Chapter 14

Amino acids

L. Eggeling
Research Centre Jülich, Germany

W. Pfefferle
Degussa AG, Germany

H. Sahm
Jülich GmbH, Germany

14.1 Introduction
14.2 Commercial uses of amino acids
14.3 Production methods and tools
14.4 L-Glutamate
14.5 L-Lysine
14.6 L-Threonine
14.7 L-Phenylalanine
14.8 L-Tryptophan
14.9 L-Aspartate
14.10 Outlook
14.11 Further reading
Acknowledgements

14.1 Introduction

The story of amino acid production started in Japan in 1908 when the chemist, Dr K. Ikeda, was working on the flavouring components of kelp. The specific taste of the kelp preparations, kombu and katsuobushi, is traditionally very popular with the Japanese (Fig. 14.1). After acid hydrolysis and fractionation of kelp, Dr Ikeda discovered that one specific fraction he had isolated consisted of glutamic acid which, after neutralization with caustic soda, developed an entirely new delicious taste. This was the birth of the use of monosodium glutamate as a flavour-enhancing

Biotechnology, third edition, eds. Colin Ratledge and Bjorn Kristiansen. Published by Cambridge University Press. Cambridge University Press 2006.

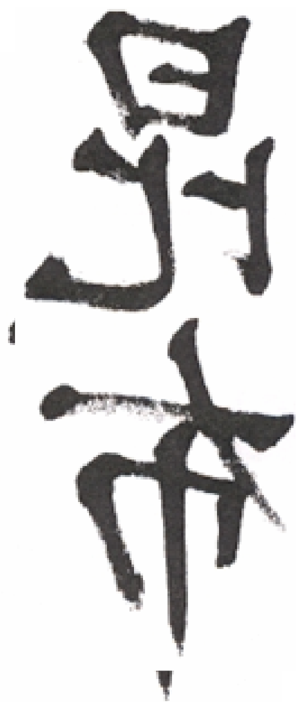


FIGURE 14.1 The ideogram for kombu as it appears on kelp preparations used as a food component. The painting was kindly provided by Dr T. Ikeda (Ajinomoto), the grandson of Dr K. Ikeda.

compound. The production of monosodium glutamate (MSG) was soon commercialised by the Ajinomoto Co. Ltd based on its isolation from vegetable proteins such as soy or wheat protein. However, with this process the waste fraction was high, and also the chemical synthesis of D,L-glutamate was of little use since the sodium salt of the D-isomer is tasteless.

The breakthrough in the production of MSG was the isolation of a specific bacterium by Dr S. Udaka and Dr S. Kinoshita at Kyowa Hakko Kogyo in 1957. They screened for amino-acid-excreting microorganisms and discovered that their isolate, No. 534, on a mineral salt medium excreted L-glutamate. It soon became apparent that the isolated organism needed biotin and that L-glutamate excretion was triggered by an insufficient biotin supply. A number of bacteria with similar properties were also isolated, which are today all known by the species name *Corynebacterium glutamicum* (Fig. 14.2). *Corynebacterium glutamicum* is a Gram-positive bacterium which can be isolated from soil. Together with genera like Streptomyces, Propionibacterium or Arthrobacter, it belongs to the actinomycetes sub-division of Gram-positive bacteria. The successful commercialisation of MSG production with this bacterium provided a big boost for amino acid production with *C. glutamicum* and later with other bacteria like *E. coli* as well. Nucleotide production also developed rapidly in the 1970s with *C. ammoniagenes*, which is closely related to *C. glutamicum*. The production mutants and the processes developed also resulted in a demand for sophisticated fermentation devices. Consequently, the development of amino acid technology was an incentive for the fermentation industry in general.

14.2 Commercial uses of amino acids

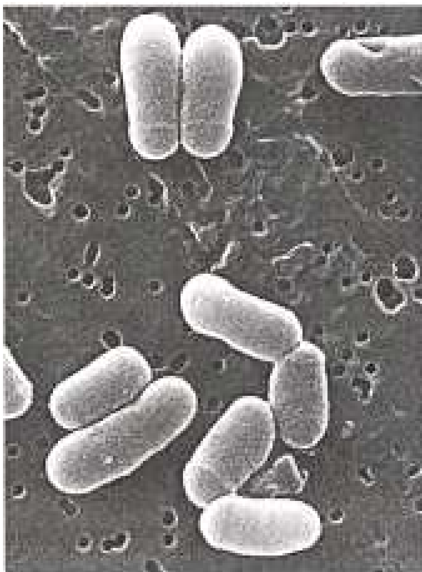


FIGURE 14.2 Electron micrograph of *Corynebacterium glutamicum* showing the typical V-shape of two cells as a consequence of cell division.

Amino acids are used for a variety of purposes. The food industry requires L-glutamate as a flavour enhancer, or glycine as sweetener in juices, for instance (Table 14.1). The pharmaceutical industry requires the amino acids themselves in infusions - in particular the essential amino acids - or in special dietary foods. And last, but not least, a large market for amino acids is their use as feed additives. The reason is that typical animal feed, like soybean meal for pigs, is poor in some essential amino acids, like methionine and lysine. This is illustrated in Fig. 14.3 where the nutritive value of soybean meal is depicted by a barrel but the use of the total barrel is limited by the shortest stave, that is by the stave representing methionine. Amino acids are added therefore to increase the effectiveness of the feed. The addition of as little as 10 kg methionine per tonne of feed increases the protein quality of the feed just as effectively as adding 160 kg soybean meal or 56 kg fish meal. The first limiting amino acid in feed based on plant crops and oil seeds is usually L-methionine, followed by L-lysine, and then by L-threonine. Another important aspect of feed supplementation is that with a balanced amino acid content the manure from the animals contains less nitrogen because more of the nitrogen in

Table 14.1 Amounts of amino acids being currently produced

Production scale (tonnes y^{-1})	Amino acid	Preferred production method	Main use
1200000	L-Glutamic acid	Fermentation	Flavour enhancer
600000	L-Lysine	Fermentation	Feed additive
550000	D,L-Methionine	Chemical synthesis	Feed additive
40000	L-Threonine	Fermentation	Feed additive
16000	Glycine	Chemical synthesis	Food additive, sweetener
14000	L-Aspartate	Enzymatic catalysis	Aspartame, polymer
13000	L-Phenylalanine	Fermentation	Aspartame
4500	L-Cysteine	Reduction of cystine Fermentation	Food additive, pharmaceutical
3500	L-Cystine	Extraction, fermentation	Cysteine, pharmaceutical
2000	t-Arginine	Fermentation, extraction	Pharmaceutical
1500	L-Alanine	Fermentation, extraction	Sweetener, building block
1200	L-Tryptophan	Fermentation	Feed, pharmaceutical
1200	L-Leucine	Fermentation, extraction	Pharmaceutical
1000	L-Valine	Fermentation, extraction	Pesticides, pharmaceutical
500	L-Isoleucine	Fermentation, extraction	Pharmaceutical

the improved feed has been used by the animal thus reducing environmental pollution.

Over the past three decades, the demand for amino acids has increased dramatically. The market is growing steadily by about 5-10% per year. Thus, within ten years the total market has approximately doubled (Fig. 14.4). Some amino acids, such as L-lysine, which is required as a feed additive, display a particularly great increase. The world market for this amino acid has increased more than 20-fold in the past two decades. Other amino acids have appeared on the market such as L-threonine, L-aspartate and L-phenylalanine, the last two being required for the synthesis of the sweetener Aspartame. Estimates for current worldwide demand for the most relevant amino acids are given in Table 14.1. L-Glutamate continues to occupy the top position followed by L-lysine together with D,L-methionine, while the other amino acids trail behind at a considerable distance.

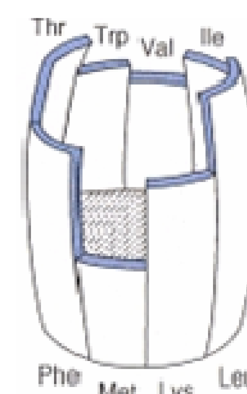


FIGURE 14.3 The barrel represents the nutritive value of soybean meal, which is first limited by its methionine content.

There is a dose interaction between the prices of the amino acids and the dynamics of the market. More efficient fermentation technology can provide cheaper products and hence boost demand. This in turn will lead to production an a larger scale with a further reduction of costs. However, since the supply of some amino acids, e.g. L-lysine, as a feed additive is directly competitive with soybean meal (the natural L-lysine source) there are considerable fluctuations in the amino acid demand depending on the crop yields. The amino acids produced in the largest quantities are also the cheapest (Fig. 14.5). The low prices in turn dictate the location of the production plants. The main factors governing the location of production plants are the price of the carbon source and the local market. Large L-glutamate production plants are spread all over the world, with

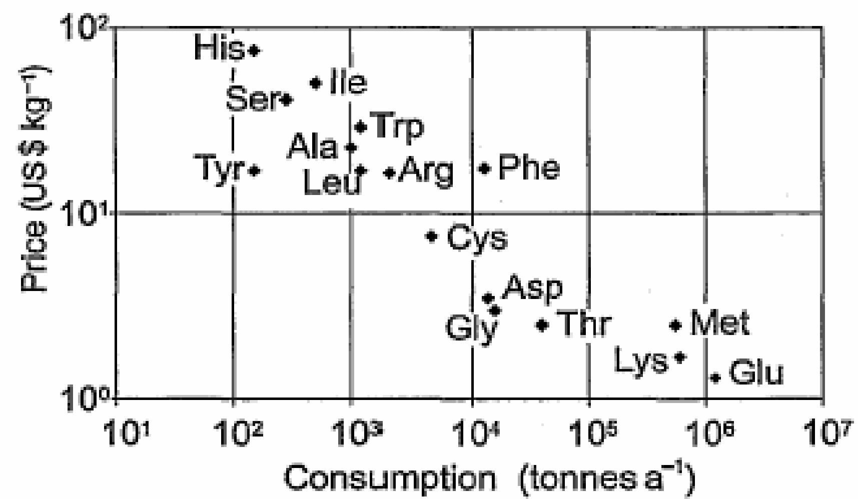


FIGURE 14.5 The amino acid with the largest market are the cheapest

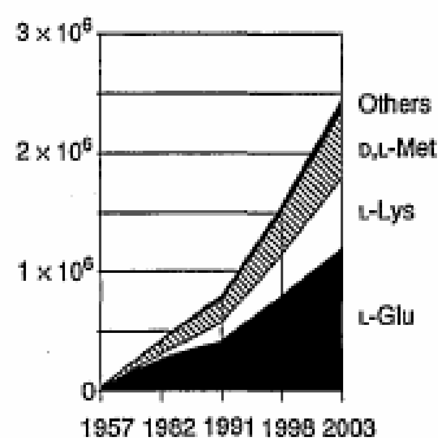


FIGURE 14.4 The amino acid market doubles about every ten years. Numbers are in tonnes per year.

a significant presence in the Far East, e.g. Thailand and Indonesia. For L-lysine the situation is different. Since one-third of the world market is in North America and there is convenient access to maize as a feedstock material for the fermentation process, about one-third of the L-lysine production capacity is located there. In almost all cases, the companies producing L-lysine are associated with the maize milling industry, either as producers, in joint ventures or as suppliers of cheap sugar. This illustrates the fact that the commercial production of amino acids is a vigorously growing and changing field with many global interactions.

