5
Enzymatic Production of Amino Acids

HUMG-YU HSIAO, JAMES F. WALTER*, DAVID M. ANDERSON AND *BRUCE K. HAMILTON

*ChemGen Corporation, 2501 Research Boulevard, Rockville, MD 20850, USA, and *W.R. Grace & Company, Washington Research Center, 7379 Route 32, Columbia, MD 21044, USA*

Introduction

Amino acid sales currently constitute a multibillion dollar annual worldwide market (Hamilton and Jackson, 1985). Amino acids are manufactured by a number of methods, including fermentation, chemical and enzymatic synthesis, and extraction. In some cases hybrid methods are used: for example, chemical synthesis followed by enzymatic resolution of optical isomers (Chibata and Tosa, 1976). This report focuses on enzymatic production of amino acids, a topic that has received increasing attention in recent years.

Three themes, all involving new technological developments, are recurrent throughout the discussion of specific enzymatic means to produce particular amino acids. These three themes are: (1) the diversity of enzymatic routes and substrates that can be used to synthesize amino acids, (2) the issue of immobilization of biocatalysts employed for enzymatic production, and (3) the application of genetic engineering to produce efficiently biocatalysts used for enzymatic manufacture of amino acids. Selected case studies have been chosen to illustrate these themes, and the coverage is not intended to be comprehensive.

Abbreviations: AAC, D,L-α-amino 2-caprolactam; DCW, dry cell weight; DOPA, dihydroxyphenylalanine; EDTA, ethylenediamine tetra-acetic acid; HPLC, high pressure liquid chromatography; PAL, phenylalanine ammonia lyase (EC 4.3.1.5); PLP, pyridoxal-5'-phosphate; PMP, pyridoxamine-5'-phosphate; SHMT, serine hydroxymethyl transferase (EC 2.1.2.1); NAD, nicotinamide adenine dinucleotide.

Biotechnology and Genetic Engineering Reviews — Vol. 6, September 1988
0264-8725/88/06/179-219 $20.00 + $0.00 P.O. Box 402, Wimborne, Dorset BH22 9TZ, UK

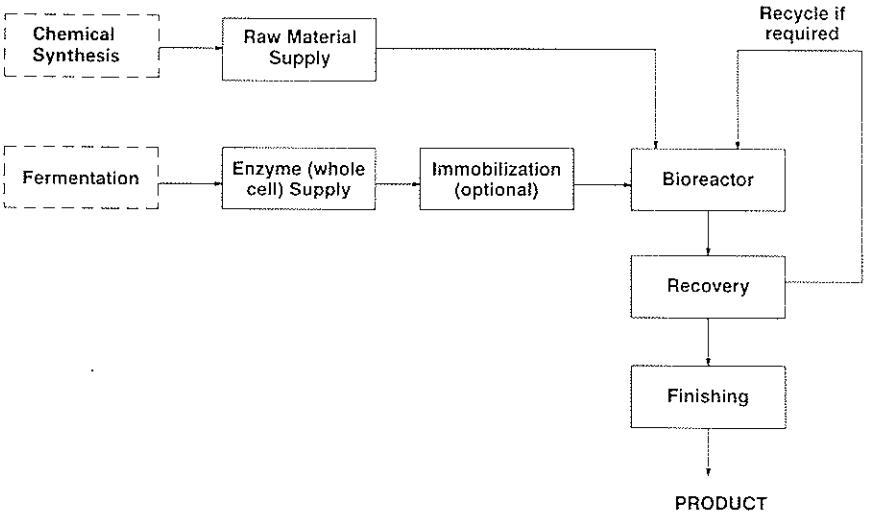


Figure 1. Elements of a typical manufacturing process for the enzymatic production of amino acids.

Elements of an enzymatic production process

The elements of a typical manufacturing process for the enzymatic production of an amino acid are shown in *Figure 1*. Raw materials for the enzymatic step are usually supplied by chemical synthesis. The enzyme itself, either in isolated or whole cell form, is prepared by microbial fermentation. The enzyme or whole cells may be immobilized for the bioreactor step. In the bioreactor step, the raw materials supplied by chemical synthesis are converted by the biocatalyst to the desired product. Depending upon reaction kinetics and thermodynamics, some portion of the raw materials may remain unreacted, in which case separation of them in the recovery section of the process may be appropriate to permit their recycle. Finally, the product is purified to specification for sale or captive use.

Commercial status

Several enzymatic processes for manufacture of amino acids that have been commercialized are listed in *Table 1*.

In 1973, Chibata and co-workers, at the Tanabe Seiyaku Co. Ltd, Osaka, Japan, commercialized the continuous enzymatic production of L-aspartic acid from fumaric acid and ammonia. This process uses immobilized *Escherichia coli* whole cells with high aspartase (EC 4.3.1.1) activity (Chibata, Tosa and Sato, 1986). In addition, it is said that L-aspartic acid has been produced industrially at Kyowa Hakko Kogyo Co. Ltd, Japan, since 1974 through use of aspartase extracted from *E. coli* and immobilized by ionic binding on an ion exchange resin (Chibata, Tosa and Sato, 1986).

