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REVIEW ARTICLE

Our microbes not only produce antibiotics, they also overproduce amino acids

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Fermentative production of amino acids is an important goal of modern biotechnology. Through fermentation, micro-organisms growing on inexpensive carbon and nitrogen sources can produce a wide array of valuable products including amino acids. The amino acid market is $8 billion and mainly impacts the food, pharmaceutical and cosmetics industries. In terms of tons of amino acids produced per year by fermentation, L-glutamate is the most important amino acid produced (3.3 million), followed by L-lysine (2.2 million). The bacteria producing these amino acids are among the top fermentation organisms with respect to titers. *Corynebacterium glutamicum* is the best producer.
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INTRODUCTION

The worldwide amino acid market is $8 billion and is projected to reach $20.4 billion by the year 2020. The US market for amino acids represents about 20% of the global market, at nearly $1.6 billion in 2011. Global amino acid production was 4.5 million tons in 2012,[1] 6.68 million tons in 2014 and is expected to reach 10 million tons by 2022, growing at a CAGR (Compound Annual Growth Rate) of 5.6% from 2015 to 2022. The growing demand for amino acids includes markets for animal feed, health foods, pharmaceutical precursors, dietary supplements, artificial sweeteners and cosmetics.[2,3] Among them, the animal feed supplement segment (involving L-lysine, DL-methionine, L-threonine and L-tryptophan) constitutes the largest share (56%) of the total amino acid market and it is expected to keep fueling market growth in the coming years. The annual demand for feed-grade amino acids globally is about 2.4 million tons with an estimated value of $\$6 \times 10^9$.[4] Furthermore, there is strong commercial interest in developing new amino acid applications.

Some companies are major players in the amino acid production industry. Among them are Ajinomoto, Archer Daniels Midland, Cargill Inc., Daesang Corporation, Evonik Industries AG, Kyowa Hakko Kogyo, Nippon Soda, Prinova U.S. LLC, Royal DSM, Showa Denko KK and Zhejiang Chemicals. Small-scale participants include Iris Biotech GmbH, Nanjing Liang Chemical, Sunrise Nutrachem Group, Tokyo Chemical Industry, Novus International Inc., Anaspec Inc., CJ Cheil Jedang Corporation and Adisseo France SAS.

Amino acid production is a multi-million ton-scale industry (Table 1). More than five million tons were produced in 2013 for the fermentative production of L-glutamate and L-lysine alone.[5] The microbial methods for production of amino acids are either fermentative or enzymatic. Produced mainly by fermentation were 3.3 million tons of L-glutamate/monosodium glutamate, 2.2 million tons of L-lysine-HCl, 330 000 tons of L-threonine, 30 000 tons of L-phenylalanine (including that made by chemical synthesis), 3000 tons of L-glutamine, 1500 tons of L-arginine, 1000 tons of L-valine, 3000 tons of L-leucine (including that made by extraction), 2000–3000 tons of L-isoleucine (including extraction), 500 tons of L-histidine, 500 tons of L-proline, 400 tons of L-serine and 200 tons of L-tyrosine. Enzymatically produced were 17 000 tons of L-aspartic acid (from fumarate and ammonia), 4000 tons of cysteine (from DL-2-amino-2-thiazoline-4-carboxylate) and 600 tons of L-alanine (from aspartate). Produced by fermentation and enzymatic methods were 14 000 tons of L-tryptophan. DL-methionine is made chemically at 850 000 tons per year.

Organisms currently used for industrial production of amino acids have been developed by mutation programs followed by selection or 'brute force' screening. Such efforts often start with organisms having some capacity to make the desired compound but which require multiple mutations leading to deregulation in a particular biosynthetic pathway before high productivity can be obtained. This approach to strain improvement has been remarkably successful in producing organisms that make industrially significant concentrations of amino acids.[6] Among the most common mutant strains employed for amino acid production are auxotrophic mutants, regulatory mutants and auxotrophic regulatory mutants. Using these bacterial mutants, all the essential amino acids except L-methionine can be produced at economically meaningful levels by 'direct fermentation' from cheap carbon sources such as carbohydrate materials or acetic acid. Progress

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Dedication: We are delighted to dedicate this manuscript to Professor Hamao Umezawa, a great scientist and a great man. It is indeed a pleasure to have our contribution included as part of the Special Issue devoted to him.
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Table 1 Worldwide production of amino acids by fermentation

Amino acids	*Titer (g l^{-1})*	*Tons per year*	*Market ($US)*	*Reference*
L-Alanine	275	500	—	5
L-Arginine	96	1 500	150 million	5,46,47
L-Glutamic acid	150	3 300 000	1.5 billion	5,7,17
L-Glutamine	49	3 000	—	5
L-Histidine	42	500	—	5
L-Isoleucine	40	3000	—	28,29
L-Leucine	38	3000	—	20,27
L-Lysine-HCl	170	2 200 000	1.5 billion	5,7,16
L-Phenylalanine	57	30 000	1.0 billion	1,5,42
L-Proline	108	500	—	5
L-Serine	65	400	—	5
L-Threonine	132	330 000	270 million	5,23,24,25
L-Tryptophan	60	14 000	150 million	1,5,40,41
L-Tyrosine	55	200	50 million	543
L-Valine	227	2,000	45 million	5,30

is even being made in the production of L-methionine by fermentation.

Several problems are commonly faced with this 'brute force' approach, which include: (i) the necessity of screening large numbers of mutants for the rare combination of traits sequentially obtained that lead to overproduction, and (ii) the weakened vitality of the producing strain after several rounds of mutagenesis. More recent approaches utilize the techniques of modern genetic and metabolic engineering to develop strains overproducing amino acids.[7] Both approaches have made an impact by use of the following strategies: (i) upregulation of the rate-limiting (controlling) enzyme of the pathway, (ii) amplification of the first enzyme after a branch point, (iii) amplification of the first enzyme leading from central metabolism to increase carbon flow into the pathway followed by sequential removal of bottlenecks caused by accumulation of intermediates, (iv) cloning of a gene encoding an enzyme with more or less feedback regulation, and (v) introduction of a gene encoding an enzyme with a functional or energetic advantage as replacement for the normal enzyme. Transport mutations are also useful, that is, mutations decreasing amino acid uptake often allow for improved excretion and lower intracellular feedback control. In cases where excretion is carrier-mediated, increase in activity of these carrier enzymes increases production of the amino acid.[6]

Metabolic reconstruction via functional gene annotation has revealed fascinating insights into *Corynebacterium glutamicum*, including functional predictions for over 60% of the identified genes. Development of specific DNA microarrays has been directed to investigate gene expression during the growth of *C. glutamicum*. Expression profiles of selected genes involved in central metabolism and amino acid production have been determined.[7] For proteomic analysis, two-dimensional gel electrophoresis has been used to identify different proteins and to study the influence of nitrogen starvation on the proteome. For the quantification of metabolic fluxes (the 'fluxome'), comprehensive approaches combining ^{13}C-tracer experiments, metabolite balancing and isotopomer modeling have been developed and applied to *C. glutamicum*.[8] They involve comparative fluxome analysis during growth on different carbon sources, and glutamate and lysine production in batch cultures by different mutants. With this information, it has been possible to conclude that the decrease in glucose uptake rate causes the metabolic shift from cell growth towards L-lysine biosynthesis and that a high flux of the tricarboxylic acid cycle is favorable for amino acid production. Additional examples of amino acids whose production has been improved by this information include L-valine and L-threonine.