14.3 Production methods and tools

Some amino acids are chemically synthesised, such as glycine which has no stereochemical center, or D,L-methionine. This latter sulphur containing amino acid can be added to feed as a racemic mixture, since animals contain a D-amino acid oxidase, which together with a transaminase activity converts D-methionine to the nutritively effective L-form. The classical procedure of amino acid isolation from acid hydrolysates of proteins is still in use for selected amino acids with a low market volume (Table 14.1). Other methods use precursor conversion with bacteria, or enzymatic synthesis. However, for L-amino acids required in large volume, fermentative production by engineered bacteria is the method of choice.

Classical strain development

Bacteria do not normally excrete amino acids in significant amounts because regulatory mechanisms control amino acid synthesis in an economical way so that the needs of the cell (for protein synthesis) are exactly matched by the synthetic processes. There are no surplus amino acids and only a small pool of them exists within the cell to meet its immediate needs. Therefore, mutants have to be generated that oversynthesize the respective amino acid. A great number of amino-acid-producing bacteria have been derived by mutagenesis and

TABLE 14.2 A genealogy of strains obtained by classical mutagenesis and screening, showing improved yield and some of the phenotypic characters of the mutants.

Strain	Character	Yield of L-lysine (%)
AJ 1511	Wild type	0
AJ 3445	AEC^r	16
AJ 3424	AEC^r Ala$^-$	33
AJ 3796	AEC^r Ala$^-$ CCL^r	39
AJ 3990	AEC^r Ala$^-$ CCL^{r}ML^r	43
AJ 1204	AEC^r, Ala$^-$ CCL^{r}ML^r FP^s	50

AEC^r, resistant to s-(β-aminoethyl)-l-cysteine; Ala$^-$, l-alanine-requiring; CCL^r resistant to a-chlorocaprolactam; ML^r, resistant to γ-methyl-l-lysine; FP^s, sensitive to β-fluoropyruvate.

screening programmes. This has involved the consecutive application of:

- undirected mutagenesis,
- selection for a specific phenotype, and
- selection of the mutant with the best amino acid accumulation.

Taking the best resulting strain, the entire procedure was repeated in several additional rounds to increase the productivity each time and, eventually, resulted in an industrial producer (Table 14.2). Due to this iterative optimisation over decades, excellent high-performance strains are now available. However, due to the repeated mutagenesis steps, such producer strains might carry, in addition to the necessary mutations, also disadvantageous mutations influencing growth and reducing the speed of sugar conversion to amino acid. Speed is of course essential to reduce individual fermentation times and thus to increase the number of total fermentations per unit time to achieve most profitable use of the fermentation equipment.

Genomic techniques

To get rid of disadvantageous mutations, it is now common practice first to compare the genome sequence of the producer with that of the wild type and then to introduce those mutations by genetic engineering that are really necessary. This *re-building* ensures high productivity of the final mutant, together with high speed of sugar conversion, to give the simplest and most effective strain possible (see Fig. 14.6). Other genomic tools are *transcriptomics* using DNA microarry technology to characterize producers of different efficiency rapidly, or to qualify variations in fermentation processes, thus resulting in still further improvements and consolidations of the entire production processes.

Intracellular flux analysis

An entirely different approach in strain development is the reliable quantification of the carbon fluxes in the living cell. A great deal

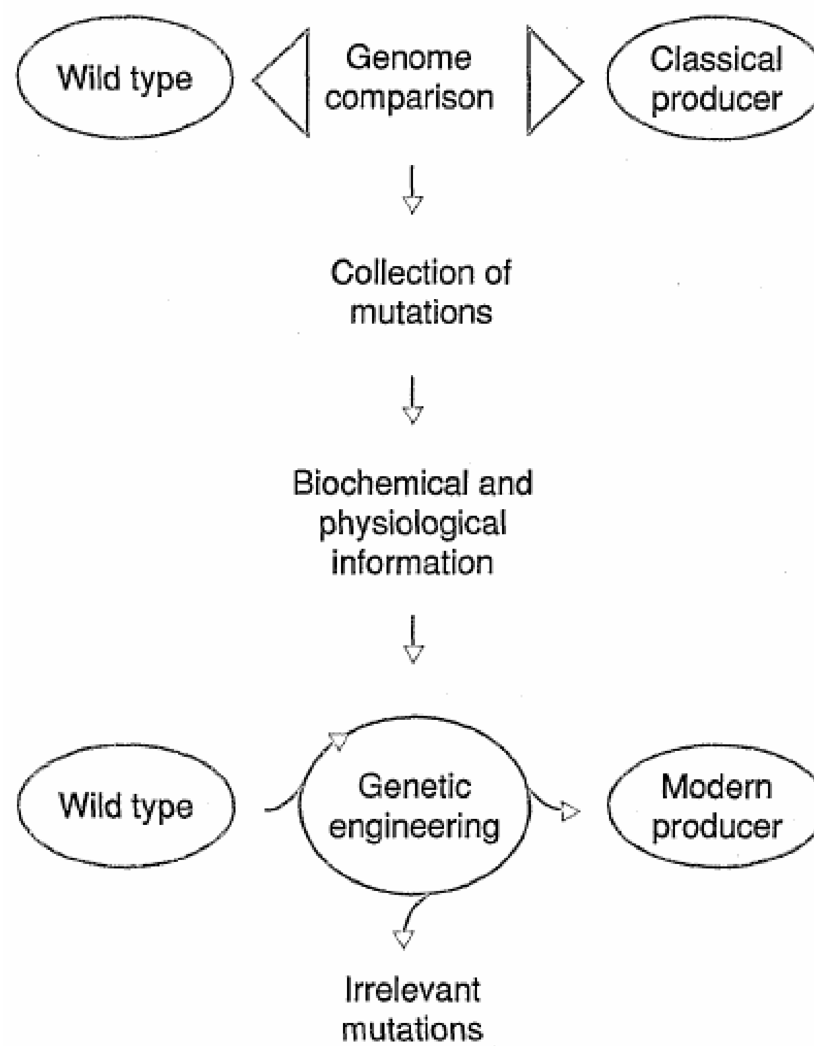


FIGURE 14.6 Comparison of the wild-type geriome with that of the classical producer allows identification of the necessary mutations, and the construction of a producer without the inherent mutations of the classical strain detrimental for high sugar consumption and product excretion rates.

of progress has been made recently in developing to a high level of sophistication the old isotope labelling technique. In particular, with ^{13}C-NMR spectroscopy the intracellular fluxes can now be quantified with very high resolution. For instance, in *C. glutamicum* it has even been possible to quantify the back fluxes as present in the anaplerotic reactions. The method is described in detail in Chapter 2 of this book. Such flux quantifications are of major assistance in selecting the reactions in the central metabolism to be modified by genetic engineering.

14.4 L-Glutamate

14.4.1 Biochemistry

As already mentioned, L-glutamate was the first amino acid to be produced. Production invariably uses *C. glutamicum*. For its fuelling pathways, this bacterium uses the glycolysis, the pentose phosphate

pathway, and the citric acid cycle to generate precursor metabolites and reduced pyridine nucleotides (Fig. 14.7).

This bacterium displays a special feature in the anaplerotic reactions, however. Since L-glutamate is directly derived from α-ketoglutarate, a high capability for replenishing the citric acid cycle is, of course, a pre-requisite for high-glutamate production. It was originally assumed that only the phosphoenolpyruvate carboxylase (PEPC) was present as a carboxylating enzyme serving this purpose. However, molecular research, in close conjunction with ^{13}C labelling studies, showed that an additional carboxylating reaction must be present. The pursuit of this enzyme activity resulted in the detection of pyruvate carboxylase activity, PyrC, and the identification of its gene. Therefore, *C. glutamicum* has the pyruvate dehydrogenase (PyrDH) shuffling acetylCoA into the citric acid cycle, and two enzymes supplying oxaloacetate: pyruvate carboxylase (PyrC) together with a phosphoenolpyruvate carboxylase (PEPC; Fig. 14.7). Both carboxylases can basically replace each other to ensure conversion of carbon-three units to oxaloacetate. This is different from *E. coli*, which has exclusively the phosphoenolpyruvate carboxylase serving this purpose, or *Bacillus subtilis*, where only the pyruvate carboxylase is present. Since it is in possession of both enzymes, *C. glutamicum* has an enormous flexibility for replenishing citric acid cycle intermediates upon their withdrawal.

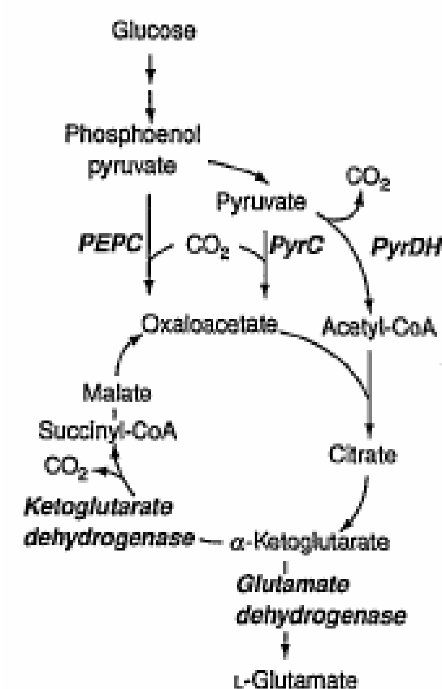


FIGURE 14.7 Sketch of main reactions of *C. glutomicum* connected with the citric acid cycle and of relevance for t-glutamate production. PyrDH, pyruvate dehydrogenase; PyrC, pyruvate carboxylase; PEPC, phosphoenol-pyruvate carboxylase; GluDH, glutamate dehydrogenase; KetogluDH, ketoglutarate dehydrogenase.