Chibata's group at Tanabe also commercialized production of L-alanine

Table 1. Some enzymatic processes for manufacture of amino acids that have been commercialized

Amino acid	Enzyme(s)	Firm	Country	Process status
L-asp	aspartase	Tanabe	Japan	Immobilized *E. coli* whole-cell process commercialized since 1973.
L-asp	aspartase	Kyowa Hakko	Japan	Immobilized *E. coli* extracted-enzyme process said to be practised industrially since 1974.
L-ala	L-aspartate β-decarboxylase	Tanabe	Japan	Immobilized *Pseudomonas dacunhae* whole-cell process commercialized since 1982.
L-lys	L-AAC hydrolase and AAC racemase	Toray	Japan	Annual production of L-lys via this route reported to exceed 4 000 tons, 1981.
L-phe	phenylalanine ammonia lyase	Genex	USA	Several hundred tons per year manufactured and sold during 1984/1985. Shutdown October 1985.
L-phe	transaminase	PEI	USA	Several tons manufactured during 1984/1985. Shutdown 1985.

from L-aspartic acid through use of microbial L-aspartate β-decarboxylase (EC 4.1.1.11) (Chibata, Tosa and Kakimoto, 1986). The process uses immobilized whole cells of *Pseudomonas dacunhae*, and was industrialized in 1982.

The Japanese firm Toray produces L-lysine from D,L-α-amino-ε-caprolactam (AAC) by using two enzymatic reactions in series: the selective hydrolysis of L-AAC (in a mixture of D, L-AAC) to L-lysine by a hydrolase (EC 3.5.2.11) from *Cryptococcus laurendii*, and the transformation of D-AAC to L-AAC by a racemase found in *Achromobacter obae*. The annual production of L-lysine via this route was reported to exceed 4000 tons in 1981 (Tosaka, Enei and Hirose, 1983).

Genex Corporation (USA) commercialized the enzymatic production of L-phenylalanine from *trans*-cinnamic acid and ammonia with phenylalanine ammonia lyase (EC 4.3.1.5) as biocatalyst (Hamilton *et al.*, 1985; Anderson, 1987). Several hundred tons per year of L-phenylalanine were manufactured and sold during 1984 and 1985, but the plant was shut down when Genex lost a supply contract with G.D. Searle.

Purification Engineering, Inc. (PEI), recently acquired by Rhone Poulenc, also developed an enzymatic process for making L-phenylalanine. Phenylpyruvate and aspartic acid were converted to L-phenylalanine and by-products (pyruvate and CO_2) with a transaminase. Several tons of product were made with this process before it, too, was shut down (Calton *et al.*, 1986; Wood and Calton, 1987).

Factors influencing process selection

Some of the factors that can influence the selection of a particular process for the enzymatic manufacture of a specific amino acid from among several

Table 2. Factors influencing selection of a process for manufacturing amino acids

(1) Manufacturing economics
(2) Patent position
(3) Ability to produce and/or control raw material
(4) Excess plant capacity
(5) Corporate experience with fermentation, enzyme immobilization, genetic engineering
(6) Corporate philosophy

alternative routes are listed in *Table 2*. Low-cost manufacturing economics are, of course, likely to play a dominant part. Patent position is of obvious importance. The ability of a firm to produce and/or control key raw materials can be a deciding factor. If excess plant capacity can be advantageously employed with a particular route, then that route may become favoured. A company whose manufacturing base is focused on fermentation may be more likely to employ biological than chemical routes. Finally, corporate philosophy may facilitate development of new technological approaches.

Key bioprocess performance parameters

The assembly of a successful bioprocess for the enzymatic manufacture of an amino acid is dependent upon the achievement of economically satisfactory values for certain key bioprocess performance parameters. Key bioprocess performance parameters are given in *Table 3*. The list in *Table 3* is divided into two categories that have to do with the sections of the bioprocess (*see Figure 1*) that embody all the process biology, namely: (1) the bioreactor, and (2) the preparation of biocatalyst for the bioreactor.

For the bioreactor section, the unit costs of substrates (dollars per pound) greatly impact manufacturing economics. Likewise, so do bioreaction yields (pounds of product per pound of each substrate). Furthermore, selective conversion of substrates to the desired product rather than by-products is important so that costly substrates are not wasted and product purification is not made more difficult. Production of product at high titre facilitates efficient recovery. A high rate of product production means that reactor size and therefore capital cost can be kept small. Long-term biocatalyst stability is

Table 3. Bioprocess keys: enzymatic production of amino acids

A. Bioreactor
(1) Low unit cost of substrates
(2) High substrate yields
(3) High selectivity for product production
(4) High product titre
(5) High rate of product production (high volumetric productivity)
(6) Long-term biocatalyst stability
B. Biocatalyst preparation
(1) High enzyme fermentation peak titre and lengthy peak titre stability
(2) Low fermentation medium cost
(3) Short fermentation time
(4) High enzyme recovery yield
(5) If enzyme is immobilized:
(a) high immobilization yield
(b) low immobilization cost (both for support and immobilization process)

advantageous for keeping down operating costs. What is particularly desirable is the production of many, many pounds of product per pound of biocatalyst over the useful lifetime of each pound of that biocatalyst.

Biocatalyst for the bioreactor is prepared by a microbial enzyme fermentation. In the enzyme fermentation, peak titre (units/litre) should be high, and also stable (at least for several hours in the fermentation broth, preferably longer) to enable enzyme recovery at high yield. Fermentation medium cost (dollars per litre) should be low; in particular, medium components should be available in large quantity and reproducibile quality at low cost (typically, laboratory media are high cost and unavailable in large quantity). The shorter the fermentation time, the more productive will be a fermentor of given volume. If the enzyme is immobilized in either isolated or whole-cell form, high immobilization yield with low cost immobilization chemistry and procedures is important.

