biologically required L-form of amino acids from cheap carbon and nitrogen sources. Bearing that in mind, extensive research has been made in order to improve the fermentation process not only from the point of lowering production costs but also of increasing the

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productivity. Improvements have included, for example, increased yield of desired metabolites, removal of unwanted cometabolites, improved utilization of inexpensive carbon and nitrogen sources, or alteration of the cellular morphology to a form better suited for separation of the organism from the product [6].

Attempts in strain improvement have mainly been directed towards regulating the corresponding pathways via classical mutagenesis and screening methods. Nowadays, most amino acids are in fact produced by the use of mutants that contain combinations of auxotrophic and regulatory mutations [7].

2. L-Lysine

2.1. L-Lysine biosynthetic pathways

Lysine is an essential amino acid and belongs to the aspartate biosynthetic pathway, which is responsible for biosynthesis of aspartate, asparagine, methionine, threonine, lysine and isoleucine. The regulation of lysine synthesis, as expected, has a close relationship with that of other amino acids in the aspartate family. In bacterial cells there are three different biosynthetic pathways for L-lysine. Initially aspartate is phosphorylated by aspartate kinase, this being the most important reaction in feedback control. *E. coli* and also other Gram-positive species proceed from this point via the succinylase pathway, leading directly to D,L-diaminopimelate, which is a lysine precursor [8]. Other bacteria, e.g. *Bacillus stabilis*, use a pathway with acetylated intermediates [8], although *Bacillus sphaericus*, carries out a single step synthesis of D,L-diaminopimelate by a specific dehydrogenase [9]. An outstanding characteristic of *C. glutamicum*, however, is that it uses both the succinylase and the dehydrogenase variant (Fig. 1). These two pathways exist side by side, allowing D,L-diaminopimelate and L-lysine synthesis, however their functions other than synthesis of D,L-diaminopimelate for cell walls and lysine synthesis are not known. Mutants with an inactive dehydrogenase pathway are still prototrophic but in over-producers lysine secretion is reduced to 50–70%. Although this dehydrogenase pathway is not essential for growth on mineral salt medium, it is however, required to handle an increased flow of metabolites to diaminopimelate, and finally to lysine [10,11].

The contribution of the dehydrogenase pathway was determined to be about 30% of the total lysine synthesized, irrespective of whether lysine-accumulating mutants or wild-type strains were tested. This diversity was generally unexpected since the specific dehydrogenase activity is always very much higher than that of succinylase enzymes, and moreover, it turns out that the partition coefficient between the dehydrogenase and the succinylase pathways varies with cultivation time. At the initiation of the culture, the dehydrogenase pathway contributes about 72% of the flux, while the contribution ceases completely at the end of lysine accumulation. The main reason for this phenomenon was found to be linked directly to ammonium availability in the culture medium, and more specifically the low affinity of dehydrogenase toward ammonium (K_m of 34 mM). When free ammonium is replaced by an organic nitrogen source only the succinylase pathway is found to be operative. So, in *C. glutamicum* the flux distribution over the two pathways of lysine synthesis is governed by the ammonium availability [11–13]. The dehydrogenase enzyme is constitutively formed in *C. glutamicum* and operates at high concentrations of free ammonium. On the other hand when the environment is rich in organic nitrogen the succinylase variant operates although it is energetically more costly [14]. The luxury of having both variants together gives *C. glutamicum* an increased flexibility in response to changing environmental conditions. Since the succinylase variant is energetically more expensive than the dehydrogenase variant, the use of the later could be more favourable in a situation where energy is limited [15].