The reductive amination of α-ketoglutarate to yield L-glutamate is catalysed by the glutamate dehydrogenase. The enzyme is a multimer, each sub-unit having a molecular weight of 49 100. It has a very high specific activity of 1.8 mmol min^{-1} (mg $protein)^{-1}$, and L-glutamate is present in the cell in a rather high concentration of about 150 mM. In the case of other amino acids, in contrast, the intracellular concentrations are usually below 10 mM. The high concentration serves to ensure the supply of L-glutamate directly required for cell synthesis and also for the supply of amino groups via transaminase reactions for a variety of cellular reactions. As much as 70% of the amino groups in cell material stems from L-glutamate.

14.4.2 Production strains

For the biotechnological production of L-glutamate, the intracellularly synthesised amino acid must be released from the cell. This requires specific treatments to result in export of the amino acid by a presumed carrier. A specific carrier must be present since otherwise, in addition to the charged L-glutamate, other metabolites and ions would also leak from the cell and the cell would not be viable. IIowever, L-glutamate formation is still not fully understood. The reason for this is that a wide ringe of treatments lead to the secretion of glutamate. These include: (i) growth under biotin limitation, (ii) addition of penicillin, (iii) addition of lysozyme, (iv) addition of surfactants, (v) use of oleic acid auxotrophs, and (vi) use of glycerol auxotrophs. All these treatments apparently have the cell wall or the lipid membrane as the target in some way or another. Furthermore, the phospholipid

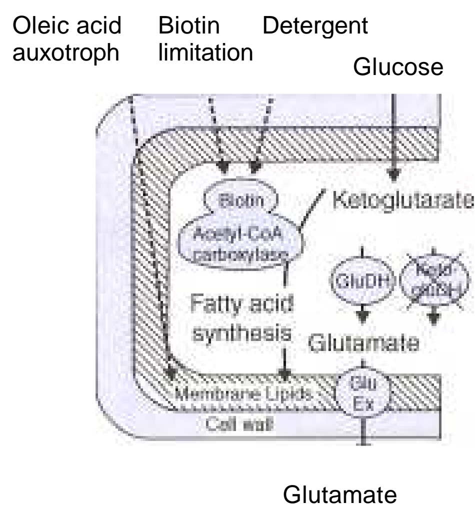


FIGURE 14.8 Model of the action of a selection of techniques (dashed arrows) to induce L-glutamate excretion and which are linked to the cell envelope. Also shown is the flux from glucose to extracellular L-glutamate involving an export carrier.

composition is significantly changed in the case of biotin limitation. Accordingly, a relation thus exists between:

- a disorder of the cell wall,
- the lipid composition of the membrane, and
- the putative carrier localised in the membrane.

A plausible model is shown in Fig. 14.8 that makes a causal relation between the classic biotin effect and glutamate export. Biotin is a coenzyme of acetyl-CoA carboxylase and thus directly involved in fatty acid synthesis. Under biotin limitation the phospholipid content of the membrane is drastically decreased from 32 to 17 nmol mg^{-1} $(DW)^{-1}$, and the content of the unsaturated oleic acid increased relative to the saturated palmitic acid by 45%. This altered lipid composition results in a favourable lipid environment of the carrier and thus in high export of L-glutamate. The membrane composition is similarly affected by oleic acid or glycerol auxotrophic mutants. Surfactant addition also acts on the acetyl-CoA carboxylase activity, since its addition results in dissociation of the multi-enzyme complex. The altered fatty acid composition of the membrane then provokes the carrier to become active and L-glutamate is excreted.
Apart from the export process and high glutamate dehydrogenase activity, a third component in L-glutamate production is α-ketoglutarate dehydrogenase (Fig. 14.7). Those unnatural conditions resulting in L-glutamate efflux also reduce the activity of this enzyme. Exposing the cell to either penicillin, surfactants or biotin-limitation reduces the α-ketoglutarate dehydrogenase activity up to a residual activity of only 10%, whereas the activity of the glutamate dehydrogenase is hardly affected. The competing α-ketoglutarate dehydrogenase activity is therefore lowered, thus preventing an excess conversion of α-ketoglutarate to succinyl-CoA and, therefore, favouring its conversion to L-glutamate.

14.4.3 Production process

The most relevant factors influencing L-glutamate formation are the ammonium concentration, the dissolved 0_2 concentration and the pH. Although, in total, a large amount of ammonium is necessary for sugar conversion to L-glutamate, a high concentration is inhibitory to growth as well to the production of L-glutamate. Therefore, ammonium is added in a low concentration at the beginning of the fermentation and is then added continuously during the course of the fermentation. The oxygen concentration is controlled, since under conditions of insufficient oxygen, the production of L-glutamate is poor and lactic acid as well as succinic acid accumulates, whereas with an excess oxygen supply the amount of α-ketoglutarate as a by-product accumulates. A flow diagram of the process is shown in Fig. 14.9.

For the actual fermentation, the production strains are grown in fermenters as large as 500 m^3 (Fig. 14.10). After pre-cultivation, the onset of L-glutamate excretion is controlled by the addition of

dt.

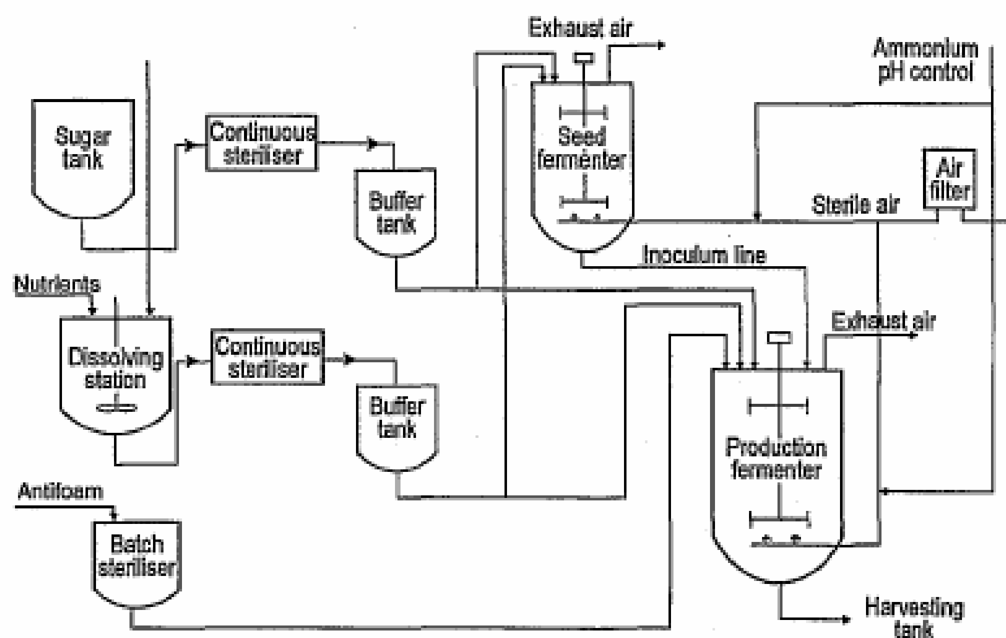


FIGURE 14.9 A scheme of the material flow in an L-glutamate production plant.

FIGURE 14.10 Amino acid production plant of Kyowa Hakko in Japan showing on the right seven large fermenters each 240 m^3 in size, suitable for L-glutamate production.

surfactants such as polyoxyethylene sorbitan monopalmitate (Tween 40). Yields of between 60 and 70% L-glutamate, based on the glucose used, have been reported. At the end of the fermentation the broth contains L-glutamate in the form of its ammonium salt. In a typical downstream process, the cells are separated and the broth is passed through a basic anion exchange resin. Glutamate anions will be bound to the resin and ammonia will be released. This ammonia can be recovered via distillation and reused in the fermentation. Elution is performed with NaOH to form monosadium glutamate (MSG) directly in the solution and to regenerate the basic anion exchanger. From the eluates, MSG may be crystallised directly followed by further conditioning steps like decolorisation and sieving to yield a food-grade quality.

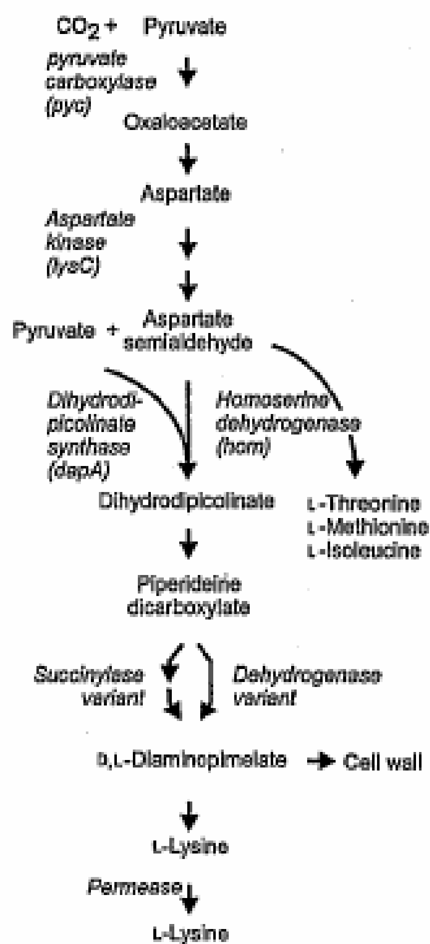


FIGURE 14.11 L-Lysine synthesis in *C. glutamicum* with the carboxylation reaction supplying oxaloacetate. Also shown is the central role of aspartate semialdehyde distribution and the link to cell-wall sythesis.