Case study 1—enzymatic routes to amino acids that involve SHMT

BACKGROUND ON SHMT

Serine hydroxymethyltransferase (SHMT) (L-serine:tetrahydrofolate 5,10-hydroxymethyltransferase, EC 2.1.2.1) is an enzyme containing pyridoxal-5-phosphate, with the physiological function of providing one-carbon units in the form of 5,10-methylenetetrahydrofolate (m-FH_4) for the synthesis of purines, thymidylate, methionine, etc. SHMT achieves this function by interconverting serine and glycine as shown in the following scheme:

$$\text{Serine} + \text{tetrahydrofolate}\,(FH_4) \xrightleftharpoons{\text{SHMT}} \text{glycine} + \text{m-}FH_4$$

SHMT is a versatile enzyme (Schirch, 1982) that can carry out several types of reactions, as shown in *Table 4*. It has the ability to catalyse aldol-condensation reactions (reactions 1–7, *Table 4*), a transamination reaction (reaction 8, *Table 4*) and a decarboxylation reaction (reaction 9, *Table 4*). It can accept unusual substrates such as formaldehyde and benzaldehyde. For retro-aldol reactions, SHMT has a very high stereo-selectivity at the α-carbon of the 3-hydroxy-amino acid but has a much lower preference at the β-carbon of the substrate (reactions 3–6, *Table 4*). For aldol-condensation reactions, SHMT preferen-

Table 4. Some reactions catalysed by SHMT (Schirch, 1982)

1. L-Serine + H_4-folate⇌glycine + 5,10-methylene-H_4-folate
2. L-α-Methyl serine + H_4-folate⇌D-alanine + 5,10-methylene-H_4-folate
3. Allothreonine⇌glycine + acetaldehyde
4. L-Threonine⇌glycine + acetaldehyde
5. *erythro*-β-Phenylserine⇌glycine + benzaldehyde
6. *threo*-β-Phenylserine⇌glycine + benzaldehyde
7. ϵ-Trimethyl-3-hydoxylysine⇌glycine + γ-butyrobetaine aldehyde
8. D-Alanine + pyridoxal-P⇌pyruvate + pyridoxamine-P
9. Aminomalonate⇌glycine + CO_2
10. (2S) − ^{3}[H]glycine + ^1H_2O⇌(2S) − ^{1}[H]glycine + ^3H_2O

tially removes the pro-*S*-proton of the α-carbon from glycine and the α-proton of D-alanine that has an *S*-configuration (reactions 2 and 10, *Table 4*). The proposed enzyme mechanism has been described by Schirch (1982).

SHMT STRAIN CONSTRUCTION

SHMT is widely present in mammalian, yeast and bacterial systems. Although most of the studies on the enzyme reactions listed in *Table 4* involved mammalian enzyme, the construction of a biocatalyst source in a bacterial strain is needed in order to produce inexpensive SHMT for the synthesis of amino acids.

The gene *glyA* that encodes SHMT in *E. coli* has been cloned and sequenced (Stauffer, Plamann and Stauffer, 1981). Plasmid pGX139, carrying the *glyA* gene, was constructed by the following steps (Hamilton *et al.*, 1985). The *glyA* gene was first cloned into pBR322 on an *Eco*RI-*Sal*I restriction fragment, then subcloned as a *Sma*I-*Bcl*I fragment on to a high copy number plasmid derived from pK01 (McKenny *et al.*, 1980) to produce pGX139. The *glyA* gene in plasmid pGX139 expressed well in *E. coli*, especially when the host strain was grown to stationary phase. However, a simple cloning of the desired gene was not good enough to achieve the goal of obtaining an inexpensive biocatalyst. The host strain and the plasmid were further modified to meet the requirements described below.

Plasmid stabilization

Plasmids are often not stable, especially when the host cells are grown in a rich medium. This problem is normally solved by applying a selective pressure on the host cell to retain the plasmids. The most common way to apply selective pressure is to include an antibiotic resistance gene in the plasmid and then to grow the host cells in the presence of the same antibiotic. Only the cells retaining the plasmid are able to grow. This strategy works well for low-volume high-priced pharmaceutical proteins. However, it is not suitable for the production of industrial enzymes, due to environmental concerns and cost constraints. In the case of SHMT production, a selective pressure based on a nutritional requirement was used to stabilize the plasmid (Hamilton *et al.*, 1985). This was done by creating a *trp* mutation on the chromosome of the host strain. Then the *trp* operon was placed on plasmid pGX139 to create plasmid pGX2236. When the host strain containing pGX2236 was grown in a medium without tryptophan, only the cells which retained plasmid (and, therefore, the *glyA* gene), were able to grow. This method works well because tryptophan synthesis is highly regulated (Platt, 1980). Even in the presence of a multicopy tryptophan operon, overproduction and excretion of tryptophan, which could cause a problem of cross-feeding plasmid-less cells, was not observed. The same method has been used to construct several other production strains and has been shown to be very effective (Anderson, Young and Kalk, 1986).

Autolysis

Because SHMT is an intracellular enzyme, cell breakage and the partial purification of the enzyme became necessary. These steps might have been avoided had the reaction been run with an immobilized whole-cell biocatalyst. However, the utilization of whole-cell immobilization was unnecessary because of the configuration of the bioreactor that was developed (described below).

The use of mechanical means to break cells open on a large scale was not advantageous. Mechanical breakage not only deactivated 10–20% of the SHMT activity but also increased the solution viscosity by releasing nucleic acids. Thus, extra steps would have been necessary to purify the desired enzyme partially in order to avoid contaminating the amino acid product.

A pseudo-extracellular system was developed to solve the mechanical breakage problem just described. The idea was to create a gentle lysis of the strain so that the desired enzyme could leach into solution and leave behind the still intact cells. Subsequently, simple centrifugation could separate the enzyme from the cell debris. The enzyme thus obtained was relatively free from cellular contaminants.