After strain generation, culture conditions must be designed for each particular strain to get the best microbial performance. For a process to be realized economically, basic research has to be successfully translated into operations on the industrial scale. Scale-up is a procedure in which the results of small-scale experiments are used as the basis for the design, testing and implementation of a larger scale system. The workhorse of the fermentation industry is the conventional batch fermenter, an agitated jacketed pressure vessel with cooling coils, baffles and a sparger ring to introduce vapor into the fermentation process. Most of the amino acids are produced by fed-batch processes using the best performing mutants. The fermentation process involves, at least, the following steps: (i) a fermentation tank is charged with culture medium and sterilized. The medium contains a suitable carbon source (such as sugar cane syrup), as well as the required nitrogen, sulfur and phosphorus sources, plus some trace elements; (ii) a seed culture of the production strain, previously grown in a smaller fermenter, is added to the fermentation tank and stirred under specified conditions (temperature, pH, aeration); (iii) depending on the culture requirements, additional nutrients are added during the fermentation in a controlled manner to allow for optimal yields; and (iv) the amino acid is released by the micro-organism into the fermentation solution and, after separation by ion exchange, is isolated by crystallization. Today, large industrial plants are in use for amino acid production and the amino acids produced by microbial process are the L-forms. Such stereo-specificity makes the processes advantageous as compared to synthetic processes.

PRODUCTION OF L-GLUTAMIC ACID

L-Glutamic acid has emerged as the largest amino acid product segment and accounted for over 40% of total market volume in 2014. Monosodium glutamate is a potent flavor enhancer, a crucial component of the taste of cheese, seafood, meat broths and other foods. Professor Kikunae Ikeda, a Japanese scientist, identified the unique taste of umami, attributed to glutamic acid, as the fifth basic taste after sweet, sour, salty and bitter in the tongue.[9] The umami receptor taste is located in aggregates of neuroepithelial cells embedded in the stratified epithelia of the oral cavity.[10] The umami taste receptor is a heteromeric complex of two class C G-protein-coupled receptors, T1R1 and T1R3. A cooperative ligand-binding model involving the Venus flytrap domain of T1R1, where L-glutamate binds to the hinge region has been proposed.[11] In addition to glutamate, there are two more umami substances derived from primary metabolites, that is, inosinic and guanylic acids.[9] Although glutamate is naturally occurring in many foods, it is frequently added as a flavor enhancer. Glutamate was first made by fermentation in Japan in the late 1950s.

Many organisms, belonging to a wide range of taxonomically related genera, including *Brevibacterium, Corynebacterium*, *Microbacterium* and *Micrococcus*, are capable of over-producing glutamate. *Brevibacterium lactofermentum* and *Brevibacterium flavum* were reclassified as subspecies of *C. glutamicum*.[12] These organisms were shown to possess the Embden-Meyerhof Parnas glycolytic pathway, the hexose monophosphate pathway, the tricarboxylic acid cycle and the glyoxylate bypass (Figure 1). The tricarboxylic acid cycle, also known as the Krebs cycle, requires a continuous replenishment of oxaloacetate in order to replace the intermediates withdrawn for the synthesis of biomass and other amino acids. During growth on glucose and other glycolytic intermediates, the anaplerotic function is fulfilled by phosphoenolpyruvate carboxylase and pyruvate carboxylase.[6]

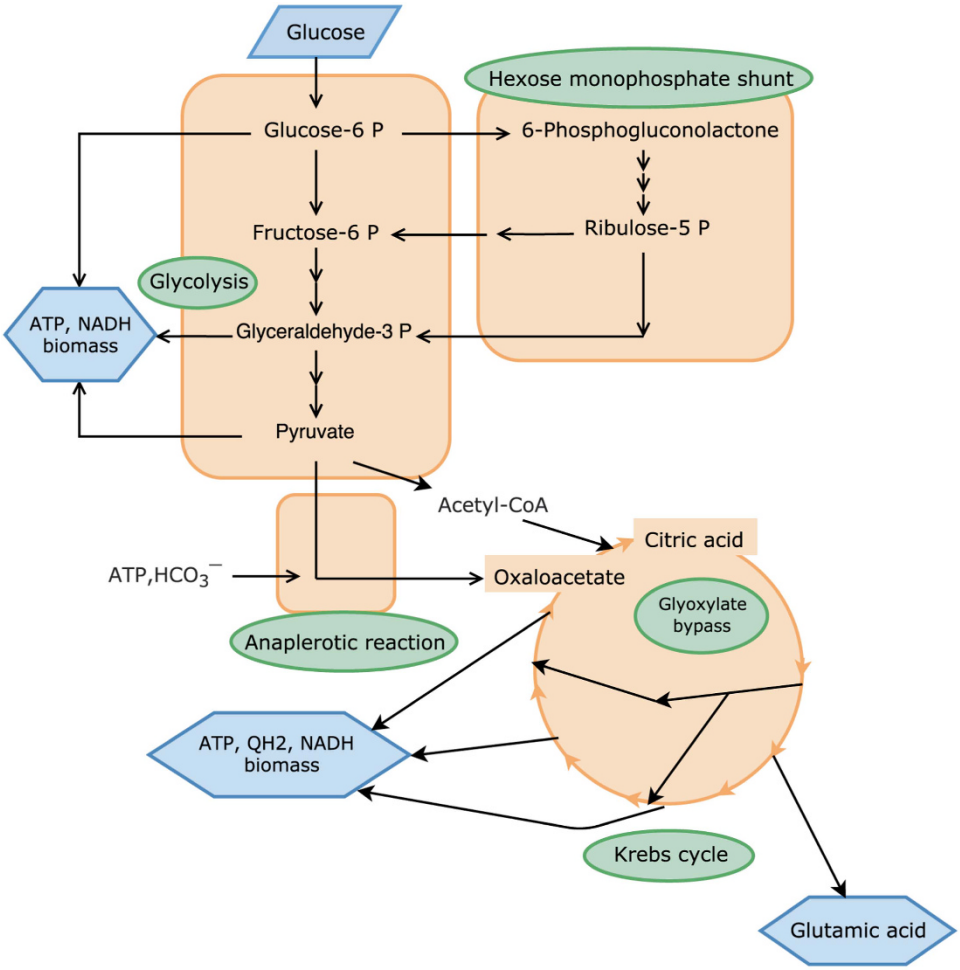

Figure 1 Biosynthesis of glutamic acid from glucose.

In normal conditions, glutamic acid over-production would not be expected to occur due to feedback regulation. Glutamate feedback controls include repression of phosphoenolpyruvate carboxylase, citrate synthase and NADP-glutamate dehydrogenase; the last-named enzyme is also inhibited by glutamate.[13] However, by decreasing the effectiveness of the barrier to outward passage, glutamate can be pumped out of the cell, thus allowing its biosynthesis to proceed unabated. The excretion of glutamate relieves the glutamate pathway from feedback control until a very high level is accumulated; commercial L-glutamate titer has reached 150 g l^{-1} (Table 1).

Glutamate excretion can be intentionally influenced by manipulations of growth conditions as follows: (i) since all glutamate over-producers are natural biotin auxotrophs, biotin limitation brings about glutamate over-production in *C. glutamicum* by decreasing the cell membrane permeability barrier that restricts the excretion of glutamate. (ii) Addition of penicillin or fatty acid surfactants (for example, tween 60) to exponentially growing cultures alters the permeability properties of the cell membrane and allows glutamate to flow out easily. Apparently, all of these manipulations result in a phospholipid-deficient cytoplasmic membrane, which favors active excretion of glutamate from the cell. This view was further substantiated by the discoveries that oleate limitation of an oleate auxotroph and glycerol limitation of a glycerol auxotroph also bring about glutamate excretion. Furthermore, glutamate-excreting cells have a very low level of cell lipids, especially phospholipids. In addition, it was shown that the various manipulations leading to glutamate over-production cause increased permeability of the mycolic acid layer of the cell wall. The glutamate over-producing bacteria are characterized by a special cell envelope containing mycolic acids which surrounds the entire cell as a structured layer and is thought to be involved in permeation of solutes. The mycolic acids esterified with arabinogalactan and the non-covalently bound mycolic acid derivatives form a second lipid layer of the cell; with the cytoplasmic membrane being the first. Over-expression or inactivation of enzymes that are involved in lipid synthesis alters the chemical and physical properties of the cytoplasmic membrane and changes glutamate efflux dramatically.[7]