2.2. Lysine secretion

Several hypotheses have been put forward explaining the mechanism of lysine efflux in *C. glutamicum*. One such makes the assumption of increased membrane permeability due to physical changes. More specifically, Hanel et al. in 1981 observed that limited aeration can change the composition of the membrane fatty acids and thus the membrane permeability is increased [16]. In 1986, however, Luntz et al., gave another possible explanation for lysine secretion, where the bacteria may potentially excrete lysine through channels whose opening is triggered by the lysine concentration [17]. When lysine concentration in the cell surpasses a threshold concentration, these channels open and lysine is excreted, thereafter these channels close again in order to retain the amount of amino acid necessary for the cell's metabolism. This lysine secretion model describes a specific secretion system and rules out the two alternative hypotheses discussed above. Here the lysine efflux in *C. glutamicum* is mediated by a specific energy-dependent secondary carrier system, which is fundamentally different from the uptake system [18,19]. The efflux of the cationic lysine is facilitated by co-transport with two OH^- ions, which is energetically equivalent to an antiport against two protons. Due to this mechanism, the carrier cycle and therefore the secretion activity is mainly regulated by the membrane potential, the pH gradient and the chemical gradient of lysine. Although high concentrations of glutamate (approximately 200 mM) and lysine (approximately 50 mM) could be detected in cells of *C. glutamicum*, only lysine was secreted into the culture medium. This, combined with the high K_m value for lysine (20 mM), indicated that the secretion system is only specific for lysine and that lysine secretion may be induced by dipeptide uptake when high internal concentrations are observed [20]. Since *C. glutamicum* is not capable of metabolising

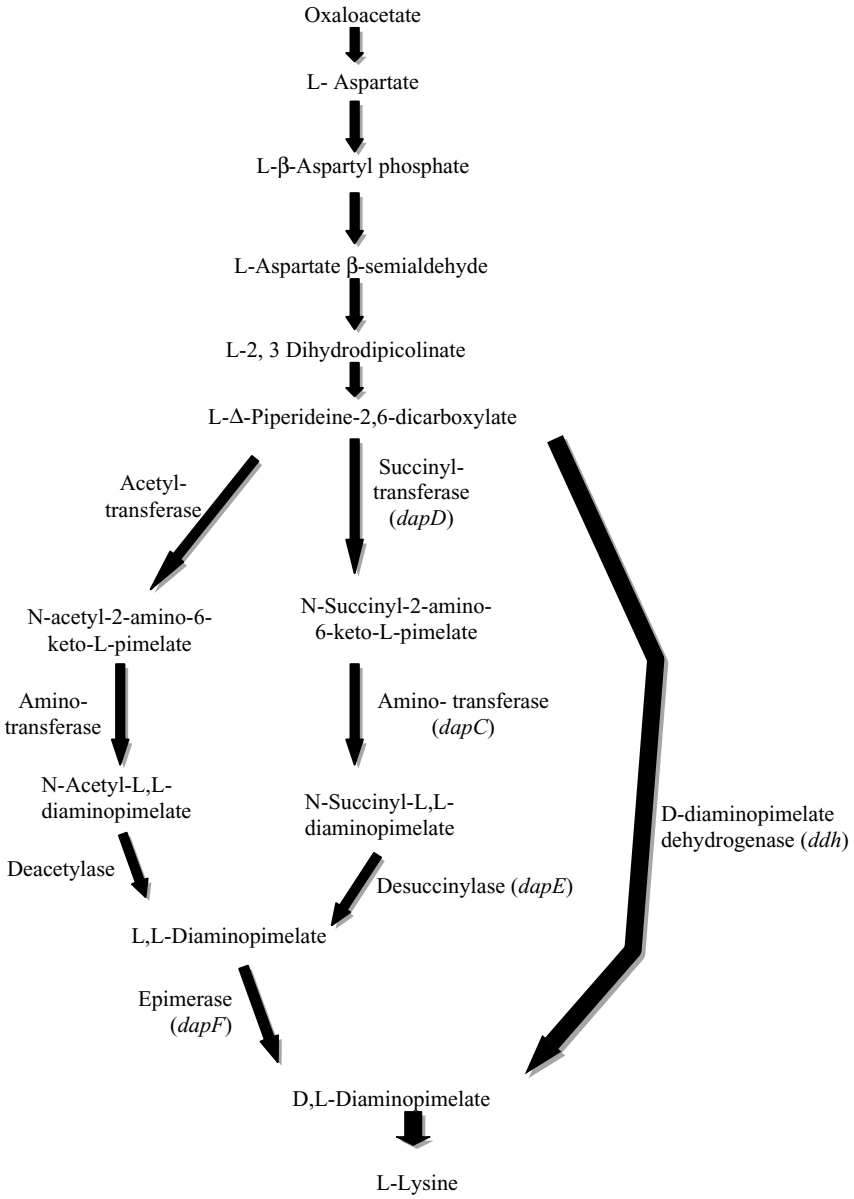

Fig. 1. L-Lysine biosynthetic pathways in prokaryotes, adapted from [8].