14.5 L-Lysine

14.5.1 Biochemistry

The second amino acid made exclusively with *C. glutamicum* is L-lysine. The carbons of L-lysine are derived in the central metabolism from pyruvate and oxaloacetate (Fig. 14.11). In contrast to the special situation with L-glutamate, where practically only a single reaction represents the synthesis pathway, L-lysine is synthesised via a long pathway. Moreover, the first two steps of L-lysine synthesis are shared with that of the other members of the aspartate family of amino acids: L-threonine, L-methionine and L-isoleucine.

The kinase-initiating lysine synthesis is feedback-inhibited

The first reaction initiating L-lysine synthesis is catalysed by aspartate kinase. As is typical of an enzyme at the start of a lengthy synthesis pathway, the activity of aspartate kinase is tightly controlled. The enzyme is inactive when L-lysine plus L-threonine together are present in excess, thus providing a feedback signal (see Chapter 2) concerning the availability of these two major metabolites of the aspartate family of amino acids. The kinase has an interesting structure (Fig. 14.12). It consists of two α-sub-units of 421 amino acid residues each, and two ß-sub-units of 171 amino acid residues. An exciting discovery was that the amino acid sequence of the ß-sub-unit is identical to that in the carboxyterminal part of the α-sub-unit. The molecular basis is that the gene for the smaller ß-sub-unit, *lysCβ*, is an in-frame constituent part of the larger α-sub-unit. Thus two promoters are present at this locus: one driving *lysCα* expression together with that of the downstream gene, *asd*, and one driving *lysCβ* and *asd* expression. The regulatory features of the kinase reside in the ß-sub-unit. Thus specifically altering the ß-sub-unit structure, or those of both sub-units together in their carboxy-terminal part, results in a kinase which is always active and no longer inhibitable. With such an insensitive kinase, *C. glutamicum* already excretes some lysine, showing the rather simple type of flux control.

The synthase limits flux

A further important step of flux control within lysine biosynthesis is at the level of aspartate semialdehyde distribution. The dihydrodipicolinate synthase activity competes with the homoserine dehydrogenase for the aspartate semialdehyde (Fig. 14.11). Graded overexpression of the synthase gene, *dapA*, together with enzyme activity measurements have shown that with an increasing activity of synthase a graded flux increase towards L-lysine is the result. Therefore, the synthase acts as a barrier to control the flux of aspartate semialdehyde towards L-lysine. This barrier can also be overcome when an increased aspartate semialdehyde concentration is available, as can easily be obtained by reducing the flux towards the homoserine-derived amino

acids. This can be achieved with mutated homoserine dehydrogenase enzymes which have very weak catalytic activity.

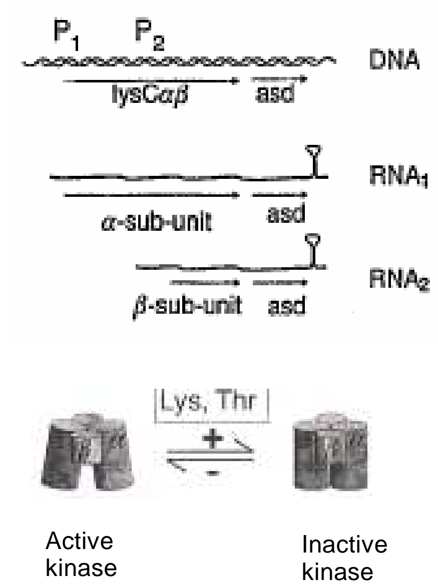


FIGURE 14.12 The *lysCasd* operon ot *C. glutamicum* and allosteric control of the kinase. The second promoter within *lysC* results in formation of the β-sub-unit constituting the regulatory sub-unit of the kinase protein of α2β2-structure.

Lysine synthesis is split which ensures proper cell-wall formation

A remarkable feature of *C. glutamicum* is its possession of a split pathway for L-lysine synthesis. At the level of piperideine-2,6-dicarboxylate, flux is possible either via the succinylase variant of D,L-diaminopimelate synthesis or the dehydrogenase variant (Fig. 14.11). In contrast, *E. coli*, for example, has only the succinylase variant and *Bacillus macerans* only the dehydrogenase variant. The flux distribution via both pathways has been quantified in a study using NMR spectroscopy and [1-^{13}C1-glucose as the substrate. Surprisingly, the flux distribution is variable (Fig. 14.13). Whereas at the start of the cultivation about three-quarters of the L-lysine is made via the dehydrogenase variant, at the end of the process the newly synthesised L-lysine is almost exclusively made via the succinylase route. There is a mechanistic reason for this. As kinetic characterisations have shown, the dehydrogenase has a weak affinity towards its substrate, ammonium, with a Km of 28 mM. Thus at low ammonium concentrations, as are present at the end of the fermentation, the dehydrogenase cannot contribute to L-lysine formation. Instead, flux via the succinylase variant is favoured, where after succinylation of piperideine-2,6-dicarboxylate, a transaminase incorporates the second amino group into the final L-lysine molecule.

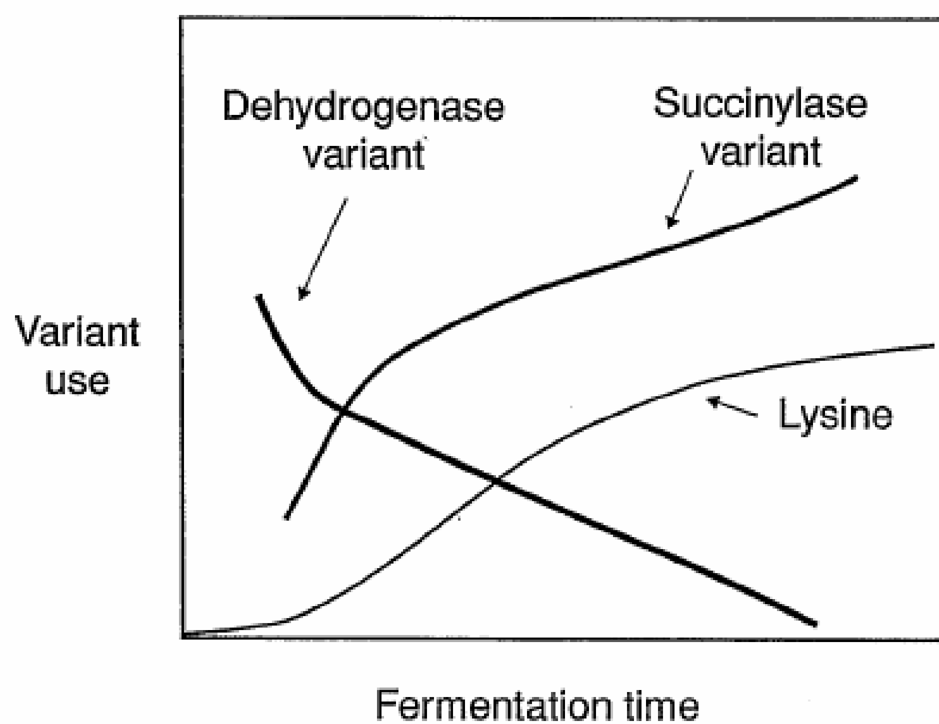


FIGURE 14.13 At the beginning of the Llysine fermentation use prevails of the dehydrogenase variant over that of the succinylase variant, whereas at the end the succinylase variant is used almost exclusively. Variant use is in per cent

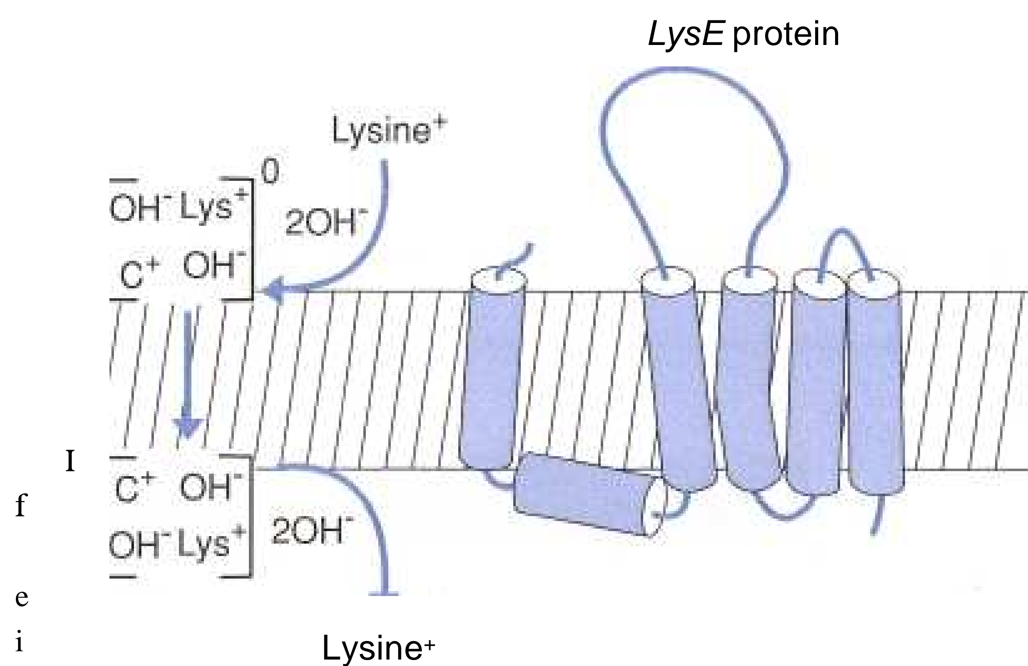


FIGURE 14.14 Topology of the L-lysine exporter showing its five membrane spanning helices and the additional hydrophobic segment. The formally distinct steps of the translocation process driven by the membrane potential are included

If either the succinylase or the dehydrogenase variant is inactivated, L-Lysine accumulation is reduced to 40%. Thus both variants together ensure in fermentations a high flux towards L-Iysine. The natural function of this split pathway is always to provide a proper supply of the penultimate intermediate of L-lysine synthesis. This is D,L-diaminopimelate, which is a crucial linking unit within the peptidoglycan of the cell wall. The split pathway in *C. glutamicum* is an example of an important principle in microbial physiology: pathway variants are generally not redundant but evolved to provide key metabolites under different environmental conditions.