Polyglutamic acid (PGA) is made by bacilli. When made by *Bacillus subtilis* and *Bacillus licheniformis*, it contains repeated units of D-glutamic acid and L-glutamic acid. However, when made by *Bacillus anthracis*, it contains D-glutamic acid exclusively. PGA is potentially useful as a drug delivery agent for drugs against cancer, for example, as a carrier of doxorubicin. It is also being considered for (a) vaccination where high molecular weight PGA can stimulate the immune system against bound antigens, (b) tissue engineering where PGA/chitosan hydrogels serve as tissue scaffolds, and (c) as medical adhesives. For the food industry, PGA improves the appearance and texture of products, and promotes the absorption of minerals, such as Ca^{++}, which are beneficial for treatment of osteoporosis. A titer of 101 g l^{-1} was achieved using *B. subtilis*.[14]

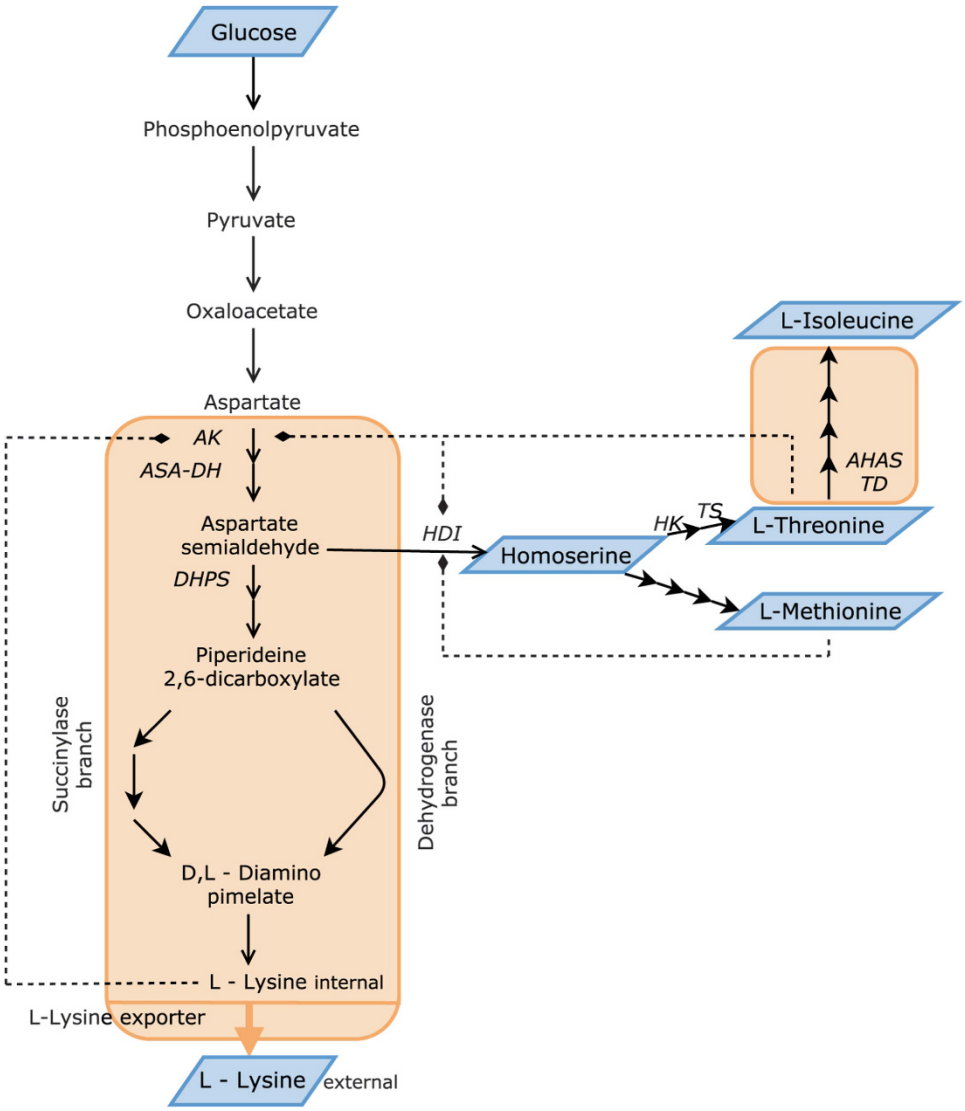

Figure 2 Biosynthetic pathway to L-lysine, L-threonine and L-isoleucine in *C. glutamicum*. AK, aspartate kinases; ASA-DH, aspartate-semialdehyde dehydrogenase; HDI, homoserine dehydrogenase; HK, homoserine kinase; TS, threonine synthetase; TD, threonine dehydratase, AHAS, acetohydroxy acid synthase.

PRODUCTION OF L-LYSINE

L-Lysine represents the fastest growing amino acid segment. The bulk of the cereals consumed in the world are deficient in L-lysine. It is an essential ingredient for the growth of animals and an important part of a billion-dollar animal feed industry. Lysine supplementation converts cereals into balanced food or feed for animals including poultry, swine and other livestock. In addition to animal feed, lysine is used in pharmaceuticals, dietary supplements and cosmetics.[4] The global market for L-lysine has increased by almost 20-fold in the past 20 or so years and several companies like Ajinomoto and Archer Daniels Midland have expanded their facilities with strong investments in Brazil, China and the US.

Lysine is a member of the aspartate family of amino acids (Figure 2). It is made in bacteria by a branched pathway that also yields methionine, threonine and isoleucine. This pathway is controlled very tightly in organisms such as *Escherichia coli*, which contains three aspartate kinases (AKs), each of which is regulated by a different end product.[6]

In addition, after each branch point, the initial enzymes are inhibited by their respective end product(s) and no overproduction usually occurs. However, *C. glutamicum*, the organism used for the commercial production of L-lysine, contains a single AK that is regulated via concerted feedback inhibition by threonine plus lysine. The relative contribution of carbon flux through the pentose phosphate pathway varies depending on the amino acid being produced, for example, while it only contributes 20% of the total flux in the case of glutamate formation, it contributes 60–70% in the case of lysine production. This is evidently due to the high level of NADPH required for lysine formation. Use of recombinant DNA technology has shown that the factors that significantly limit the overproduction of lysine are: (i) feedback inhibition of AK by lysine plus threonine, (ii) the low level of dihydrodipicolinate synthase, (iii) the low level of phosphoenolpyruvate carboxylase and (iv) the low level of aspartase.[15] *C. glutamicum* produced 1 500 000 tons of L-lysine in 2013.[16]

Much work has been done on auxotrophic and regulatory mutants of glutamate over-producing strains for the production of lysine. By genetic removal of homoserine dehydrogenase (HDI), a glutamate-producing wild-type *Corynebacterium* strain was converted into a lysine over-producing mutant that cannot grow unless methionine and threonine are added to the medium. As long as the threonine supplement is kept low, the intracellular concentration of threonine is limiting and feedback inhibition of AK is bypassed, leading to excretion of over 70 g l^{-1} of lysine in culture fluids. In some strains, addition of methionine and isoleucine to the medium led to the increase in lysine over-production. Selection for S-2-aminoethylcysteine (AEC; thialysine) resistance blocks feedback inhibition of AK. Other anti-metabolites useful for deregulation of AK include a mixture of α-ketobutyrate and aspartate hydroxamate. Leucine auxotrophy can also increase lysine production. L-Lysine titers are known to be as high as 170 g l^{-1},[7] (Table 1) with a productivity of 4 g l^{-1} h^{-1}.[15] The selling price of L-lysine was $1.50 per kg in 2015.[17]

Excretion of lysine by *C. glutamicum* is by active transport reaching a concentration of several hundred millimolar in the external medium. Lysine, a cation, must be excreted against the membrane potential gradient (outside is positive) and the excretion is carrier-mediated. The system is dependent on electron motive force, not on ATP.