lysine [21], the role of the specific export carrier is to maintain the internal lysine concentration at a specified level when peptides are used as carbon and energy sources. Under these circumstances, the bacterium catabolizes only peptides related to central metabolic pathways, such as glutamate, aspartate and alanine for instance, where other amino acids, such as lysine must be excreted [20]. In addition, analysis on the hyperproducing strain MH20-22B of *C. glutamicum*, with feedback-resistant aspartate kinase, revealed that, apart from its ability to accumulate large amounts of L-lysine, the mutant also has an extraordinary secretion activity. Schrumpf et al. (1992) [22] suggested that the secretion carrier might have strong influence on the overproduction of L-lysine. Subsequent experiments carried out on strains with ranging lysine productivities revealed that, although the excretion carrier is present in both mutants and wild type strains, it has a higher expression rate in mutants [23].

The lysine export carrier has recently been cloned [24]. The lysE gene product was found to be a membrane protein, only 236 amino acids in size which spanned the membrane five times. Although six hydrophobic domains were identified on the basis of hydrophobic analyses, only five of them span the membrane. It has been postulated that the additional hydrophobic segment may dip into the membrane or be localized in the surface (Fig. 2). The large loop is located in the cytosol along with the amino terminus, whereas the carboxyl terminal end is directed outside the membrane. This topology enables a direct interaction of the two negative charges of the free loop with the positive charges of L-lysine. In fact, LysE belongs to a new superfamily of translocators whose

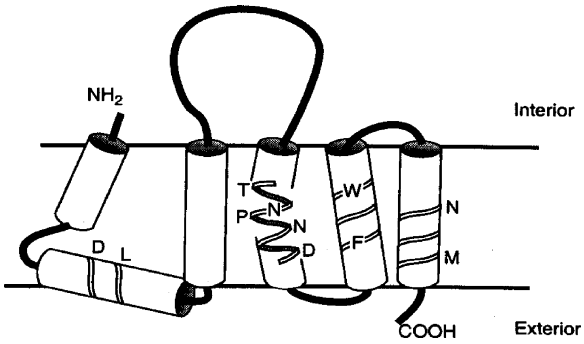

Fig. 2. Model of the topology of the *lys*E product encoding the L-lysine exporter of *Corynebacterium glutamicum* [2].

members are probably all involved in the export of small solutes [25]. Localized immediately adjacent to *lys*E is a regulatory gene *lys*G [24], whose function is the prevention of L-lysine loss. Induction requires the presence of a coinducer, which is either intracellular L-lysine or L-arginine (LysE exports both L-lysine and L-arginine). Furthermore, L-histidine and L-citrulline may act as additional coinducers of *Lys*E, although they are not exported by LysE themselves [26]. Studies on the physiological role function of lysE showed that, in the absence of the carrier, L-lysine may reach an intracellular concentration of more than 1100 mM, which in fact prevents cell growth. Therefore, further to regulation of amino acid synthesis, the export system also represents an important means to regulate the intracellular concentration [24] (Fig. 3). This hypothesis might better represent the natural situation, such as in soil from which *C. glutamicum* has been isolated and where protein degradation products are present.

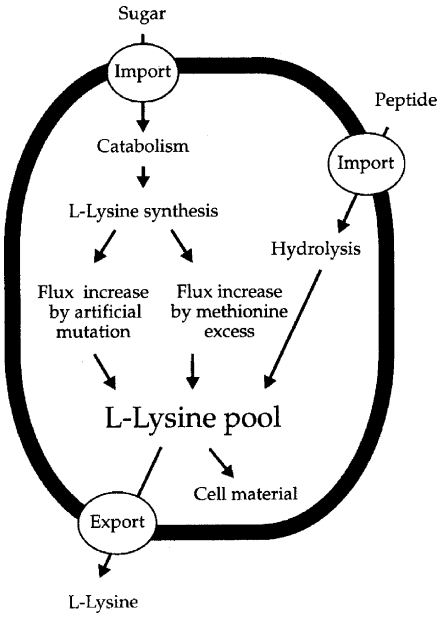

Fig. 3. Physiological function of the lysine exporter in *Corynebacterium glutamicum*. The exporter serves to excrete an excess of L-lysine either after artificial deregulation of lysine biosynthesis, as a result of natural flux imbalances or due to peptide hydrolysis [16].