Gentle lysis was achieved by cloning the endolysin genes from the *E. coli* phage lambda into the host strain (Anderson, 1987). The endolysin genes include the lambda *R* gene, which codes for lysozyme (EC 3.2.1.17), and the lambda *Rz* gene, which codes for endopeptidase (EC 3.4.21). These two gene products, in conjunction with the lambda *S* gene product, have been suggested to be responsible for the destruction of *E. coli* cell wall and membrane (Taylor, 1971; Bienkowsku-Szewezyk and Taylor, 1980; Garretty *et al.*, 1981; Crabtree and Cronan, 1984). It was also known that an organic solvent such as chloroform could replace the lambda *S* gene product to permeabilize membrane.

The *R* and *Rz* genes were obtained from phage lambda through an *Eco*RI-*Cla*I restriction fragment (Anderson, 1987). These two genes were cloned on to plasmid pGX2236, which already contained the *gly*A gene and the tryptophan operon, to yield plasmid pGX2302. Although no promoter was included in this two-gene fragment, the phenotype of easy cell lysis was observed in the host strain. This lysis system worked especially well with the host micro-organism *Klebsiella aerogenes* (see 'SHMT Fermentation' below).

Host strain

The ideal host strain should be able to grow to a high cell density without difficulty and it should not make enzymes which can degrade desired substrates or product. Otherwise, those degradation enzymes would have to be removed before the SHMT extract could be used for amino acid production. After several Gram-negative bacteria were examined, a strain of *Klebsiella aerogenes* was chosen as the host strain (Anderson *et al.*, 1984). This strain showed its

ability to grow to 30–40 g DCW/l in a minimal medium with soluble starch as a carbon source. A mutant (LV025) lacking L and D serine dehydratases (EC 4.2.1.13 and 4.2.1.14), which degrade serine to pyruvate, H_2O and ammonium ion, was readily available (Vining and Magasanik, 1981). The strain LV025 did not show any detectable activity for glycine degradation (e.g. no glycine synthase (EC 1.4.4.2) (Feldman and Gunsalus, 1950)). LV025 was further mutagenized to obtain a stable tryptophan synthase mutant for complementation by plasmid pGX2302. Plasmid pGX2302 was inserted into *Klebsiella aerogenes* to yield the final strain GX3036 as the production strain.

SHMT FERMENTATION

Production strain GX3036 was grown in a minimal salt medium with a continuous feed of soluble starch (Anderson *et al.*, 1984). *Klebsiella aerogenes* was known to produce pullulanase (EC 3.2.1.41) to degrade polysaccharides to glucose for growth. Curves for growth and SHMT production are shown in *Figure 2*. By controlling dissolved oxygen and glucose at low levels (glucose through a slow feed of starch), log-phase growth was prolonged, although at a reduced rate, and SHMT production was induced. SHMT production peaked at early stationary phase with some degree of stability. The induction of SHMT has been suggested to be linked to the end products of C-1 metabolites such as methionine (Dev and Harvey, 1984a, b). The cell concentration at the end of fermentation routinely reached 25 g DCW/l and about 27% of the total protein was SHMT (Anderson, 1987).

At the end of fermentation, about 10–15% of the SHMT activity was already in the broth. By the introduction of organic solvents such as chloroform or

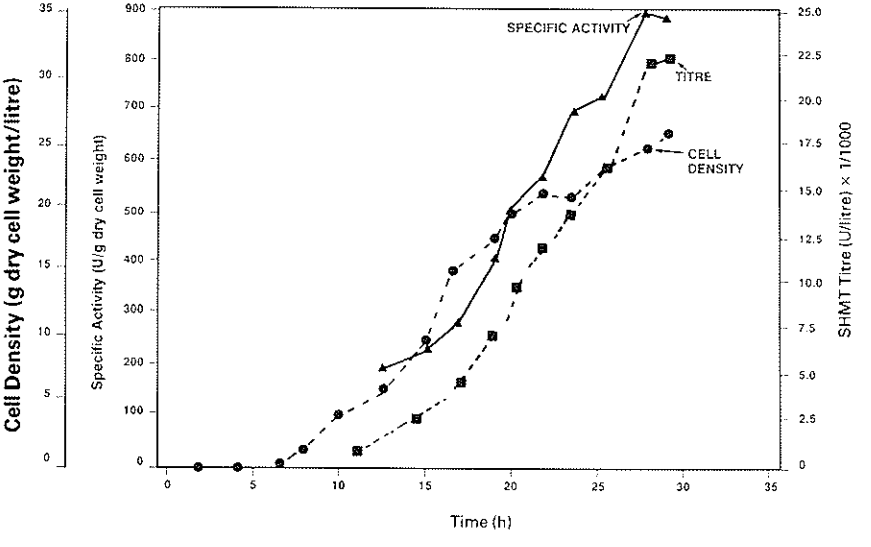


Figure 2. Production strain (*Klebsiella aerogenes* strain GX3036) growth and SHMT production in a 14 l fermentor (Anderson, 1987). 900 U/g dry cell weight; 13·5% of dry cell weight is SHMT (27% of total protein; 4 g SHMT/litre).

methylene chloride, or by leaving the cell mass in the fermentor overnight, almost all SHMT activity was released into the broth. SHMT was recovered by a simple centrifugation to remove cell debris. About 50% of the total protein in the broth was SHMT and, more importantly, contaminants such as cell wall and nucleic acids in the enzyme solution were only about 2% of the level of the solid matter recovered in the the crude extract when mechanical cell disruption and centrifugation were employed.