Genome-based strain reconstruction has been used to improve the lysine production rate of *C. glutamicum* by comparing a high producing strain (production rate slightly less than 2 g l^{-1} h^{-1}) with the wild-type strain. Comparison of 16 genes from the production strain, encoding enzymes of the pathway from glucose to lysine, revealed mutations in five of the genes. Introduction of three of these mutations (*hom*, *lysC* and *pyc*, encoding HDI, AK and pyruvate carboxylase, respectively) into the wild-type created a new strain that produced 80 g l^{-1} in 27 h, at a rate of 3 g l^{-1} h^{-1}. An additional increase (15%) in L-lysine production was observed by introduction of a mutation in the 6-phosphogluconate dehydrogenase gene (*gnd*). Enzymatic analysis revealed that the mutant enzyme was less sensitive than the wild-type enzyme to allosteric inhibition by intracellular metabolites. Isotope-based metabolic flux analysis demonstrated that the *gnd* mutation resulted in an 8% increase in carbon flux through the pentose phosphate pathway during L-lysine production. Finally, by introducing the *mqo* (malate:quinone oxidoreductase) mutation, it was possible to increase both the rate of production and the L-lysine titer to 95 g l^{-1} by fed-batch culture.[18]

With the use of systems metabolic engineering, 12 defined genome-based changes in genes encoding central metabolic enzymes redirected the major carbon flux towards the optimal L-lysine pathway usage, as predicted by *in silico* modeling. The engineered *C. glutamicum* strain was able to produce lysine with a high yield of 0.55 g per gram of glucose, a titer of 120 g l^{-1} lysine and a productivity of 4.0 g l^{-1} h^{-1} in fed-batch culture.[7,19]

PRODUCTION OF L-THREONINE

L-Threonine is used in the agricultural, pharmaceutical and cosmetic industries. It is the second major amino acid used for feeding pigs and poultry. The pathway of threonine biosynthesis is similar in all micro-organisms. Starting from L-aspartate, the pathway involves five steps catalyzed by five enzymes: AK, aspartate-semialdehyde dehydrogenase (ASA-DH), HDI, homoserine kinase, and threonine synthetase.

Production of L-threonine has been achieved with the use of several micro-organisms. In *Serratia marcescens*, construction of a high threonine producer was done by transductional crosses that combined several feedback control mutations into one organism. Three classes of mutants were obtained from the parental strain as the source of genetic material for transduction: (i) one strain in which both the threonine-regulated AK and HD were resistant to feedback inhibition by threonine. It was selected on the basis of β-hydroxynorvaline resistance; (ii) a second strain, also selected for β-hydroxynorvaline resistance, in which HDI was resistant to both inhibition and repression and the threonine-regulated AK was constitutively synthesized; and (iii) a third strain that was resistant to thialysine, in which the lysine-regulated AK was resistant to feedback inhibition and repression. Since at least one of the three key enzymes in threonine synthesis was still subject to regulation in these strains, each produced only modest amounts of threonine (4.1 to 8.7 g l^{-1}). Recombination of the three mutations by transduction yielded a strain that produced higher levels of threonine (25 g l^{-1}), had AK and HDI activities that were resistant to feedback regulation by threonine and lysine, and was a methionine bradytroph (leaky auxotroph). Another six regulatory mutations derived by resistance to amino acid analogues were combined into a single strain of *S. marcescens* by transduction. These mutations led to desensitization and derepression of AKs I, II, and III and HDIs I and II. The resulting transductant produced 40 g l^{-1} of threonine, which was further improved to 63 g l^{-1} through over-expression of phosphoenolpyruvate carboxylase.[20] When sucrose was continuously fed to the medium, production of 100 g l^{-1} was obtained after 96 h of incubation.[21]

Production of L-threonine by *E. coli* is limited by formation of acetate. By genetically decreasing acetate production, Xie *et al.*[22] increased L-threonine production to 111 g l^{-1}. They did this by simultaneously deleting some genes encoding key glycolytic enzymes. The genes were *pfk*, encoding phosphofructokinase, and *pyk*, encoding pyruvate kinase.

Determination of the key enzymes involved in L-threonine production in *E. coli* by proteomics indicated that the isoenzyme LysC, which catalyzes the first step, is the key enzyme of the pathway from aspartate to threonine.[23] Over-expression of LysC increased the threonine titer by 30% to 132 g l^{-1} (Table 1) and the yield was increased from 40 to 50%.

In *E. coli*, threonine production was increased to 76 g l^{-1} by conventional mutagenesis and selection/screening techniques. Of major importance were mutations to decrease both regulation of the pathway and degradation of the amino acid. An *E. coli* fed-batch process with methionine and phosphate feeding yielded 98 g l^{-1} L-threonine at 60 h. Another *E. coli* strain was developed via mutation and genetic engineering and optimized by inactivation of threonine dehydratase (TD), resulting in a process yielding 100 g l^{-1} after 36 h of fermentation.

By using combined feeding strategies, L-threonine levels reached 124 g l^{-1}.[24] Threonine production by *E. coli* is limited by the formation of acetate. Threonine excretion by *C. glutamicum* is mainly (>90%) effected by a carrier-mediated export mechanism dependent on membrane potential. Cloning of extra copies of threonine export genes into an *E. coli* strain producing threonine led to increased production. Also increased was resistance to toxic anti-metabolites of threonine. Another means of increasing threonine production is reduction in the activity of serine hydroxytransferase, which breaks down threonine to glycine. In *C. glutamicum* ssp. *lactofermentum*, threonine production reached 58 g l^{-1} when a strain producing both threonine and lysine (isoleucine auxotroph resistant to thialysine, α-amino-β-hydroxyvaleric acid and S-methylcysteine sulfoxide) was transformed with a recombinant plasmid carrying its own *hom* (encoding HDI), *thrB* (encoding homoserine kinase) and *thrC* (encoding threonine synthetase) genes.[25]

PRODUCTION OF L-LEUCINE

L-Leucine belongs to the branched chain amino acids. Together with L-valine and L-isoleucine, branched chain amino acids are widely used as fitness supplements and for patients with hepatic encephalopathy. Besides, L-leucine can be used as a lubricant in the pharmaceutical industry. Its market is continuously growing and strategies to improve L-leucine are required.

Production of L-leucine was initially reported in analogue-resistant mutants selected by random mutagenesis from the glutamate-producer *Bacillus lacto-fermentum* 2256 and further optimized by additional mutagenesis steps.[26] By applying metabolic engineering approaches to *C. glutamicum*, a titer of 24 g l^{-1} with a yield of 0.3 moles per mole of glucose, and a volumetric productivity of 4.3 mmol per l h^{-1} in a defined minimal medium was attained.[20] It has been possible to increase the production levels of the amino acid, as well as the genetic stability of the producer strain.[27] The constructed mutant overexpressed the rate-limiting enzyme 2-isopropylmalate synthase, and its amino acid precursors were redistributed to increase L-leucine production. The strain accumulated 38 g l^{-1}, in a 50-l fermentor under fed-batch conditions, with a molar product yield of 0.42 mol L-leucine per mole of glucose (Table 1).