2.3. L-Lysine uptake

As discussed previously, the uptake system in *C. glutamicum*, differs from the secretion system. Bröer and Krämer in 1990 showed that lysine uptake is mediated by an antiport system, using a lysine auxotrophic strain (because *C. glutamicum* does not consume lysine) [27] and that this antiporter can function effectively in both an electroneutral and in an electrogenic mode. When lysine-lysine exchange takes place (homologous exchange), the antiporter functions in an electroneutral mode where no net charge is translocated and so the antiporter does not sense the membrane potential. On the contrary, when the antiporter exchanges lysine against alanine, valine or isoleucine (heterologous exchange), vectorial movement of charge occurs and consequently the antiporter senses the membrane potential. Therefore, the exchange system is electrogenic and the cells may switch between electroneutral homologous exchange and electrogenic heterologous exchange [27]. A year later, the gene encoding lysine uptake system was cloned and it was found that this *lysl* gene encodes a protein of 501 amino acids (Lysl), which is an integral membrane protein with 13 transmembrane segments [28,29].

3. Metabolic flux analysis

Wild strains of Coryneform bacteria are able to produce only small amounts of amino acids extracellularly. For that reason several methods have been employed to alter the cellular metabolism and regulatory controls of the bacteria, such as mutation, cell fusion and genetic manipulation techniques [30]. However, information on steady-state metabolic fluxes and on flux dynamics is essential for interpreting the metabolic network in amino acid production. This refers to both the central metabolism, which provides the building blocks and energy, as well as to those anabolic pathways that lead to the production of a specific amino acid.

Metabolic engineering, as defined by Stephanopoulos, is the combination of analytical methods to quantify fluxes and their control with molecular biological techniques to implement suggested genetic modifications. In addition, the metabolic pathway is defined to be any sequence of feasible and observable biochemical reaction steps connecting a specified set of input and output metabolites. The determination and mathematical analysis of movement of metabolic biochemichals in vivo has been termed metabolic flux analysis [31]. Metabolic fluxes can generally provide essential information about bottlenecks in the production of a desired metabolite, and facilitate design of engineered high production strains, for example.

Metabolic engineering has been applied to improve the yield and productivity of native products synthesized by

microorganisms, such as in the production of amino acids by *C. lactofermentum*, where the introduction of a feed-back insensitive homoserine dehydrogenase enabled redirection of the flux from lysine towards either threonine or isoleucine [32]. Often the industrial strain employed for a given process has a substrate spectrum and it is therefore necessary to extend the substrate range, as in the case of *C. glutamicum* in which the entire *E. coli* lactose operon was expressed to enable the utilization of lactose [33]. Metabolic engineering approaches can also be used in the construction of pathways leading to novel products, which may be new to the host cell, like the production of novel polyketides by gene shuffling of polyketide synthases [34]. In industrial processes there are often by-products formed, which might be a problem due to their toxicity, or due to the fact that they interfere with the purification of the product. In these cases the by-product can be eliminated through gene disruption, while in other cases the formation of the by-product is essential for the overall cellular function. In these instances a complete analysis of the metabolic network is useful in order to design a strategy for reduction of by-product formation [36]. Metabolic engineering can also be applied to the improvement of physiological cellular properties, such as the sensitivity to high glucose concentrations or the ability to tolerate low oxygen concentrations. A good example of the latter case is the cloning of bacterial haemoglobin from *Vitreoscilla* (VHb) and its expression in *E. coli*, which exhibits improved product synthesis at low dissolved oxygen concentrations [35].

Several concepts have been developed to obtain a quantitative and sufficiently complete picture of the metabolic network in the cell. The first of these was the "metabolic control theory", which attempted to determine the significance of a given reaction within a complex metabolic pathway by calculating the flux control coefficients [36]. The subsequent values of the flux control coefficients can be calculated from the effect that the changes of the enzymatic activity have on the flux and metabolite concentrations. Savageau et al., however, later generalized this hypothesis to form the "biochemical systems theory" [37], but a more recent acceptable concept is the "metabolite balance technique" [38]. Where, the yield and the productivity of metabolite production can be enhanced by modifications of the product pathway that only happen at the principal nodes of the network. Once the principal nodes for the product of interest have been identified in the metabolic network and their degree of rigidity has been assessed, the flux distribution in these nodes can be determined by the quantification of all input (e.g. substrate, oxygen) and output. If the split-ratio of the principal node remains unchanged during a perturbation, then the node is potentially rigid and conversely, if the node split-ratio significantly alters under perturbations, then the node is potentially flexible, as exemplified during lysine synthesis by *C. glutamicum* [39]. Another essential factor in the analysis of a metabolic network is the identification of independent pathways, which are the smallest set of reactions connecting a single network output with the necessary network inputs in a manner that permits the levels of internal species to reach a steady state. Recently Simpson et al. developed a method for the identification of all independent pathways in a network regardless of its size or complexity [40], and these have been widely used in subsequent metabolic flux studies.