ENZYMATIC SYNTHESIS OF SERINE

The reaction scheme used for synthesis of serine is shown below (Hsiao, Wei and Campbell, 1986):

$$\text{HCHO} + \text{FH}_4 \xrightarrow{\text{non-enzymatic}} \text{m-FH}_4$$

$$\text{Glycine} + \text{m-FH}_4 \xrightarrow{\text{SHMT}} \text{Serine} + \text{FH}_4$$

$$\text{Glycine} + \text{HCHO} \xrightarrow{\text{SHMT}} \text{Serine}$$

The overall reaction is composed of two subsidiary reactions. The formation of N^5-N^{10}-methylene tetrahydrofolic acid (m-FH_4) is a non-enzymatic reaction that has been studied by Kallen and Jencks (1966). An equilibrium constant of $3{\cdot}2 \times 10^4 M^{-1}$ in favour of m-FH_4 formation has been reported (Blakley, 1960a). The pH optimum of this reaction is about 6 (Kallen and Jencks, 1966). Serine formation is catalysed by SHMT and the pH optimum of the reaction is between 8 and 8·5 (Hsiao, Wei and Campbell, 1986). The equilibrium constant of the overall reaction has been calculated to be about $3{\cdot}0 \times 10^3 M^{-1}$ in favour of serine formation (Alexander and Greenberg, 1956). The K_m for glycine is 11·6 mM at 37°C and pH 8. No substrate inhibition was detected and this allowed the use of a high initial concentration of glycine in the reaction for serine synthesis. The exogenous addition of two co-factors (pyridoxal-5-phosphate (PLP) and tetrahydrofolic acid (FH_4)) is required for maximum activity (Hsiao, Wei and Campbell, 1986).

FH_4 is not commercially available in large quantity but it can easily be prepared by reducing folate (Blakley, 1960b; Hamilton *et al.*, 1985), which is available on a large scale, with a quantitative yield. FH_4 is known to be very sensitive to oxygen (Kallen and Jencks, 1966). A strong reducing reagent such as β-mercaptoethanol has been used to stabilize FH_4 (Kallen and Jencks, 1966). In a reactor with constant agitation, a continuous monitoring system was developed to ensure FH_4 stability. This was done by monitoring the redox potential of the reaction solution with a redox probe and maintaining the redox potential below 340 mV by the addition of β-mercaptoethanol. FH_4 was stable under this condition for more than 24 h (Hsiao, Wei and Campbell, 1986).

SHMT in solution was quite stable and it was also active in the presence of 200 mM β-mercaptoethanol (Hsiao, Wei and Campbell, 1986). SHMT was quickly inactivated in the presence of a few millimolar formaldehyde. Glycine or serine provided some protection for SHMT, but still could not prevent the

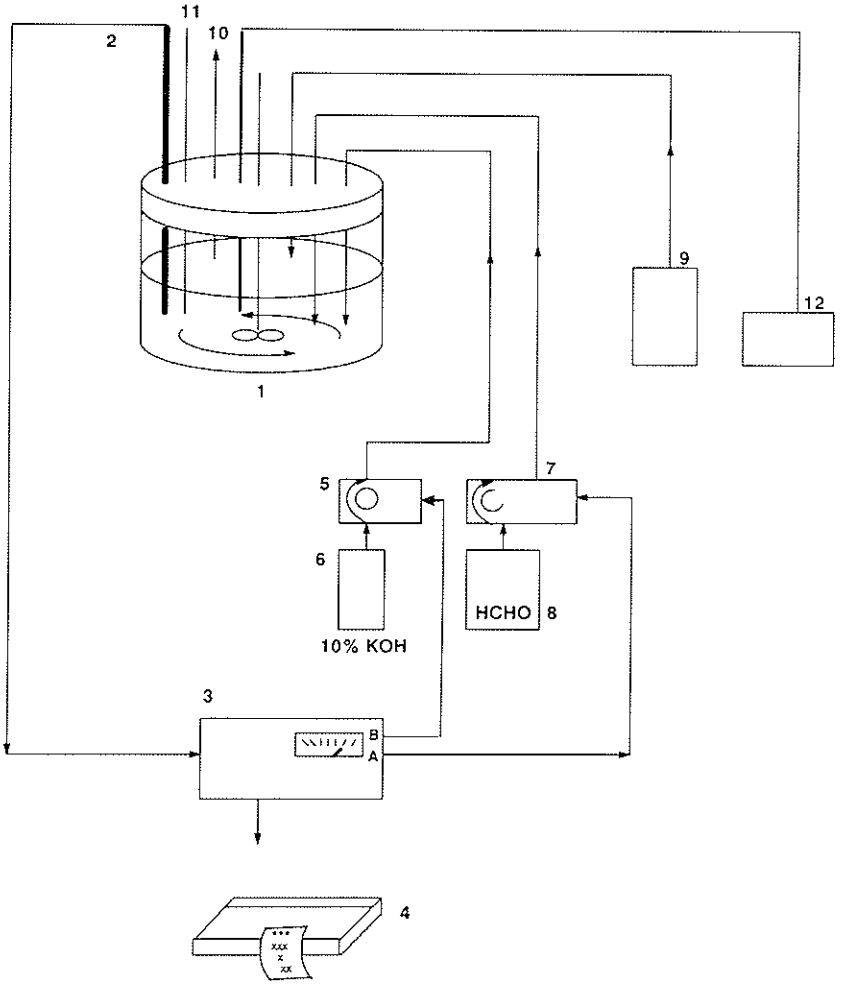


Figure 3. Bioreactor configuration used for enzymatic synthesis of serine with SHMT: 1. Bioreactor; 2. pH electrode; 3. pH controller; 4. Recorder; 5. P-1 pump; 6. 10% KOH; 7. P-1 pump; 8. Formaldehyde solution (37%); 9. N_2 tank; 10. N_2 outlet; 11. Sampling tube; 12. Temperature control. From Hsiao and Wei, 1986.

eventual inactivation of the enzyme. When whole cells were used as biocatalyst, only a small fraction of the SHMT activity was observed, presumably due to the size of m-FH_4, which might have imposed a diffusional limitation on the whole-cell biocatalyst.