Export of L-lysine

The molecular basis for bacterial amino acid export was completely unknown until 1996 since a specific export process appeared nonsensical. The breakthrough was achieved by the cloning of the lysine export carrier from *C. glutamicum,* which at one blow enabled amazing discoveries concerning the nature and relevante of such a new type of exporter. The L-lysine carrier, LysE, is a comparatively small membrane protein of 25.4 Da. It has the transmembrane spanning helices typical of carriers and is probably active as a dimer (Fig. 14.14). Several distinct steps are involved in the translocation mechanism. These are: (i) the loading of the negatively charged carrier with its substrate L-lysine together with two hydroxyl ions, (ii) substrate translocation via the membrane, (iii) the release of L-lysine and the accompanying ions at the outside of the membrane, and, finally, (iv) the reorientation of the carrier. The driving force for the entire translocation process is the membrane potential.

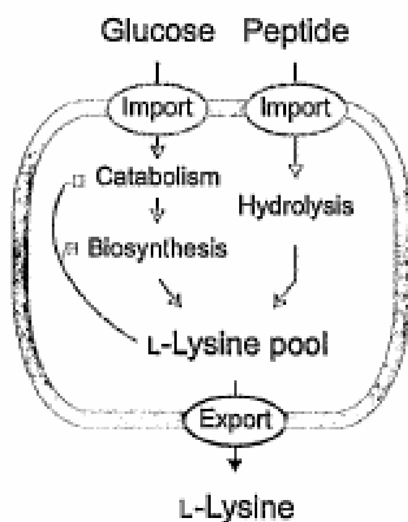
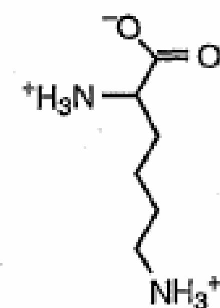


FIGURE 14.15 Amino acid exporters serve as a valve to release an excess of amino acid present, as can be the case in producers or in the natural situation during growth an peptides.

Access to the lysine-exporter gene, *lysE,* has also made it possible to solve the puzzle as to why *C. glutamicum* has such an exporter at all. In a *lysE* deletion mutant supplied with glucose and 1 mM of the dipeptide, lysyl-alanine, an extraordinarily high intracellular L-lysine concentration of more than 1 M accumulates, abolishing growth of the mutant (Fig. 14.15). Thus, the exporter serves as a valve to excrete

any excess intracellular L-lysine that may arise in the natural environment in the presence of peptides. As in the case of other bacteria, too, *C. glutamicum* has active peptide-uptake systems as well as hydrolysing enzymes giving access to the amino acids as valuable building blocks. However, *C. glutamicum* has no t-lysine-degrading activities and therefore must prevent any piling up of L-lysine. As genome projects have now shown, there are indeed numerous similar carriers present in various Gram-negative and Gram-positive bacteria. Therefore, this type of intracellular amino acid control by an exporter is expected to be present in other bacteria, too.



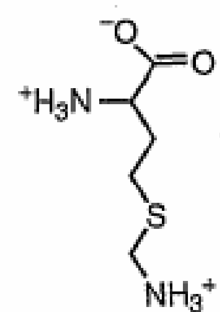


FIGURE 14.16 Aminoethyl cysteine is a sulphur-containing analogue of L-lysine for generating mutants deregulated in L-lysine synthesis.

14.5.2 Production strains

L-Lysine producer strains have been derived over the decades by mutagenesis to give strains excreting more than 170 g 1^{-1} L-lysine. lt is clear that these strains may carry an extensive List of phenotypic characters to achieve this massive flux directioning (Table 14.2). Typically, the strains are resistant to some analogue of lysine or diaminopimelate. A typical feature of L-lysine producers is their resistance to the lysine analogue S-(2-aminoethyl)-L-cysteine (Fig. 14.16). In these mutants, the aspartate kinase (see Fig. 14.12) is mutated so that it is no longer inhibited by L-lysine. Dozens of other chemicals structurally related to t lysine, such as γ-methyl-L-lysine or α-chlorocaprolactam, have been used in screenings to obtain improved producers. At this stage of strain development it was often not known how phenotypic characters correlate with overproduction and which was the molecular basis for that. However, with the advent of whole-genome sequencing classical producers can be sequenced now, and the mutations recognised can be used to assay for their importance and relevance to derive a good producer (see Fig. 14.6). Using this genomic-based approach, just three point mutations were introduced into the genome of the wild type to derive an excellent L-lysine producer. By introducing alleles of the genes coding for aspartate kinase (*lysC*-Thr311I1e), pyruvate carboxylase (*pyc*-Pro458Ser) and homoserine dehydrogenase (*hom*-Va159A1a) production of 80 g 1^{-1} L-lysine with a productivity of 3.0 g 1^{-1} h^{-1} was achieved.

14.5.3 Production process

The most common carbon sources for L-lysine fermentation and also other amino acids are molasses (cane or sugar beet molasses), high test molasses (inverted cane molasses), or sucrose and starch hydrolysates. In contrast to *E. coli*, the wild type of *C. glutamicum* can utilise both glucose and sucrose. In the past, molasses was mostly used for production since it is a relatively cheap carbon source. However, the utilisation of molasses has severe disadvantages:

- waste is exported from the sugar company to the fermentation plant and causes additional costs there;
- the seasonal availability of molasses causes ageing effects in its quality during storage.

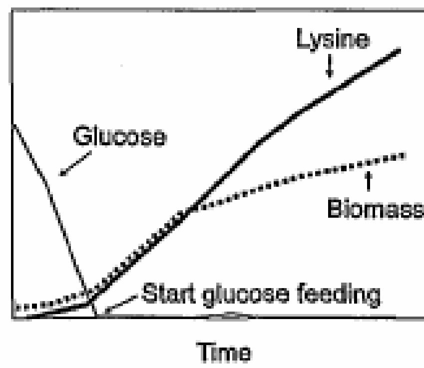


FIGURE 14.17 Time course of L-lysine accumulation in a production process. There are three phases of growth and L-lysine accumulation.

Therefore, there is a clear tendency away from molasses towards refined carbon sources such as hydrolysed starches. Profitable nitrogen sources are ammonium sulphate and ammonia (gaseous or ammonia water). The growth factors required are provided from plant protein hydrolysates, cornsteep liquor or by the addition of the defined compounds. A typical lysine fermentation is shown in Fig. 14.17. After consumption of the initial sugar, the substrates are added continuously and L-lysine accumulates up to 170 g 1^{-1}. Ammonium sulphate provides the counter-ion to neutralise the accumulating basic amino acid. Therefore, L-lysine is present in the fermentation broth as its sulphate. As a convention in the literature, lysine is usually given as lysine·HC1. Due to the high sugar cost, the conversion yield is a very important criterion for the entire production process. Technical processes have been published with a yield of 45-50 g lysine 100 g 1^{-1} carbon source.

For the recovery of L-lysine, several basically different processes have been developed. Three processes are currently in use to supply L-lysine in a form suitable for feed purposes:

- A crystalline preparation containing 98.5% L-lysine•HC1. It can be made by ion exchange chromatography, evaporation and crystallisation. Also direct spray-drying of the ion exchange eluate is possible.
- An alkaline solution of concentrated L-lysine containing 50.7% L-lysine. It is obtained by biomass separation, evaporation and filtration.
- A granulated lysine sulphate preparation consisting of 50% L-lysine. It consists of the entire fermentation broth conditioned by spraydrying and granulation

These processes differ significantly in investment costs, losses during downstreaming, amount of water volume and user friendliness. All this, together with the fermentation itself, decides the success of the various competitors producing L-lysine.

14.6 L-Threonine

14.6.1 Biochemistry

The commercial production of L-threonine is done with *E. coli* mutants. The synthesis of L-threonine proceeds via a short pathway comprising only five steps (Fig. 14.18). As already mentioned, the first steps are shared with that of L-lysine and L-methionine synthesis. Furthermore, L-threonine is also an intermediate in the L-isoleucine synthesis. This naturally requires special melabolic regulation. In *C. glutamicum* this was solved in such a way that the Sole aspartate kinase present was only inhibited by the joint presence of L-lysine and L-threonine. In the case of *E. coli*, however, three isoenzymes are present each of which is separately inhibited by a different endproduct: one by L-threonine, one by L-lysine and one by L-methionine. There are furthermore two homoserine dehydrogenase activities: one

is inhibited by L-threonine and one by L-methionine. Additionally, the corresponding genes are grouped into transcriptional units, thereby ensuring a balanced synthesis of the appropriate amino acid at the level of gene expression. The relevant operon for L-threonine synthesis in *E. coli* is *thrABC*. It encodes three polypeptides, with *thrA* encoding an apparently fused polypeptide of aspartate kinase plus homoserine dehydrogenase. A strong expression control of this operon is provided by a transcription attenuation mechanism. The corresponding leader peptide at the beginning of the transcription unit is Thr-Thr-Ile-Thr-Thr-Thr-Ile-Thr-Ile-Thr-Thr, serving to sense the availability of L-threonine and L-isoleucine. When the corresponding tRNAs are uncharged, the leader peptide formation does not occur, and transcription of the operon is increased at least ten-fold.

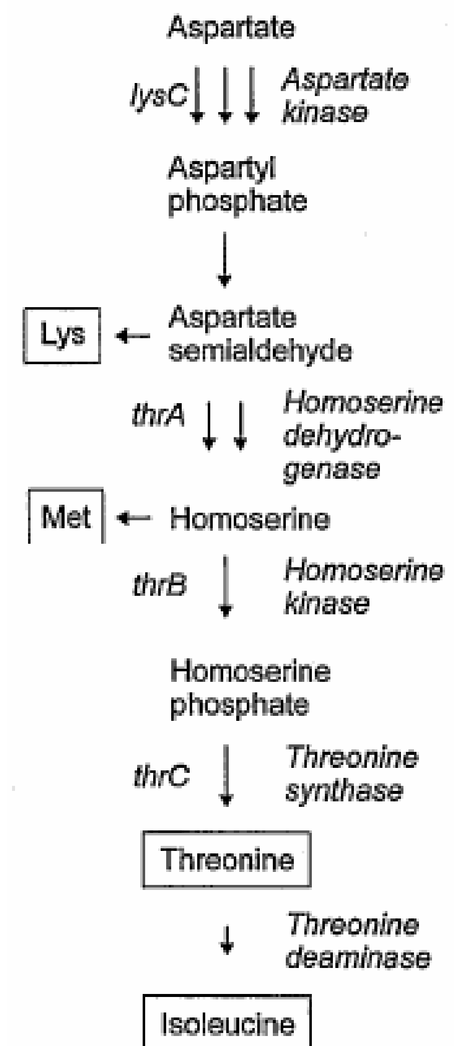


FIGURE 14.18 The short pathway of L-threonine synthesis linked with that of L-lysine, L-methionine and L-isoleucine synthesis. *Escherichia coli* has isoenzymes, as indicated by the parallel arrows, each of them separately regulated by either inhibition or gene expression involing the individual amino acids of this pathway.