PRODUCTION OF L-ISOLEUCINE

L-Isoleucine is of commercial interest as a food and feed additive and for parenteral nutrition infusions. This branched chain amino acid is currently produced both by extraction of protein hydrolysates and by fermentation with classically derived mutants of *C. glutamicum.* The biosynthesis of isoleucine by *C. glutamicum* involves 11 reaction steps, of which at least five are controlled with respect to activity or expression. L-isoleucine synthesis shares reactions with the lysine and methionine pathways. In addition, threonine is an intermediate in isoleucine formation, and the last four enzymes also carry out reactions involved in valine, leucine and pantothenate biosynthesis.[28] Therefore, it is not surprising that multiple regulatory steps identified in *C. glutamicum*, as in other bacteria, are required to ensure the balanced synthesis of all these metabolites for cellular demands. In *C. glutamicum,* flux control is exerted by repression of the *homthrB* and *ilvBNC* operons. The activities of AK, HDI, TD, and acetohydroxy acid synthase are controlled by allosteric transitions of the proteins to provide feedback control loops, and homoserine kinase is inhibited in a competitive manner. Isoleucine increases the km of TD from 21 to 78 mM whereas valine reduces it to 12 mM. The acetohydroxy acid synthase is 50% feedback inhibited by isoleucine plus valine and leucine.[28]

Isoleucine processes have been devised in various bacteria such as *S. marcescens, C. glutamicum* ssp. *flavum,* and *C. glutamicum*. In *S. marcescens*, resistance to isoleucine hydroxamate and α-aminobutyric acid led to derepressed levels of TD and acetohydroxy acid synthase and production of 12 g l^{-1} of isoleucine. Further work involving transductional crosses into a threonine-over-producer yielded isoleucine at 25 g l^{-1}. The *C. glutamicum* ssp. *flavum* work employed resistance to α-amino-β-hydroxyvaleric acid and the resultant mutant produced 11 g l^{-1}. Mutation to D-ethionine resistance yielded a mutant making 33.5 g l^{-1} isoleucine in a fermentation continuously fed with acetic acid.A threonine-over-producing strain of *C. glutamicum* was sequentially mutated to resistance to thiaisoleucine, azaleucine and α-aminobutyric acid; it produced 10 g l^{-1} of isoleucine. An improved strain was obtained by cloning multiple copies of *hom* (encoding HDI), and wild-type *ilvA* (encoding TD) into a lysine-over-producer, and by increasing homoserine kinase (encoded by *thrB*); 15 g l^{-1} isoleucine was produced. Independently, cloning of three copies of the feedback-resistant HDI gene (*hom*) and multicopies of the deregulated TD gene (*ilvA*) in a deregulated lysine producer of *C. glutamicum* yielded an isoleucine producer (13 g l^{-1}) with no threonine production and reduced lysine production. Application of a closed-loop control fed-batch strategy raised production to 18 g l^{-1}, which was further amplified using metabolic engineering strategies to 40 g l^{-1} of isoleucine (Table 1).

PRODUCTION OF L-VALINE

L-Valine is another branched chain amino acid used in human and animal nutrition, manufacture of pharmaceuticals, as a moisturizing agent in cosmetics, and for chemical synthesis of antibiotics, antiviral agents and herbicides. Metabolic engineering has increased microbial production of L-valine.[30] Engineered *E. coli* has produced 60 g l^{-1} with a productivity of 2 g l^{-1} h^{-1} and a yield of 0.34 moles of valine per mole of glucose. Engineered *C. glutamicum* has made 172 g l^{-1} of valine with a yield of 0.63 moles per mole of glucose. Recently, the valine titer has reached 227 g l^{-1} (Table 1).

PRODUCTION OF L-METHIONINE

L-Methionine deficiency is involved in vascular disease, Alzheimer's disease and cancer. This amino acid is used in pharmacy, the food industry and in feed additives.[31] An excellent review on L-methionine production is that of Willke.[32] Natural L-methionine can be produced at 5 g l^{-1} by normal or mutated microbes but high level production involves recombinant techniques, for example, genetically modified *E. coli* or *C. glutamicum* producing 35 g l^{-1}. Most of the methionine is used for animal feed in livestock production, that is, over 600 000 tons per year. It is also used as a flavor in food additives. In pharmaceuticals, L-methionine is used in hepatic therapeutics and drugs for the prevention of hepatic impairments. It is also used as a nutritive supplement in infant milk preparations, parenteral nutrition, health foods and in sports supplements. DL-methionine has been made for over 50 years by chemical synthesis and has an annual capacity of about one million tons. In 2013, the DL-methionine market was 850 000 tons, selling for $2.85 billion. Food-grade DL-methionine in 2014 sold for $4.70–$4.83 per kg.

Methionine is commercially produced by chemical synthesis, by protein hydrolysis, or by microbial biosynthesis. A review of the production of L-methionine by fermentation is that of Kumar and Gomes.[33] No methionine process by fermentation has been commercialized. Methionine biosynthesis requires ATP as an energy source, and thus organisms produce just enough methionine for their own growth. No wild-type microbe is known to overproduce methionine.

Annual sales of DL-methionine have exceeded $3 billion. It had a global production of 800 000 tons in 2013, of which over 600 000 tons were used as feed additives. Chemical synthesis has been preferred due to its content of sulfur which makes it difficult for engineered microbes to make it in large quantities. In 2017, it was produced chemically as DL-methionine, using methyl mercaptan, acrolein and HCN. However, this involves poisonous substrate intermediates, waste and high consumption of energy. Also, chemical synthesis suffers by the fact that it yields both D- and L-methionine and the process involves hazardous chemicals such as acrolein, methylmercaptan, ammonia and cyanide. An alternative method employs microbes, such as *E. coli*, converting aspartate to aspartyl-4-phosphate with aspartokinase, using aspartate-semialdehyde dehydrogenase to convert aspartyl-4- phosphate to L-aspartate-semialdehyde, which is then reduced by homoserine dehydrogenase to homoserine. Homoserine is then converted to O-succinyl-homoserine by homoserine

transsuccinylase. The O-succinyl-homoserine is converted to homocysteine which is then methylated to methionine.

Metabolic engineering of wild-type *E. coli* W31 resulted in production of L-methionine at 5.6 $g\,l^{-1}$ in a 15-l fermentor.[31] In parallel studies, *E. coli* was engineered via deletion of a negative transcriptional regulator MetJ and overexpression of homoserine O-succinyltransferase MetA, together with efflux transporter YjeH, resulting in L-methionine overproduction. Additional modifications have included tolerance to high L-ethionine concentrations and blockage of the lysine biosynthetic pathway. After optimization, the metabolically tailored *E. coli* strain produced 9.75 $g\,l^{-1}$ of L-methionine with a productivity of 0.20 $g\,l^{-1}\,h^{-1}$ in a 5-l bioreactor.[34]

The French company METabolic EXplorer (METEX) developed and patented a multi-recombinant *E. coli* strain able to over-produce methionine. Some of the strain properties are an increased expression of genes involved in the synthesis of methionine, serine and glycine, transport and metabolism of glucose, and transport of sulfate and thiosulfate. In addition, several enzymes were detected with a reduced feed-back sensitivity to S-adenosylmethionine and threonine. Genetic engineering of *E. coli* has led to the production of 35 $g\,l^{-1}$ of L-methionine at the company.[32] An attenuated expression of genes involved in methionine regulation has been described by Figge *et al.*[35] Methionine efflux was enhanced by overexpressing the homologous genes *ygaZ* and *ygaH* from *E. coli*, which originated from different bacteria (*Citrobacter koseri, Citrobacter freundii, Enterobacter sp., Photorhabdus luminescens, Raoultella ornithinolytica, Shigella flexneri, Yersinia enterocolitica*). This process was transferred to Evonik Industries AG of Germany for commercial exploitation. The transferred package included METEX's entire technology for methionine production by fermentation, as well as the patents and essential bacterial strains. Therefore, the commercial fermentative production of methionine is expected to start in the near future. Evonik Industries AG is a major producer of amino acids for animal nutrition and has several complexes for the chemical production of methionine.

S-Adenosylmethionine (SAM) is a key part of sulfur amino acid metabolism and is made from methionine and ATP by methionine adenosyltransferase. It functions as a major donor of methyl groups in the transmethylation of proteins, nucleic acids, hormones, polysaccharides, phospholipids and fatty acids. It is involved in the synthesis of ergosterol and is an intermediate in synthesis of polyamines. It is the main biological methyl donor because of its active methylthioether group. SAM is a chemotherapeutic agent in alcoholic liver disease, depression, osteoarthritis, Alzheimer's disease, colon cancer and AIDS.