3.1. Metabolic flux analysis of lysine production

The application of the "metabolite balance technique" to lysine production by *C. glutamicum* elucidated the flux partitioning in the central metabolism of this organism [40]. Microbial fermentation for L-lysine production can be broken down to four phases (Table 1), but Phase I can be effectively removed by the use of a strain of *C. glutamicum* resistant to *S*-(β-aminoethyl)-L-cysteine (AEC), and fed-batch bioprocessing techniques can be employed to remove Phase IV [41–43]. Thus, from the metabolic engineering point of view only Phases II and III are directly relevant. In *C. glutamicum*, flux distributions constructed from fermentation data revealed that the pentose phosphate pathway (PPP) and phosphoenolpyrunate carboxylase (PPC) shunt, support substantial flux during growth and lysine overproduction.

Although almost 35 branch points are involved in lysine biosynthesis, flux distributions in the *C. glutamicum* metabolic network indicate that significant changes in flux partitioning occur at only three principal nodes: glucose-6-phosphate (G6P); phosphoenolpyruvate (PEP) and pyruvate (Pyr), while the carbon partitioning at all other nodes remain independent of product yield [38]. Partitioning of carbon at the principal nodes must be optimal for maximum L-lysine yield, however, if any of these principal nodes is rigid then sub-maximal L-lysine yield will result. Metabolic flux modelling based on fermentation data revealed that lysine yield is not pyruvate-limited and that the

Table 1
Phases of microbial fermentation during lysine production, adapted from [44]

Growth phases	Characteristics
Phase I	Balanced growth with little or no byproducts. The duration and the biomass concentration of this phase depend on the initial supply of threonine
Phase II	High lysine and biomass production rates, exhaustion of threonine supply, constant respiration
Phase III	High lysine production rates, growth plateaus and respiration decreases
Phase IV	Gradual reduction of lysine production, decrease in biomass concentration, redirection of glucose to byproduct formation (i.e. pyruvate, acetate, alanine and valine)

Pyr branch point might be flexible [44]. Since this may be the case, limitations in lysine yield must therefore result from rigidity at either the G6P or PEP branch points.

G6P is the branch point between glycolysis and the pentose phosphate pathway and therefore it is possible that glucose flow reaching that point is preferentially entering glycolysis over the PPP branch point, thus limiting lysine yield due to low NADPH availability. In order to investigate the G6P branch point flexibility, Vallino and Stephanopoulos [45] constructed two experimental perturbations. The first involved fermentation and flux analysis of a *C. glutamicum* mutant, which had weak G6P isomerase (the first branch point enzyme of glycolysis) activity and the second examined the effect of cultivating *C. glutamicum* on gluconate (a metabolite that enters the PPP directly and effectively bypasses the G6P branch point) as the sole carbon source. The results revealed that lysine yield is not limited by suboptimal flux partitioning at the G6P branch point caused by the dominance of the glycolytic branch and that lysine yield is also not limited by NADPH production, since gluconate catabolism makes NADPH readily available. Thus, the G6P branch point is flexible and lysine yield limitations must be attributed to suboptimal flux partitioning at the PEP branch point, thereby indicating that the PEP node is strongly rigid. Although, the G6P point has been demonstrated to be flexible under moderate lysine yields, Vallino and Stephanopoulos [45] stress that this branch point can become a limiting factor if lysine yield is dramatically increased by improving flux partitioning at the other principal branch points. In order to achieve high lysine yields, it is now clear that significant alterations in flux partitioning at the principal branch points (glucose-6-phosphate [G6P], phosphoenolpyruvate [PEP] and pyruvate [Pyr]) must occur. It is worth noting, however, that the correlation between fluxes in amino acid biosynthesis and secretion has not yet been considered.