Considering all the facts described above, especially the high toxicity of formaldehyde toward SHMT and the need to reuse FH_4 at high efficiency, the most advantageous configuration of the serine bioreactor was taken to be a stirred tank with a continuous feed of formaldehyde and SHMT used in soluble form. The formaldehyde had to be fed slowly to avoid any build-up of free formaldehyde in the reaction mixture. However, the rate of formaldehyde feed had to be high enough to support high serine productivity. The ideal

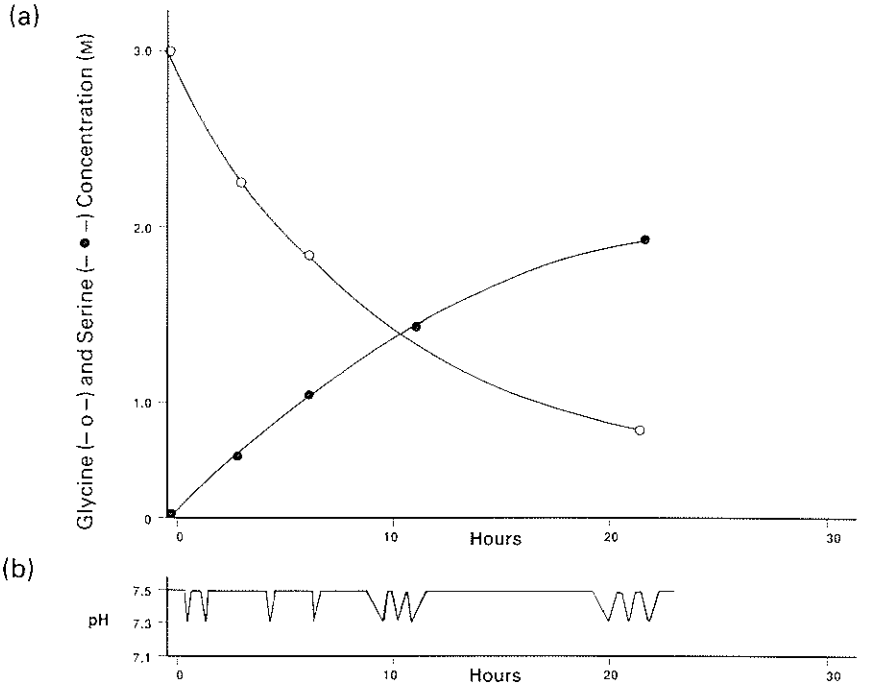


Figure 4. SHMT bioreactor results for enzymatic synthesis of serine (– • –) from glycine (– o –) and formaldehyde. The change in amino acid composition with time is shown in (a), and the pH profile is shown in (b). From Hsiao and Wei, 1986.

formaldehyde feed rate was equal to the rate of serine synthesis. The actual regulation of formaldehyde addition was complicated by the fact that product inhibition and/or reverse reaction became significant as serine accumulated (Hsiao, Wei, and Campbell, 1986).

An automatic feedback control system was developed for formaldehyde delivery utilizing the phenomenon of formal titration (Hsiao and Wei, 1986). The bioreactor configuration used is shown in *Figure 3*, and an example of results is shown in *Figure 4*. A 3 M glycine solution was prepared, and 100 ml of it was charged to the bioreactor. A pH electrode was connected to a pH controller that had a base (10% KOH) pump at the base position and a formaldehyde (37% solution) pump at the acid position. The reaction pH was adjusted to 7·5 and the temperature was maintained at 37°C. The set points on the pH controller for both the base pump and formaldehyde pump were the same as the reaction pH. A suitable amount of SHMT was added and a stream of nitrogen gas was introduced over the surface of the reaction mixture. After the addition of β-mercaptoethanol (30 mM final concentration), FH_4 was dissolved into the reaction mixture to give a final concentration of 5 mM. Formaldehyde solution was pumped into the reactor at a rate of 17 ml/l/h. The pH was followed and recorded. After an initial pH adjustment with base due to the presence of β-mercaptoethanol, the base pump was taken off the controller. After the pH had been stable for 3–4 h (during which period the rate

of serine synthesis was the same as the rate of formaldehyde addition), the pH dropped to 7·42 and the pH controller automatically turned the formaldehyde pump off. Once the continuous supply of formaldehyde was cut off, the pH rose to 7·48 and reactivated the formaldehyde pump. At this time, the rate of formaldehyde addition was reduced to 12 ml/l/h. The observed rise in pH was attributed to the reversion of Schiff base formed between formaldehyde and amino acids. In other words, the pH drop was a kinetically controlled process because serine synthesis was not fast enough to consume the incoming formaldehyde. After the formaldehyde pump was turned off, the residual formaldehyde in the reactor was redistributed between Schiff base formation and serine synthesis. Since the equilibrium constant of serine synthesis is much higher than that of Schiff base formation (Kallen and Jencks, 1966), formaldehyde in the form of Schiff base was converted back into serine, with an accompanying pH rise. The cycle of pH drop followed by reduction of formaldehyde feed rate continued until the reaction was close to equilibrium.