14.6.2 Producer strains

Based on this pathway structure and regulation there is a clear focus on two major targets for the design of a producer strain: the prevention of L-isoleucine formation and stable high-level expression of *thrABC*. Therefore, in one of the first steps of strain development, chromosomal mutations were introduced to produce an isoleucine leaky strain (Fig. 14.19). The isoleucine mutation located in the threonine deaminase is a very specific and important one. L-Isoleucine is required only at low L-threonine concentrations but, at high concentrations of L-threonine, growth is independent of added L-isoleucine. This is the case with a threonine deaminase mutated to have a low affinity. This mutation has several advantageous consequences. In the first place, it prevents an excess formation of the undesired by-product L-isoleucine. Additionally, it prevents the L-isoleucine dependent premature termination of the *thrABC* transcription due to limiting $tRNA^{Ile}$.

A third consequence of the isoleucine mutation is more subtle. It relates to the stability of the plasmid-containing producer strain in the various pre-cultivation steps. Starting from a single clone, a preculture is inoculated for each production and is then enlarged in several stages. This means that the clone is fermented for about 25 generations so that there is a great danger of the plasmid that contains the *thrABC* operon being lost. This would, of course, be a complete disaster if it happened in the final production stage. In the presence of the isoleucine leaky mutation, however, cells that have lost the plasmid now are clearly disadvantaged since they are not synthesising a high concentration of L-threonine. Their further proliferation is halted, thereby stabilising a culture where almost all the cells that are growing contain the plasmid. Further engineering during strain evolution involved the introduction of resistance to L-threonine and L-homoserine, which turned out to result in increased expression of a carrier protein exporting L-threonine from the cell into the medium. Subsequently, *tdh*, which encodes the threonine dehydrogenase, was inactivated thus preventing threonine degradation. To obtain very high activities of the *thrABC*-encoding enzymes, the operon was cloned

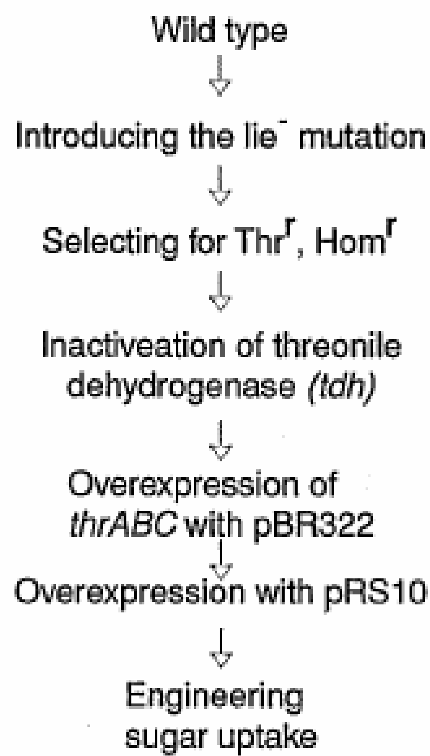


FIGURE 14.19 Relevant steps in the development of an *E. coli* strain suitable for threonine production involving undirected mutagenesis, gene inactivation and use of different plasmids.

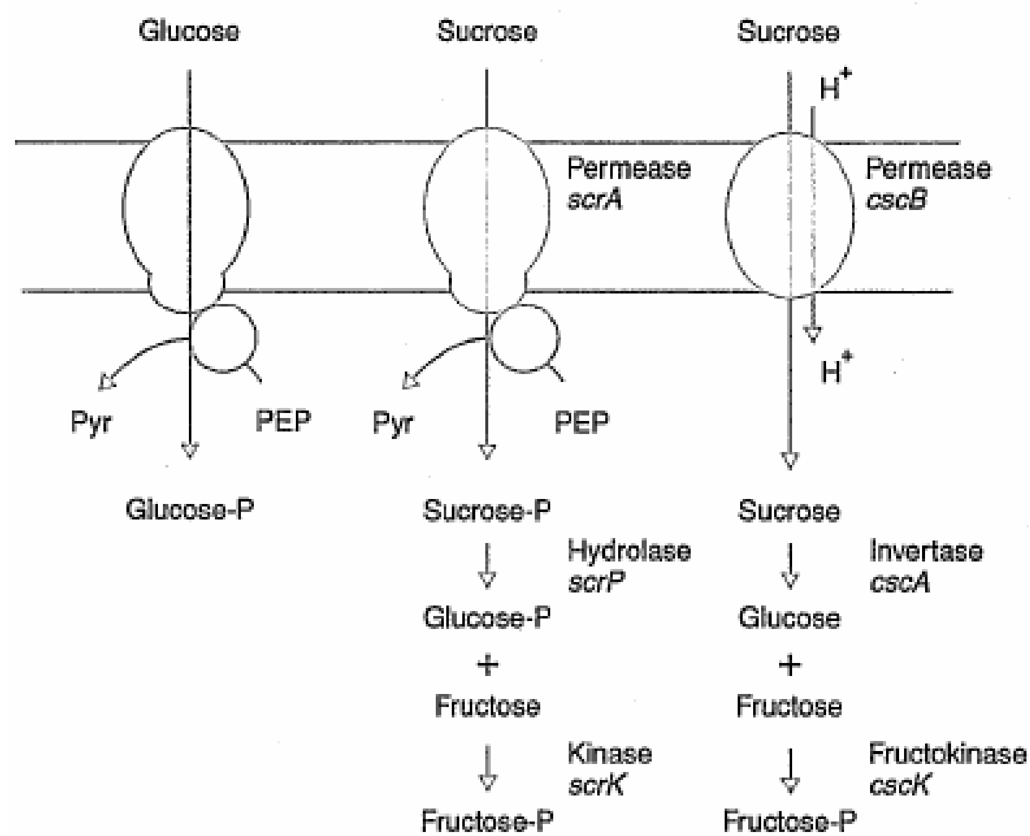


FIGURE 14.20 Mechanisms of sugar uptake and phosphorylation in *E. coli*. Translocation is coupled by phosphorylation, as is the case for the phosphotransferase system (left and middle), or occurs in symport with protons without phosphorylation (right). The phosphotransferase translocating sucrose (middle) shares one of the phosphoryl transfer domains with a component of the phosphotransferase translocating glucose. Pyr, pyruvate; PEP, phosphoenolpyruvate.

from a strain whose aspartate kinase and homoserine dehydrogenase activities are resistant to L-threonine inhibition. In addition the transcription attenuator was deleted. In fermentations the operon engineered in this way was successfully used with pBR322 as a vector, but a further improvement was obtained by replacing this plasmid by a pRS1010 derivative, resulting in an even more stable high-level expression.

Substrate uptake

Since the cost of the sugar has a decisive influence an the price of the amino acid produced, it is essential to be able to switch between glucose and sucrose as substrates. However, K-12 *E. coli* strains cannot use sucrose, as was also the case with the originally developed L-threonine producer. Fortunately, two different sucrose-utilising systems of other strains are available to engineer sugar utilization (Fig. 14.20). One of them is represented by the *scr* regulon of *E. coli* strain H155, where the actual translocator consists of the phosphoenolpyruvate : sugar phosphotransferase system (PTS). Introduction of the *scr* genes into a K-12 strain results in the uptake and phosphorylation of sucrose. Due to subsequent hydrolase and fructokinase activities, the sugar is then channelled into the central metabolism. An alternative sucrose

utilisation system is provided by the *csc* regulon of some *E. coli* strains. In this case, sucrose is translocated by the *cscB* encoded translocator in symport with protons. Using transposition, the sucrose-utilisation capability of the *csc* regulon was introduced into a glucose-utilising strain. Although originally without uptake of sucrose, this strain now imported sucrose at a rate of 9 pmol min^{-1} (mg $DW)^{-1}$. With the plasmid-encoded regulon the rate obtained was 43 pmol min^{-1} (mg $DW)^{-1}$, which was almost identical to that of the strain from which the csc regulon had been isolated.

14.6.3 Production process

The fermentation of the engineered L-threonine producer is in a simple mineral salts medium with either glucose or sucrose as the substrate with addition of a small amount of a complex medium component like yeast extract. After inoculation and consumption of the initially provided sugar, continuous feeding of sugar begins. Additionally, ammonia has to be fed in the form of gas or as NH_4OH which is regulated via pH control. Thus the feeding strategy in the case of L-threonine fermentation is quite easy as compared to L-lysine fermentation where the accumulation of the basic product requires the feeding of sulphate as the counter-ion. After 77 h of fermentation L-threonine is present at about 100 g l^{-1} wich a conversion yield of up to 60%. The fermentation is characterised by low by-product formation, which is an advantage for downstream processing. Cystallization of L-threonine is easy due to its low solubility and the low Salt concentration present. A process is described where the cells are initially coagulated by a heat- or pH-treatment step, followed by filtration. Subsequently, the broth is concentrated and crystallisation initiated by cooling. The separation and drying of the crystals leads to an isolation yield of 80-90% with the L-threonine having a purity of more than 90%. A recrystallization step may be required for high-purity L-threonine.