PRODUCTION OF AROMATIC AMINO ACIDS

The aromatic amino acids L-tryptophan and L-phenylalanine are compounds with multiple applications in the food industry. Tryptophan is an important amino acid used as a feed additive and in the pharmaceutical, cosmetic, food and health product industries. A new use for tryptophan deals with the recent increase in the feeding by farmers of dried distiller grains to animals. These grains are a by-product of the conversion of corn into ethanol, but they require tryptophan supplementation.[36] L-Tryptophan is an essential amino acid used to supplement low-protein diets for pigs with high contents

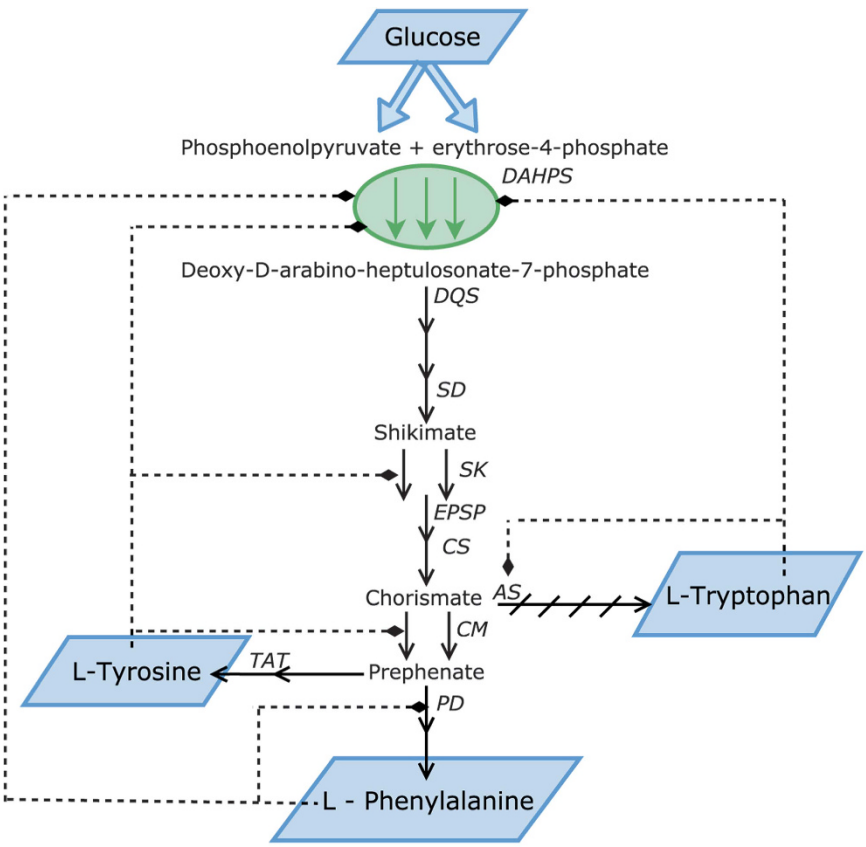

Figure 3 Biosynthetic pathways for L-tryptophan, L-phenylalanine and L-tyrosine. DAHPS, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; DQS, dehydroquinate synthase; SD, shikimate dehydrogenase (SD); SK, shikimate kinase (SK); CS, chorismate synthase; CM, chorismate mutase; TAT, tyrosine amino transferase; PD, prephenate dehydratase; AS, anthranilate synthase.

of grain that may be deficient in this amino acid. It is particularly suitable for young pigs, improving feed intake, growth and feed efficiency. In addition, tryptophan is involved as a precursor for serotonin and melatonin, and can also be degraded in the organism to nicotinic acid/nicotinamide.[37]

In *C. glutamicum* ssp. *flavum*, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS) is feedback inhibited concertedly by phenylalanine plus tyrosine and weakly repressed by tyrosine. Other enzymes of the common pathway (Figure 3) are not inhibited by phenylalanine, tyrosine and tryptophan, but the following are repressed: shikimate dehydrogenase, shikimate kinase (SK), and 5-enolpyruvylshikimate-3-phosphate synthase. Elimination of the uptake system for aromatic amino acids in *C. glutamicum* resulted in increased production of aromatic amino acids in deregulated strains.[6]

In regard to its production, a tryptophan process was improved from 8 g l^{-1} to 10 g l^{-1} by mutating the *C. glutamicum* ssp. *flavum* producer to azaserine resistance. Azaserine is an analog of glutamine, the substrate of anthranilate synthase (AS). Such a mutant showed a 2-3 fold increase in the activities of DAHPS, dehydroquinate synthase, shikimate dehydrogenase, shikimate kinase, and chorismate synthase. Another mutant, selected for its ability to resist sulfaguanidine, showed additional increases in DAHPS, dehydroquinate synthase and tryptophan production. The reason that sulfaguanidine was chosen as the selective agent involves the next limiting step after derepression of DAHPS, that is, conversion of the intermediate chorismate to anthranilate by AS. Chorismate can also be undesirably converted to *p*-aminobenzoic acid and sulfonamides which are *p*-aminobenzoic acid analogs. A sulfaguanidine-resistant mutant was obtained with *C. glutamicum* ssp. *flavum* and production increased from 10 g l^{-1} tryptophan to 19 g l^{-1}. The sulfaguanidine-resistant mutant was still repressed by tyrosine but showed higher enzyme levels at any particular level of tyrosine.

Elimination of tryptophan permeases improved L-tryptophan production by *E. coli*.[38] The level reached was 15 g l^{-1}. A genetically engineered strain of *E. coli* produced 34–40 g l^{-1} tryptophan with a yield of 0.15 g per g of glucose and a productivity of 0.6 g l^{-1} h^{-1} in a fed-batch fermentation.[39] The new strain was aided by use of a mutant over-producing L-serine, a precursor of L-tryptophan. A conventionally mutated strain of *E. coli* produced 48 g l^{-1} of L-tryptophan.[40] In another study, deletion of the phosphate acetyltransferase gene (*pta*) and the high-affinity tryptophan transporter gene (*mtr*), and increasing the level of the aromatic amino acid transporter gene (*yddG*), yielded 48.7 g l^{-1} of L-tryptophan.[40]

Gene cloning of the tryptophan branch and mutation to resistance to feedback inhibition yielded a *C. glutamicum* strain producing 43 g l^{-1} of L-tryptophan. The genes cloned were those which encode AS, anthranilate phosphoribosyl transferase, a deregulated DAHPS, and other genes of tryptophan biosynthesis. However, sugar utilization decreased at the late stage of the fermentation and plasmid stabilization required antibiotic addition. Sugar utilization stopped due to killing by accumulated indole. By cloning in the 3-phospho-glycerate dehydrogenase gene (to increase production of serine, which combines with indole to form more tryptophan), and by mutating the host cells to deficiency in this enzyme, both problems were solved. The new strain produced 50 g l^{-1} tryptophan with a productivity of 0.63 g l^{-1} h^{-1} and a yield of 20% from sucrose.[41] Further genetic engineering to increase the activity of the pentose phosphate pathway increased production to 60 g l^{-1} (Table 1).