This method of feedback control of formaldehyde addition was also used to optimize reaction conditions. As described in an earlier section, serine synthesis is composed of two reactions which have different pH optima. Since SHMT activity is less sensitive to pH than the non-enzymatic rate for formation of m-FH_4 is, and because it is difficult to recycle FH_4, reaction pH was chosen to be 6·5, in favour of m-FH_4 formation. A minimal PLP concentration, which was required to maintain SHMT activity in the bioreactor, was determined to be 0·5 mM. FH_4 at a concentration of 0·5 mM could easily handle formaldehyde addition at a rate of 17 ml/l/h. When the serine bioreactor was run at pH 6·5, 37°C, in the presence of 0·5 mM PLP and 1 mM FH_4, 90% of 3M glycine was converted to serine at a near quantitative yield. The average productivity was about 10 g/l/h. About 80% of SHMT activity was recovered after reaction. The reactor was also successfully run at a glycine concentration of 450 g/l. A final serine concentration of 452 g/l with 88% yield was obtained (Hsaio and Wei, 1986).

ENZYMATIC SYNTHESIS OF β-PHENYLSERINE

β-Phenylserine is an unnatural amino acid that contains two chiral centres, and thus has four isomeric forms. Due to these chiral centres, it is very difficult chemically to synthesize the optically pure form of any of these isomers. However, the variety of isomers makes β-phenylserine an interesting exploratory raw material for use in the synthesis of new specialty chemicals, such as pharmaceutical intermediates, pesticides, and artificial sweeteners. Phenylserine cannot be produced by fermentation, so enzymatic synthesis is the only practical choice. In particular, the SHMT-catalysed conversion of glycine and benzaldehyde to form β-phenylserine has been investigated by several workers (Nakazawa *et al.*, 1975; Ulevitch and Kallen, 1977; Walter and Bull, 1987).

Although SHMT selectivity produces only the 'L' form of β-phenylserine, the enzyme forms a mixture of the L-*erythro* (E) and L-*threo* (T) isomers. At equilibrium, the bulk of β-phenylserine is in the *threo* form (E/T ratio ~2·5/1;

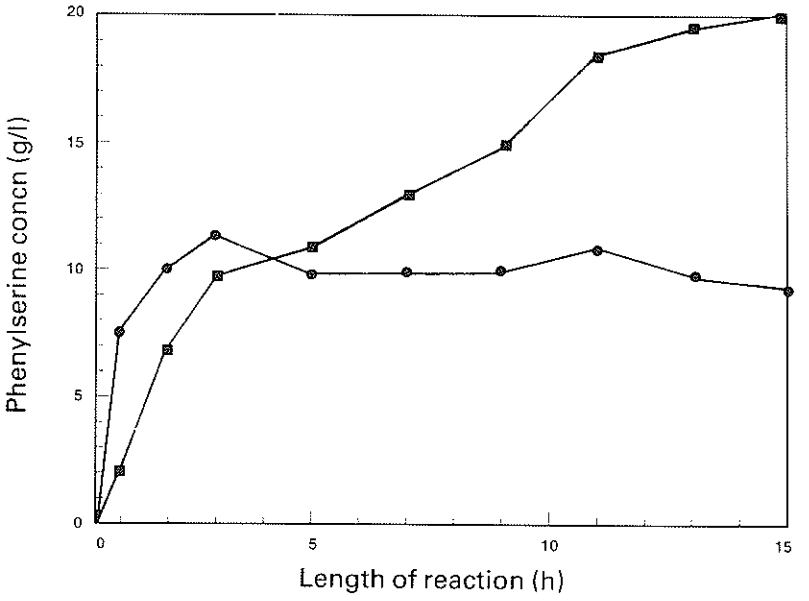


Figure 5. Fed-batch production of β-phenylserine from glycine and benzaldehyde with SHMT. Conditions: 35°C, pH 7, 100 g/l glycine, 5000 units/l SHMT (Walter and Bull, 1987). *threo* (—■—); *erythro* (—●—).

Walter and Bull, 1987). However, the initial reaction rate favours formation of the *erythro* isomer (*Figure 5*). These results suggest that in order to achieve high yields of the L-*threo* isomer the reaction should be run as close to equilibrium as possible. However, production of the L-*erythro* isomer at high yield and concentration is much more difficult. A fed batch or continuous reactor would be limited to very low L-*erythro* phenylserine concentrations and yields in order to obtain high E/T ratios.

To overcome these difficulties, an extractor-bioreactor was designed to remove products as they are produced (Walter and Bull, 1987). In this bioreactor configuration (*Figure 6*), purified SHMT was added to an agitated vessel containing 200 g/l glycine and saturated with benzaldehyde. The product from this reaction was continuously removed by an ultrafiltration membrane that retained enzyme. The aqueous permeate, containing both products and reactants, was then contacted with an immiscible organic mixture of benzaldehyde and butanol that selectively removed the phenylserines. The aqueous retentate, saturated with benzaldehyde and still containing glycine, was continuously recycled to the bioreactor. The organic stream, now containing phenylserine, was passed to a second extractor where it was contacted with an acidic aqueous stream to re-extract the phenylserines. The organic stream was then recycled to the first extractor. By properly sizing the extractors and the bioreactor, this system permitted the continuous production of phenylserine with a high ratio of L-*erythro* phenylserine at a high yield. Furthermore, the product was produced at a high concentration and was free of enzyme, co-factors, and glycine. It was only contaminated by benzaldehyde.