14.7 L-Phenylalanine

14.7.1 Biochemistry

L-Phenylalanine can be produced with *E. coli* or *C. glutamicum*. The L-phenylalanine synthesis is shared in part with that of L-tyrosine and L-tryptophan. The three aromatic amino acids have in common the condensation of erythrose 4-phosphate and phosphoenolpyruvate to 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) with further conversion in six steps up to chorismate. L-Phenylalanine is then finally made in three further steps (Fig. 14.21). There are three DAHP synthase enzymes in *E. coli* encoded by *aroF, aroG* and *aroH.* These enzymes play a key role in flux control. Their regulation of catalytic activity, in each case by one of the three aromatic amino acids, recalls the specific regulation of aspartate kinase in the synthesis of threonine. About 80% of the total DAHP-synthase activity is contributed

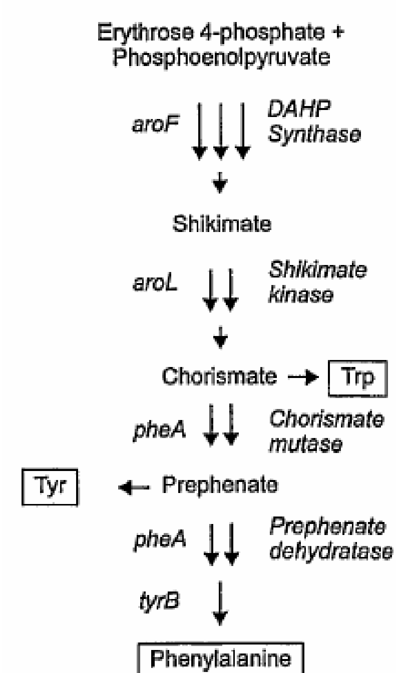


FIGURE 14.21 Simplified pathwayof L-phenylalanine synthesis involving actually ten enzyme-catalysed reaction steps. In addition, isoenzymes operate in *E. coli* as indicated by the parallel arrows. Selected key enzymes and key genes are given.

by the *aroG*-encoded enzyme. Interestingly, there are two bifunctional polypeptides, each encoding corismate mutase-prephenate dehydratase. That encoded by *pheA* is inhibited by L-phenylalanine, and that encoded by *tyrA* by L-tyrosine. The *pheA* gene expression is dependent on the level of $tRNA^{Phe}$.

14.7.2 Production strains

Producer strains have a feedback-resistant DAHP activity encoded either by *aroF* or *aroG* and a feedback-resistant chorismate mutase-prephenate dehydratase encoded by *pheA*. As a rule, the producers are L-tyrosine auxotrophic mutants. There are very good reasons for this, one of which is that enzymes of the common pathway from DAHP to prephenate are no longer regulated by L-tyrosine and enzyme activities are no longer feedback-inhibited. Another reason is that in this way tyrosine accumulation is prevented, which would otherwise undoubtedly result as a by-product since there are only two additional steps from prephenate to L-tyrosine. An essential aspect is that due to the auxotrophy: a beneficial growth limitation is possible by appropriate tyrosine feeding (see below). In some *E. coli* strains, the temperature-sensitive cI_{857} repressor of bacteriophage) has been used together with the λP_L promoter to enable inducible expression of the key genes *pheA* and *aroF*. This enables extremely high enzyme activities to be adjusted solely in the actual production runs thus eliminating the inherent problems of strain stability due to the resulting high metabolite concentrations. It enables the pre-cultivation steps up to the seed fermenter to be performed with low expression of the key genes but in the actual large production fermenter the genes are now induced to a high level of expression.

14.7.3 Production process

As with the other amino acids, effective L-phenylalanine production is the joint result of genetically engineering cellular metabolism and tight control of the production process in the fermenter. Control of metabolic regulation is necessary for two reasons. First, the carbon flux has to be optimally distributed between the four major products of glucose conversion, which are L-phenylalanine, biomass, acetic acid and CO_2. The second reason is that the cellular physiology is not constant during the course of fermentation, which correspondingly requires an adaptation of fermentation control during the process. Figure 14.22 shows the typical time curve of L-phenylalanine production. The major problem is that *E. coli* tends to produce acetic acid which has a strong negative effect on process efficiency. To prevent this, researchers have developed an ingenious sugar-feeding strategy, which first collects on-line data and fluxes such as O_2 concentration, sugar consumption and biomass concentrations. These are then counterbalanced during the process to control the optimum sugar concentration. The feeding of sugar starts when the cells enter Stage 2 of the fermentation where the glucose initially provided has almost been consumed. The trick is to prevent too high a glucose concentration occurring since this would result in acetic

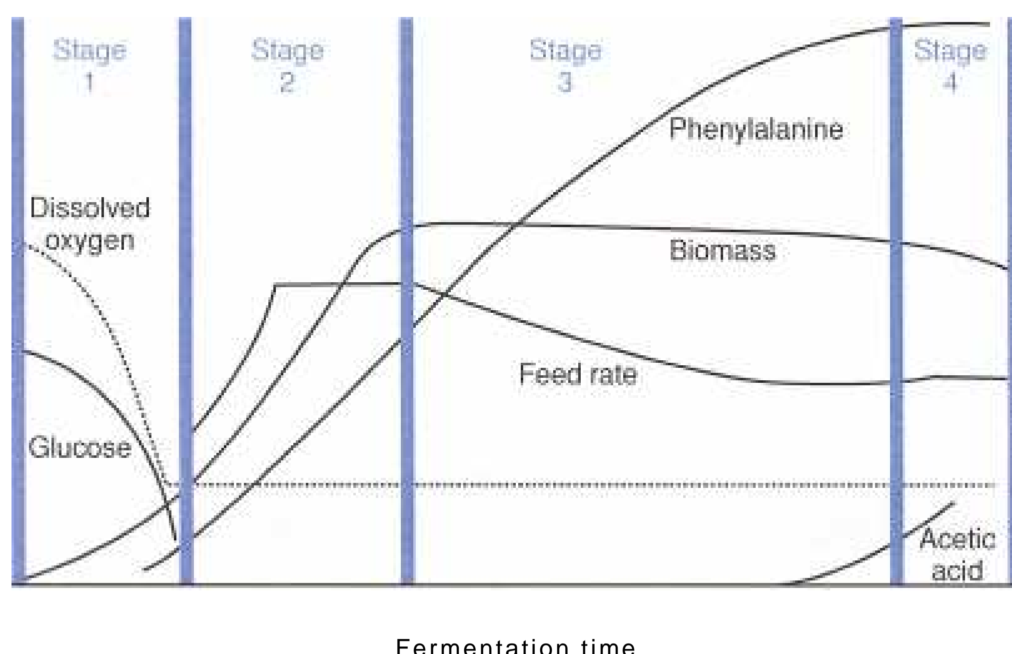


FIGURE 14.22 The four stages of L-phenylalanine production characterised by different physiology requiring different process control regimes to give he highest yields in shortest times.

acid formation and, at the same time, to prevent too low a glucose concentration since this would result in an excess of CO_2 evolution. Thus the feeding rate is a compromise where the process is run at the highest possible feeding rate which still provides a sufficiently strong limitation to prevent acetic acid excretion. When the L-tyrosine initially present has been consumed, the cells proceed to Stage 3. As already mentioned, almost all L-phenylalanine producers are tyrosine auxotroph. The L-tyrosine concentration selected at the start of the culture therefore fixes the minimum amount of biomass necessary to metabolise the pre-determined amount of glucose efficiently. In Stage 3, the metabolic capacity of the cells decreases which brings about a consequent decrease of the glucose feeding rate. At the end of Stage 3, acetic acid excretion begins and the cells enter Stage 4 where no further L-phenylalanine accumulation occurs and the process is eventually terminated. This example of amino acid production shows that by the sophisticated application of feeding strategies with adaptive control a very high L-phenylalanine concentration can be achieved with a high yield within 2.5 days. Values of 50.8 g l^{-1} L-phenylalanine with a yield of 27.5% have been reported.

14.8 L-Tryptophan

14.8.1 Biochemistry

L-Tryptophan is a high-price amino acid which still has a rather low market volume. It is one of the candidate amino acids to be used for further improvement of animal feed. Effective production processes are available with mutants of *E. coli*, *C. glutamicum* and *Bacillus subtilis*. However, still in use is the effective enzymatic synthesis of L-tryptophan from cheap precursors yielding a product of highest purity. This type of production is based an the activity of the biosynthetic tryptophan synthase (Fig. 14.23). This enzyme catalyses the last step in the tryptophan synthesis, which in fact consists of

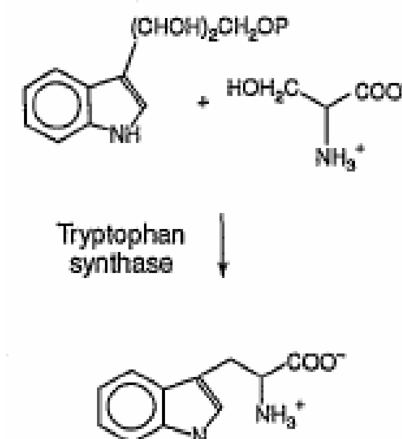


FIGURE 14.24 The tryptophan synthase uses *in-vivo* indole 3-glycerol phosphate plus Lserine, and in the production process indole plus L-serine.

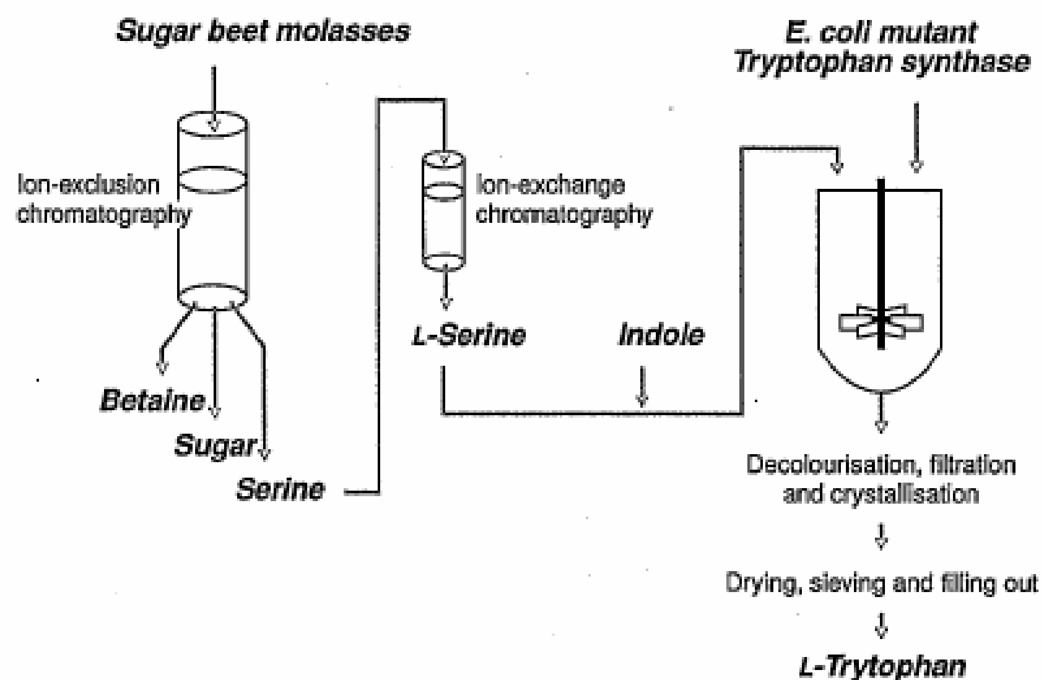


FIGURE 14.24 Flow scheme to use molasse-derived L-serine together with indole as subtrates for biotransformation into L-tryptophan. Biotransformation is done with pre-grown *E. coli* cells having high tryptophan synthase activity.

two partial reactions:

Indole-3-glycerol phosphate
→ indole + glyceraldehyde 3-phosphate (α-sub-unit)
Indole + L-serine -4 → L-tryptophan + H2O ($ß_2$-sub-unit)

These separate reactions are catalysed by separate sub-units of the enzyme: a and ß. The enzyme of E. coli is an a2ß2-tetramer, which can be dissociated into functional a-sub-units and a ß2-sub-unit. The a-subunit catalyses the cleavage of indole-3-glycerol phosphate, whereas the ß2-sub-unit catalyses the condensation of t-serine with indole to form t-tryptophan. It is this latter reaction which is advantageously used in the industrial production. .