L-Phenylalanine is another commercially important amino acid. It is used as food or feed additive. Its main demand (70%) stems from being a building block for the low calorie sweetener aspartame. A deregulated strain of *E. coli*, in which feedback inhibition and repression controls were removed, made 11 g l^{-1} phenylalanine in a fed-batch culture. Production was increased to 28 g l^{-1} when a plasmid was cloned into *E. coli* containing a feedback inhibition-resistant version of the chorismate mutase-prephenate dehydratase gene, a feedback inhibition-resistant DAHPS, and the O_RP_R and O_LO_L operator-promoter system of lambda phage. Further process development of genetically engineered *E. coli* strains brought phenylalanine titers up to 46 g l^{-1}. Independently, genetic engineering based on cloning *aroF* and feedback resistant *pheA* genes created an *E. coli* strain producing 51 g l^{-1}.[42]

A *C. glutamicum* ssp. *lactofermentum* culture, obtained by selection with *m*-fluoro-phenylalanine, produced 5 g l^{-1} phenylalanine, 7 g l^{-1} tyrosine, and 0.3 g l^{-1} anthranilate and contained desensitized DAHPS and prephenate dehydratase. DAHPS in the wild type was inhibited cumulatively by phenylalanine and tyrosine, whereas prephenate dehydratase was inhibited by phenylalanine. Cloning of the gene encoding prephenate dehydratase from a desensitized mutant and the gene encoding desensitized DAHPS increased enzyme activities and yielded a strain producing 18 g l^{-1} phenylalanine, 1 g l^{-1} tyrosine, and no anthranilate. Further cloning of a recombinant plasmid expressing desensitized DAHPS increased phenylalanine production to 26 g l^{-1}. Similarly, *C. glutamicum* strains have been developed, producing up to 57 g l^{-1} of phenylalanine (Table 1). L-Tyrosine is another aromatic amino acid, mainly utilized as a precursor in the synthesis L-3,4-dihydroxyphenylalanine, the preferred drug for the treatment of Parkinson´s disease. Around 250 metric tons of L-3,4-dihydroxyphenylalanine are produced every year via both enzymatic and chemical methods.

L-Tyrosine over-production has been achieved by cloning shikimate kinase into a tyrosine-producing *C. glutamicum* ssp. *lactofermentum* strain. Production of tyrosine increased from 17 g l^{-1} from a deregulated phenylalanine-producing *C. glutamicum* strain into the deregulated tryptophan producer, *C. glutamicum* KY 10865 (a chorismate mutase-deficient strain, phenylalanine, and tyrosine double auxotroph with a desensitized AS). This increased tyrosine production to 26 g l^{-1}. The use of *E. coli* for tyrosine over-production was achieved by replacing the *pheLA* genes of a phenylalanine-producing strain with a multi-gene cassette kanamycin resistance gene. Surprisingly, deletion of the *lacI* repressor led to an increase in *tyrA* expression and a five-fold increase in tyrosine production to more than 50 g l^{-1} at a 200-l scale.[43] Recently, the fermentation titer reached 55 g l^{-1} (Table 1). An enzymatic method yielded 131 g l^{-1}.

OTHER AMINO ACIDS

Beta-alanine

Beta-alanine, also known as 3-aminopropionic acid (3-AP), is the sole naturally occurring beta-amino acid. It has been proposed as an intermediate in formation of acrylamide and acetonitrile or as a direct precursor of poly-beta-alanine (nylon-3). Poly-beta-alanine is used for cosmetics and water purification. 3-AP is used as a precursor in synthesis of pantothenic acid. Biological production of 3-AP has been carried out by whole cell conversion of beta-aminopropionitrile by *Alcaligenes* sp., *Aminobacter aminobrance* ATCC 22314 or *Rhodococcus* sp G20, as well as by whole cell or enzymatic conversion of aspartic acid to 3-AP. Song *et al.*[44] metabolically engineered a fumaric acid-producing strain of *E. coli* to convert glucose to 3-AP. A level of 32 g l^{-1} was produced in 39 h with a yield of 0.135 g 3-AP g^{-1} glucose and a productivity of 0.828 g l^{-1} h^{-1}. This was the first report of

glucose conversion to 3-AP. The overexpressed genes were *aspA* and *panD*.

L-Arginine

A review of the use of metabolic engineering to develop producers of L-arginine and its derivatives is that of Shin and Lee.[45] Activities of L-arginine include stimulation of secretion of growth hormone, prolactin, insulin and glucagon, promotion of muscle mass, and enhancement of wound healing. High production of L-arginine has been achieved with metabolically engineered *C. glutamicum* at 92.5 g l^{-1}.[46]

Improvement of L-arginine production was achieved by Zhang *et al.*[47] Arginine has applications in health food supplements, as well as in the pharmaceutical and cosmetic industries. Previously, *C. glutamicum* had been shown to be an improved producer of L-arginine. In *Corynebacterium crenatum*, *N*-acetyl-L-glutamate kinase (NAGK) catalyzes the second step in L-arginine biosynthesis and is inhibited by L-arginine. Study of NAGK synthesis in three mutants yielded improved activity, that is, higher specific activity and thermostability. Site-directed mutagenesis of the *NAGK* gene in *C. crenatum* yielded a strain producing 45.6 g l^{-1} of L-arginine. *C. crenatum* strain SYPA-EH3, containing these mutations, plus one additional mutation, produced 61.2 g l^{-1} at 0.638 g l^{-1} h^{-1} after 96 h of fermentation. Further studies on overexpression, NADPH optimization and increased glucose consumption in fed-batch fermentations yielded a final strain producing 87.3 g l^{-1} of L-arginine, with a yield of 0.431 g l^{-1} of arginine per gram of glucose. Systems metabolic engineering further increased production to 92.5 g l^{-1} of L-arginine. More recent production is at 96 g l^{-1} (Table 1).

L-Ornithine is used as a food supplement and nutrition product. It is useful for liver diseases and wound healing, and increases serum levels of growth hormone and insulin-like growth factor-1. It protects the liver by detoxifying excess ammonia in humans. Its use in foods is to reduce bitterness in juices and other beverages. It is usually produced by extraction from hydrolyzed protein or by microbial fermentation. Use of metabolic engineering to increase availability of NADH resulted in production of L-ornithine by *C. glutamicum* at 14.8 g l^{-1}.[48] The engineering involved recombinant expression of *gapC* (encoding NADP-dependent-glyceraldehyde-3-phosphate dehydrogenase) from *Clostridium acetobutylicum*, and *rocG* (encoding NAD-dependent glutamate dehydrogenase) from *B. subtilis*. In another study, disruption of three genes, which code for $NADP^+$-dependent oxidoreductases led to disappearance of glucose dehydrogenase, along with increased activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. This led to production at a level of 15 g l^{-1}.[49] Ornithine can be produced by a genetically engineered strain of *C. glutamicum* at 51.5 g l^{-1}.[50] Another method used arginase to convert L-arginine to L-ornithine plus urea.[51] The arginase gene was cloned from *Bacillus amyloliquefaciens* Bio-127 and expressed in *B. subtilis*. Production of 356 g l^{-1} of ornithine was obtained via fed-batch fermentation in 12 h in a 5-l fermentor. The related compound putrescine is produced at 24.5 g l^{-1} with metabolically engineered *E. coli*.[52]

L-Citrulline

Development of an L-citrulline process using *C. glutamicum* has been described by Hao *et al.*[53] Wild-type strain ATCC 13032 produced 0.15 g l^{-1}. Citrulline is an intermediate in biosynthesis of L-arginine from L-glutamate in a seven-step pathway. By inactivating gene *argG*, encoding argininosuccinate synthetase (converting citrulline to argininosuccinate, which normally is converted to arginine) and gene *argR*, the repressor gene, strain CIT2 was obtained which produced 5.43 g l^{-1} of citrulline. Overexpression of the *argJ* gene, encoding the enzyme deacetylating acetylornithine to ornithine (which then is converted to citrulline), yielded strain CIT3 producing 8.5 g l^{-1} of citrulline.