In *C. glutamicum*, the cytosolic lysine pool responds to both the biosynthetic flux and the activity of the lysine secretion system [20], thereby demonstrating that secretion displays significant flux control and that improvements in membrane transport are also required in order to improve extracellular production lysine rates [21]. Metabolic flux analysis of lysine-producing *C. glutamicum* was reported by Hollander in 1994, who demonstrated that the lysine yield is strongly dependent on the way the organism generates NADPH, and that excess energy production in biosynthesis limits the yield of the amino acid [46]. Furthermore, metabolic flux modelling, based on continuous culture data, revealed that the production of ATP is not likely to be a limiting factor in L-lysine production and that a high TCA activity, coupled with the high rigidity of the PEP node, is likely to be the cause of the large discrepancy between theoretical and actual yields in L-lysine fermentations [47].

It is clear that the "metabolite balance technique" is a powerful tool that facilitates rational identification of enzymes, branch points and sub-networks that may limit product synthesis. However, the complexity of the metabolic networks combined with the insufficient knowledge base, make this method only partially reliable. The main limitation of this approach relates to the difficulty in including the particular importance of metabolite cycles, complex nodes or equilibrating reactions (forward and backward flow in the enzymatic reaction) into the calculations [48]. For that reason other methods have been included in this type of analysis, such as isotopic tracer flux analysis or NMR-techniques to provide additional information on flux distribution of complex components within the metabolic network. These techniques are ideally suited to provide the missing flux information because they allow the split ratios of flux distributions to be determined at important metabolic branch points [49]. For example, the [^{13}C]-labelling technique has been combined with metabolite balancing, to determine the flux distribution at the branch point between glycolysis and the pentose phosphate pathway. These studies revealed that the oxidative pentose phosphate pathway in *C. glutamicum* is mainly regulated by the ratio of NADPH/NADP concentrations and the specific activity of glucose-6-phosphate (G6P) dehydrogenase [49]. The integrated metabolite balancing and [^{13}C]-labelling approach was also applied to *C. glutamicum* in order to analyse its anaplerotic network. The anaplerotic reactions are of paramount importance for the synthesis of L-lysine because they supply oxaloacetate, a precursor to aspartate. *C. glutamicum* possesses two C3-carboxylating enzymes (PEP carboxylase, Pyr carboxylase) and three C4-decarboxylating enzymes (oxaloacetate decarboxylase, PEP carboxykinase and malic enzyme). With the use of [^{13}C]-labelling techniques it was revealed that carboxylation and decarboxylation occur simultaneously in *C. glutamicum* and that Pyr carboxylase and PEP carboxykinase (which were found to be the most active enzymes in the anaplerotic node) constitute a futile cycle in which oxaloacetate flows to pyruvate via phosphoenolpyrunate [21,49].

4. Improvements in L-lysine production

Large-scale production of L-lysine is generally achieved with the use of *C. glutamicum* mutants obtained through classical screening programs. These mutants are resistant to aspartate kinase [26], this reaction being the most important reaction in feedback control. Individual overexpression of all the enzymes involved in the pathway of aspartate to lysine revealed that only the gene for the feedback-resistant aspartate kinase alone is enough to achieve lysine secretion in the wild type. The deregulation of this enzyme is therefore very important in order to obtain *C. glutamicum* strains that overproduce lysine, although upregulation of the remaining enzymes involved in this biosynthetic pathway had no effect on the lysine overproduction. However, the overexpression of dehydrodipicolinate synthase may also convert the wild type into a lysine overproducer, though not to the same extent as deregulation of aspartate kinase. This implies that dehydrodipicolinate synthase is also involved in the flux

control of this pathway and consequently, the construction of a strong lysine producing strain relies on attempts to increase the activities of these two enzymes [50]. However, *C. glutamicum* also possesses both succinylase and dehydrogenase variants for L-lysine synthesis and therefore the manipulation of these two biosynthetic pathways is also critical for further strain improvement. Apart from the biosynthetic pathways in *C. glutamicum*, the overexpression of *lys*E also appears to be necessary for the construction of L-lysine overproducing strains [50].