14.8.2 Production from precursors

The production is based an *E. coli* cells which have a high tryptophan synthase activity. The a and ß-sub-units encoding genes *trpA* and *trpB*, respectively, are located an the *trpEDCBA* operon which is regulated by repression and attenuation. In the *E. coli* mutant used, the repressor of that operon has been deleted as it is part of the attenuator region together with the first structural genes of the operon. As a consequence, about 10% of the total protein is tryptophan synthase with an excess of ß-sub-units present. Although indole is not the true substrate, with a sufficiently high concentration the synthase as well as the ß-sub-units present will react with it. Indole is available from the petrochemical industry as a comparably cheap educt, whereas the second educt, L-serine, is recovered from molasses during sugar refinement using ion-exclusion chromatography, and further purification steps (Fig. 14.24). The resulting L-serine is fed to the previously cultivated *E. coli* cells, and indole is added continuously at a concentration adjusted to 10 mM, which is controlled on-line. This type of process ensures an almost quantitative conversion of indole

Table **14.3** Comparison of the productivity of immobilized *E. coli* cells for the production of L-aspartate

Immobilisation method	Aspartase activity (Ug^{-1} cells)	Half-life (days)	Relative productivity (%)[b]
Polyacrylamide	18 850	120	100
Carrageenan	56340	70	174
Carrageenan (GA)[a]	37 460	240	397
Carrageenan (GA + HA)[a]	49 400	680	1498

[a] GA, glurtaraldehyde; HA, hexamethylene diamine.
[b] Considers the initial aclivity, decay constant and operation period.

to yield L-tryptophan with a space-time yield of about 75 g 1^{-1} day^{-1}. Further processing of the L-tryptophan solution can be taken from Fig. 14.24 leading to a pyrogen-free pharmaceutical product of the highest quality.

14.9 L-Aspartate

14.9.1 Biochemistry

L-Aspartic acid is widely used as a food additive and in pharmaceuticals. Demand increased rapidly with the introduction of aspartame as an artificial sweetener. This is a dipeptide consisting of L-aspartate and L-phenylalanine which is about 200-fold sweeter than sugar and was successfully introduced into the market as a low-calorie sweetener. Although L-aspartate was originally produced fermentatively, it is currently produced exclusively using aspartase due to the high productivities and the cost effectiveness of the process. In fact, the use of aspartase to make L-aspartate represents one of the highest productivities known for an enzyme used in biotechnology (see also Chapter 24). The method developed allows re-use of the enzyme to the extent that over 220 000 kg of product can be produced per kilograms of enzyme.

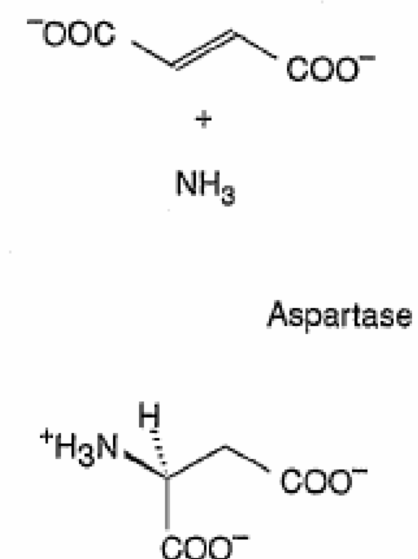


FIGURE 14.25 Fumarate and ammonium serve as substrates for the aspartase.

Aspartase catalyses the interconversion between L-aspartate and fumarate plus ammonia (Fig. 14.25). The reaction favours the amination reaction. The enzyme of *E. coli* is a tetramer with a molecular weight of 196 000, which has an absolute requirement for divalent metal Ions. A severe disadvantage at the beginning of the studies by the Tanabe Seiyaku Co. Ltd, which now successfully uses aspartase, was the instability of the enzyme. After incubation of the enzyme in solution for just half an hour at 50 °C, activity is no longer detectable. Nevertheless, a residual activity of 10% is present when the enzyme is immobilised in polyacrylamide. Such a physical confinement of cells in space turned out to be the method of choice. Table 14.3 Shows that with the natural polymer κ-carrageenan, resulting from a screening of different polymers and use of appropriate cross-linking, exceptional improvements were obtained in the relative productivity as well as

in the stability of the catalyst. The final material has a half-life of almost two years. This represents an almost unimaginable progress in comparison to the initial situation where the enzyme in free solution only had a half-life measured in minutes. An initial disadvantage of the original cells used was their fumarase activity, which results in the partial conversion of fumarate to L-malic acid. To solve this problem a heat treatment step of the cells is used which eliminates the fumarase activity almost completely. Using such conditioned cells and starting from 1 M ammonium fumarate, the final product solution contains 987 mM L-aspartate, 10.7 mM non-reacted fumarate and only trace quantities (1.9 mM) L-malic acid.

For the production process the immobilised cells are packed into a column designed as a multi-stage system. The stages introduced, each consisting of a set of parallel horizontal tubes, serve two purposes. On the one hand, they allow effective cooling to prevent decay of the catalytic activity since the aspartase reaction is exergonic. About 6 kcal heat mol^{-1} substrate evolves in the actual large-scale production process which is very close to that calculated from the Standard free energy change of the aspartase reaction of 4 kcal mol^{-1}. On the other hand, the flow properties of the column are increased. Any compacting of the bed over time is prevented, and the preferred plugflow characteristics are obtained. With such a column, flow rates of two column volumes per hour are possible. The continuous process enables full automation and control to achieve an optimum throughput with the highest product quality. Yet another advantage of such a controlled continuous process is its reduced waste production. A typical volumetric activity is about 200 mmol h^{-1} (g cells)$^{-1}$. Assuming a 1000-litre column, the yield of L-aspartate is 3.4 tonnes per day, which is 100 tonnes per month. The final product is eventually purified by crystallisation.

14.10 Outlook

Although amino acids are now among the classical products in biotechnology, their demand is increasing enormously. Constant development of the processes is required, new processes have to be established and understanding of the exceptional capabilities of producer strains deepened. Surprising new information, of general interest for cellular physiology, has been gathered such as the existence of specific export carriers or cyclic fluxes within the anaplerotic reactions. Moreover, much information has been obtained from strain development in conjunction with fermentation technology, with the new science of metabolic engineering at the interface between them. In fact, amino acid production is an outstanding example of the integration of many different techniques. In this way, the early Japanese activities an the taste of kelp laid the foundation for the continuing very successful and flourishing production of amino acids.

a

14.1.1 Further reading

Bongaerts, J., Krämer, M., Müller, U., Raeven, 1.. and Wubbolts, M. Metabolic engineering for microbial production of aromatic amino acids and derived compounds. *Metabolic* Engineering 3: 289-300, 2001.

Chibata, I., Tosa, T. and Shibatani T. The industrial production of optically active compounds by immobilized biocatalysts. In *Chirality in* Industry, eds. A. N. Collins, G. N. Sheldrake and J. Crosby. London: John Wiley & Sons, 1992.

Debabov, V. G. The threonine story. *Advances* Biochernical Engineering 79: 114-136, 2003.

de Graaf, A. A. Metabolic flux analysis of Corynebacterium glutamicum. In Bioreactor Engineering, Modeling and Control eds. K. Schügerl and K. H. Bellgardt. Berlin: Springer-Verlag, 2000, pp. 506-555.

Eggeling, L. and Bott, M. *Corynebacterium* glutamicum: Basic Biology and Application. A Handbook. Boca Raton, FL: CRC Press, 2004.

Hodgson, J. Bulk amino acid fermentation: technology and commodity trading. Bio/Technology 12: 152-155, 1994.

Eggeling, L. and Sahm, 11. New ubiquitous translocators: amino acid export by Corynebacterium *glutamicum* and Escherichia coli. Archives of Microbiology 180: 155-160, 2003.

Ikeda, M. Amino acid production processes. Advances Biochernical Engineering 79: 1-35, 2003.

Konstantinov, K. B., Nishino, N., Seid, T. and Yoshida, T. Physiologically motivated strategies for control of the fed-batch cultivation of recombinant *Escherichia coli* for phenylalanine production. *Journal of* Fermentation and Bioengineering 71: 350-355, 1990.

Ohnishi, J., Mitsuhashi, S., Llayashi, M., et al. A novel methodology employing *Corynebacterium glutamicum* genome information to generate a new r-lysine-producing mutant. Appliecl Microbiology and Biotechnology 58: 217-223, 2002.

Acknowledgements

We would like to thank the following for providing material for this article: R. Faurie, Amino GmbH; K. Ikeda, Ajinomoto Ltd; N. Kato, Kyoto University; Y. Kawahara, Ajinomoto Ltd; W. Leuchtenberger and G. Thierbach, Degussa AG; T. Shibasaki, Kyowa Hakko Kogyo; T. Tosa, Tanabe Seiyaku.