L-Aspartic acid, a four carbon amino acid, is used to make the synthetic sweetener aspartame and amino analogs of C4 building block chemicals, for example, 1,4-butanol, tetra-hydrofuran and gamma-butyrolactone. Free and immobilized thermostable aspartase of *B. subtilis* YM-55-1, expressed in *E. coli*, was used to produce 66 g l^{-1} of aspartic acid in a 24-h fermentation.[54]

Gamma-aminobutyric acid (*GABA*) is used in pharmaceuticals and functional foods. It acts in mammalians as an inhibitory neurotransmitter to modulate the overall excitability of the central nervous system. It improves brain function, has anti-anxiety effects, tranquilizer effects, boosts fertility, has diuretic effects, anti-diabetic effects and is used in the treatment of epilepsy. It is also used to produce nitrogen-containing industrial chemicals, for example, *N*-methyl pyrrolidinone and bioplastics. A recombinant strain of *C. glutamicum* produced GABA when two glutamate decarboxylase genes (*gadB1* and *gadB2*) from *Lactobacillus brevis* were co-expressed.[55] The GABA concentration reached 27 g l^{-1}. Important in this process was a urea supplementation strategy. Urea is important in production of the precursor L-glutamate, serving as a nitrogen source, and for maintenance of neutrality during glutamate production. Another strain of *C. glutamicum* produced 29.5 g l^{-1} of GABA from endogenous glutamic acid.[56] The genetic modifications were the deletion of genes *odhA* and *pyc*. Gene *odha* encodes the E1 subunit of the 2-oxoglutarate dehydrogenase complex; gene *pyc* encodes pyruvate carboxylase. The result was generation of a recombinant strain which accumulated GABA. A coupled process to produce both GABA and lactic acid was developed by Zhao *et al.*[57] The concentration of GABA produced was 44.8 g l^{-1} along with 80 g l^{-1} of lactic acid. Bioconversion of L-glutamate to GABA by resting cells of *L. brevis* TCCC 13007 has been further reviewed by Shi *et al.*[58] In the bioconversion process, the ratio between the substrates L-glutamic acid and monosodium glutamate is of great importance for the final GABA yield. At a ratio of 80 g l^{-1} L-glutamic acid to 240 g l^{-1} of monosodium glutamate, a maximum production of 201 g l^{-1} of GABA was obtained after a 10 h reaction with a high molar bioconversion ratio of 99.4%.

5-Aminovaleric acid

Genetic engineering of *C. glutamicum* LYS-12, a lysine hyperproducer, led to the production of 28 g l^{-1} of 5-aminovalerate (5-AVA) and 7 g l^{-1} of glutarate.[59] In another study, metabolically engineered *C. glutamicum* produced 33 g l^{-1} of 5-AVA from glucose.[60] 5-AVA is used for production of chemicals and polymers. These are platform chemicals, that is, building blocks for bio-based plastics such as nylon, polyamides and polyesters. The pathway from glucose to 5-AVA includes lysine, the well-known product of *C. glutamicum* metabolism.

L-Homoserine

Metabolic engineering of *E. coli* W3110 led to production of 39.54 g l^{-1} of L-homoserine from glucose.[61] L-homoserine is a non-essential amino acid and a precursor of threonine and methionine. It does not participate in protein synthesis. It can be used to induce the immune response to increase plant resistance to disease, as well as improving the growth of young pigs. Thus, it has potential applications as a fertilizer additive for crops and feed additive for stock farming. The techniques used in the metabolic engineering work were: blockage of competing and degradative pathways, overcoming the

bottleneck of carbon flux to homoserine, deregulating feedback inhibition, increasing export flux, and modifying the TdcC transporter.

L-Pipecolic acid

Synthesis of L-pipecolic acid from DL-lysine was studied by Tani *et al.*[62] They used an *E. coli* strain carrying a plasmid encoding AIP (apoptosis-inducing protein), Δ^1-piperideine-2-carboxylase reductase (Pip2C reductase) and lysine racemase from *Pseudomonas putida*, and glucose dehydrogenase from *B. subtilis*. In a one-pot process, 45 g l^{-1} of L-pipecolic acid was made from DL-lysine (87% yield) in 46 h. High optical purity (over 99.9% enantiometric excess) was achieved. L-pipecolic acid is a non-proteinogenic amino acid involved in synthesis of FL-506, rapamycin and other products.

Glutathione

Glutathione (L-gamma-glutamyl-L-cysteinyl-glycine = GSH) is involved in resistance to oxidative stress, regulation of the intracellular potassium level, maintenance of redox balance and improvement of immunity. It is the most abundant free thiol. It is a redox-active tripeptide thiol and has the following activities: anti-oxidization (maintaining redox balance), detoxification, immune booster. GSH is used in the pharmaceutical, cosmetic and food industries. Fermentation appears to be a favorable production process due to the mild conditions used, high recovery and low cost.

A review of GSH biosynthesis is that of Yang *et al.*[63] Its biosynthetic pathway contains two ATP-dependent consecutive reactions catalyzed by (1) γ-L-glutamyl-L-cysteine synthetase, and (2) L-GSH. Thus, gamma-L-glutamyl-L-cysteine is formed from L-glutamate and L-cysteine, the rate-limiting step of GSH biosynthesis, and GSH is formed by L-GSH synthetase which connects gamma- L-glutamate-L-cysteine with glycine. GSH inhibits GSH synthetase, resulting in low fermentation levels. *Saccharomyces cerevisiae* produced 317 mg l^{-1} of GSH.[64] However, a novel GSH synthetase was found to be less subject to GSH inhibition. A strain of *E. coli* was engineered to contain this GSH synthetase. The recombinant *E. coli* strain coupled with *S. cerevisiae* led to the production of 10.4 g l^{-1} GSH with feeding of the precursor amino acids and glucose.[65]

Enzyme Gsh F, which contains both γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GSH II) activities from *Streptococcus thermophilus*, has been used for GSH biosynthesis by a recombinant strain of *E. coli*. The strain produced two enzymes, one from *Actinobacillus pleuropneumoniae* and the other from *Actinobacillus succinogenes*.[66] It yielded 36.6 mM (11.3 g l^{-1}) of GSH with a productivity of 2.06 mM h^{-1} and a molar yield of 0.92 mol mol^{-1} when the precursors glutamate, cysteine and glycine were added at 75 mM each and glucose was used as the sole carbon and energy source.

DISCUSSION

The microbial production of amino acids, through fermentation, serves a market with strong prospects of growth and contributes significantly to our quality of life. Micro-organisms are capable of converting inexpensive carbon and nitrogen sources into valuable metabolites, such as amino acids, which can be added to foods as a flavor enhancer and/or to increase their nutritional value. Also, some amino acids are proving very valuable as biosynthetic precursors for the manufacture of therapeutics.

The ability of a fermentation process to produce an amino acid depends on the overproduction capacity of the strain being used. In the early years of fermentation processes, strain development depended entirely on classical strain breeding involving intensive rounds of random mutagenesis, followed by equally strenuous programs of screening and selection. However, recent innovations in molecular biology, on the one hand, and the development of new tools in functional genomics, transcriptomics, metabolomics and proteomics on the other, have resulted in more rational approaches for strain improvement. Most of the amino acids are usually produced by fed-batch processes using high performance mutants, and separation by ion exchange chromatography for crystallization.

As can be seen from a good part of this article, the actinobacterium *C. glutamicum* has been very useful for production of amino acids. A review of this organism, called 'an industrial workhorse', was published by Lee *et al.*[15] The organism started out as a producer of glutamic acid, lysine and other amino acids, but its use was extended to production of other chemicals, fuels and polymers. Its genome sequence has been elucidated and published. High throughput technologies, such as genomics, transcriptomics, proteomics and metabolomics, have been applied to study *C. glutamicum*. Its benefits are GRAS (Generally Recognized As Safe) status, rapid growth to high cell densities, genetic stability, a limited restriction modification system, lack of autolysis, maintenance of metabolic activity under growth-arrested conditions, low protease activity favoring recombinant protein production, plasticity of metabolism, strong secondary metabolism properties, broad spectrum carbon utilization (pentoses, hexoses, and alternative carbon sources), and stress tolerance to carbon sources. Other products of *C. glutamicum* include ethanol at 119 g l^{-1}, isobutanol at 73 g l^{-1}, L-lactate at 95 g l^{-1}, D-lactate at 120 g l^{-1}, succinate at 156 g l^{-1}, threonine at 52 g l^{-1}, GABA at 38 g l^{-1} and ectoine at 4.5 g l^{-1}. The organism has also been useful for producing proteins because of a low level of extracellular protease activity and the presence of two native protein secretion mechanisms.

The roles of amino acids and, in turn, microbial fermentations, stand to grow in stature, especially as we enter a new era in which the use of renewable resources is recognized as an urgent need.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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