Significant direct production of L-lysine from carbohydrate was first developed with a homoserine- or both threonine- and methionine-requiring auxotrophs of *C. glutamicum* [51]. The overproduction of L-lysine by homoserine or threonine auxotrophs is due to the release of feedback inhibition from aspartate kinase, since these strains cannot produce threonine. The inhibition of homoserine synthesis, by nullifying the activity of the homoserine dehydrogenase enzyme, results in the release of the feedback inhibition by threonine and lysine on aspartate kinase. Consequently, the aspartic semialdehyde produced can proceed to lysine through the lysine biosynthetic pathway, where no further inhibition has been detected (Fig. 4). Threonine and leucine auxotrophs can also be utilized for L-lysine production, but they are inferior to homoserine auxotrophs, which themselves may produce up to 28–30 g L-lysine l^{-1} [51].

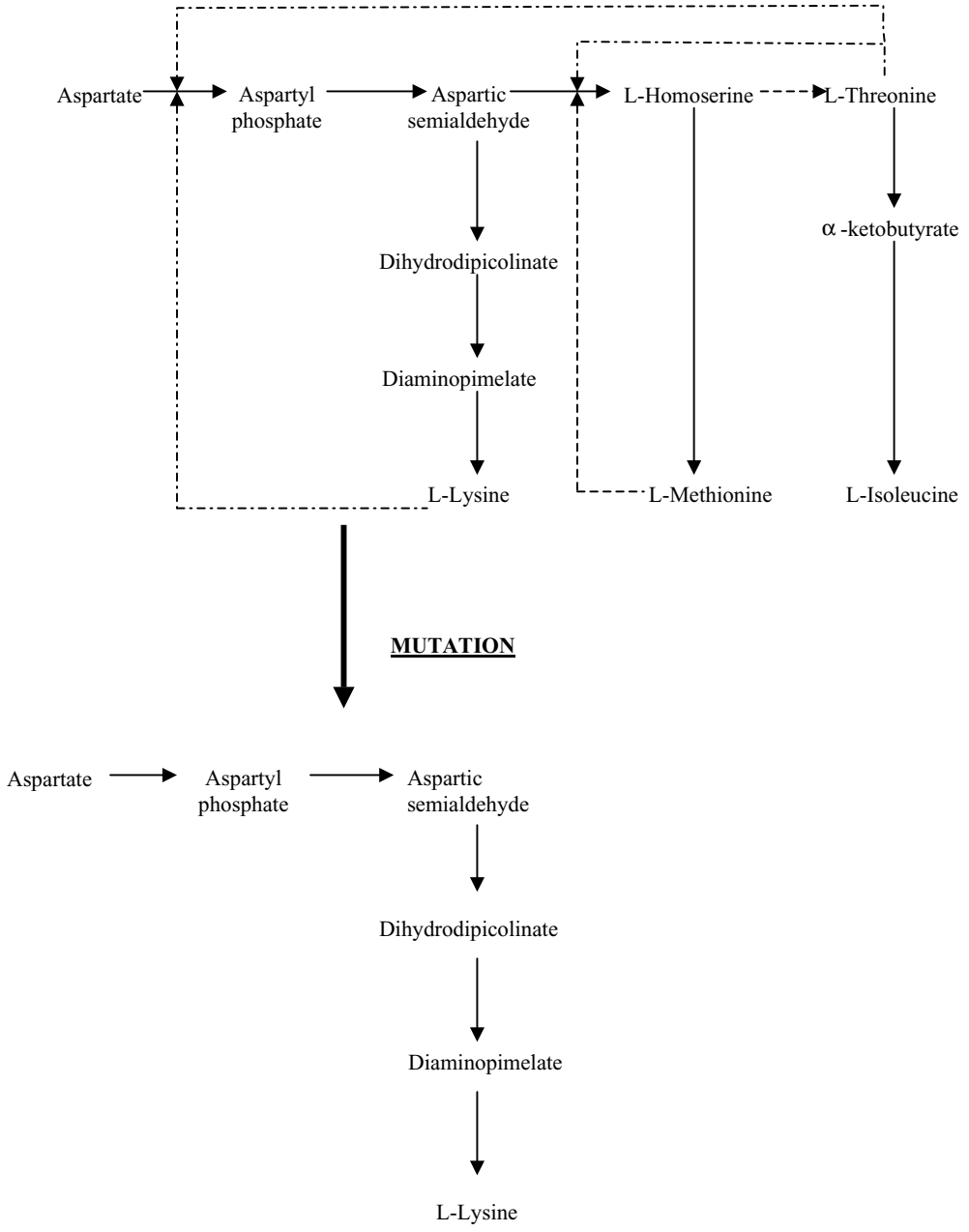

Fig. 4. Deregulation of lysine biosynthesis in homoserine auxotrophs of *Corynebacterium glutamicum*. Modified from [21].

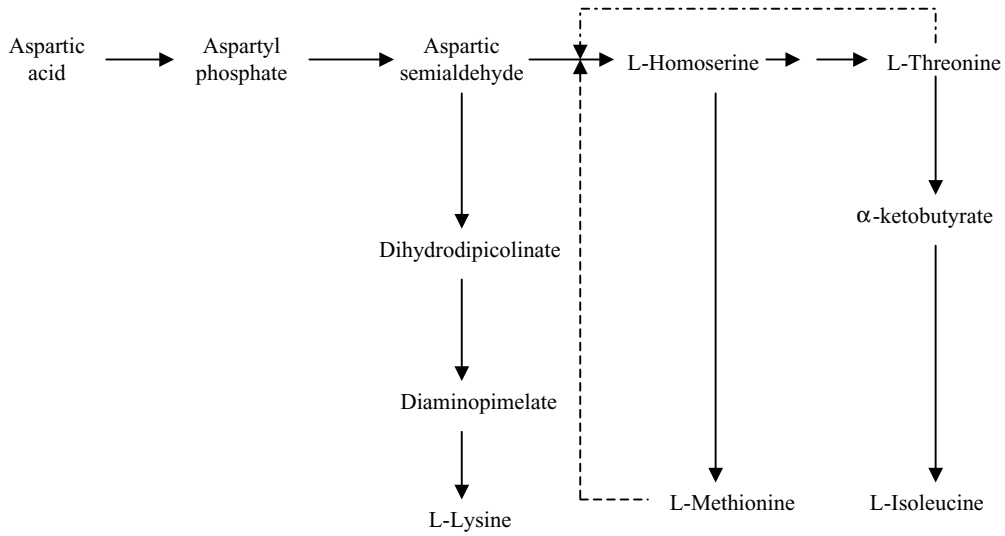

Fig. 5. Deregulation of lysine in AEC-resistant mutant of *Brevibacterium flavum*. Adapted from [21].

Improved L-lysine yield was also achieved through development of mutants with aspartokinase insensitive to feedback inhibition by the end product, but with normal catalytic activity. Regulation amino acid analogues are used that act as pseudofeedback inhibitors, slowing synthesis of the end product and consequently, the microorganism does not grow due to its inability to replace the end products within its metabolism. Deregulatory mutants are resistant to the analogue but may grow in its presence. For example, an AEC (S-2-aminoethyl-L-cystein, a lysine analogue) resistant mutant of *Brevibacterium flavum* is capable of producing large amounts of L-lysine [18]. With blockage of feedback inhibition on aspartate kinase, resistance to AEC is also achieved which releases the concerted feedback inhibition. In this way, aspartic semialdehyde cannot be converted to threonine because of the feedback inhibition of L-threonine and thus the overproduced aspartic semialdehyde is channelled to L-lysine production (Fig. 5) [18,52]. An increase in lysine yield was achieved with the further use of an AEC-resistant homoserine and leucine *C. glutamicum* auxotroph. This was observed to produce 39.5 g L-lysine l^{-1} in a medium containing 10% (w/v) reducing sugars, while homoserine plus leucine auxotrophs produced 34.5 g L-lysine l^{-1}. Furthermore, studies have detailed a *C. glutamicum* mutant which required homoserine, leucine and pantothenic acid and was resistant to AEC, and that produced 42 g L-lysine l^{-1} in a cane molasses medium containing 10% w/v reducing sugars [18]. This is currently the most popular carbon source used in industrial lysine production is cane molasses but others such as acetic acid and ethanol may also be used.

5. Conclusions

Almost half a century has passed since the discovery of *Micrococcus glutamicus* (later named *C. glutamicum*), a microorganism capable of excreting L-amino acids [3]. Since then, several attempts have been made to understand and improve amino acid production and this work has yielded significant information on the biochemistry, physiology and genetics of *C. glutamicum*.

Rapid progress in biochemistry and microbial genetics has increased our knowledge and understanding of carbon metabolism and metabolic regulation, and metabolic flux analysis has become a new powerful tool, enabling researchers to understand cellular kinetics [38]. Integration of the aforementioned techniques is expected not only to raise productivity but also to further the mechanistic understanding of amino acid production by coryneform bacteria in the artificial fermentation environment.

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