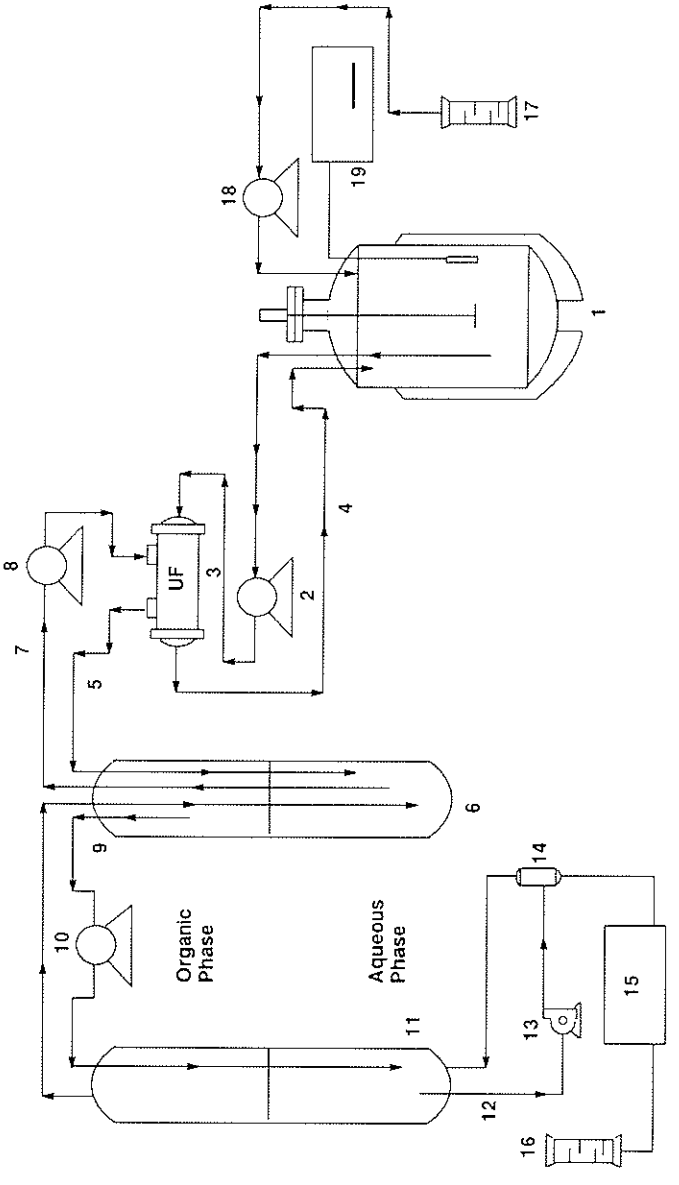


Figure 6. Extractor reactor for production of high ratio of L-*erythro*-β-phenylserine compared with L-*threo*-β-phenylserine (Walter and Bull, 1987). 1. Bioreactor; 2. Pump; 3. UF unit; 4. Aqueous retentate (with SHMT) recycle to bioreactor; 5. Permeate feed to Extractor 1; 6. Extractor 1; 7. Recirculation loop; 8. Pump; 9. Organic feed from Extractor 1 to Extractor 2; 10. Pump; 11. Extractor 2; 12. Acidic aqueous extract rich in phenylserine; 13. Pump; 14. pH adjustment vessel; 15. pH controller; 16. Acidic titrant; 17. Benzaldehyde feed; 18. Pump; 19. pH controller.

Using a 1 litre reactor, an L-*erythro* β-phenylserine concentration of 25 g/l with an E/T ratio of 11/1 and a volumetric productivity of about 1 g/l/h was achieved. This process illustrates how combining enzymatic synthesis and continuous separation processes can produce unique chemicals.

ENZYMATIC SYNTHESIS OF TRYOSINE

Tyrosine phenol lyase (TPL; EC 4.1.99.12) or β-tyrosinase, which catalyses the degradation of tyrosine to phenol, ammonium ion and pyruvate, is widely present in bacteria, especially *Erwinia herbicola* and *Citrobacter freundii*. The synthesis of the enzyme was induced when tyrosine was included in the growth medium (Kumagai, Matsui and Yamada, 1970). Under proper growth conditions, *Erwinia herbicola* or *Citrobacter freundii* can produce TPL at up to 1–2% of their dry cell weight without any genetic manipulation. TPL, like several other pyridoxal-5-phosphate-containing amino-acid-degrading enzymes (including tryptophanase (EC 4.1.99.1) and cysteine desulfhydrase (EC 4.4.1.1),), catalyses the α, β-elimination reaction with wide substrate specificity (Yamada and Kumagai, 1975). It also degrades serine to pyruvate, ammonium ion and water.

Enei *et al.* (1971) first demonstrated the reversibility of the TPL reaction. By increasing ammonium ion concentration in a reaction mixture with TPL present, tyrosine could be produced from phenol, ammonium ion, and pyruvate. The synthesis of tyrosine was possible because of the very low solubility of tyrosine in aqueous solution. As tyrosine was synthesized, it quickly reached it solubility limit and precipitated out of solution. Consequently, the reaction was driven to near completion by this thermodynamically-controlled process. Enei *et al.* (1974) also showed that serine and phenol could be converted to tyrosine by TPL. L-DOPA was synthesized when catechol instead of phenol was used.

Since TPL has been shown to be able to convert serine and phenol to tyrosine, Lee and Hsiao (1986) combined an SHMT reaction with a TPL reaction to produce tyrosine from glycine, formaldehyde and phenol as shown in the following scheme:

$$\begin{aligned}
&\text{HCHO} + \text{FH}_4 \xrightarrow{\text{non-enzymatic}} \text{m-FH}_4 \\
&+ \text{m-FH}_4 + \text{Glycine} \xrightarrow{\text{SHMT}} \text{Serine} \\
&\text{Glycine} + \text{HCHO} \xrightarrow{\text{SHMT}} \text{Serine} \\
&+ \text{Serine} + \text{Phenol} \xrightarrow{\text{TPL}} \text{Tyrosine} + \text{H}_2\text{O} \\
&\text{Glycine} + \text{HCHO} + \text{Phenol} \xrightarrow[\text{TPL}]{\text{SHMT}} \text{Tyrosine} + \text{H}_2\text{O}
\end{aligned}$$

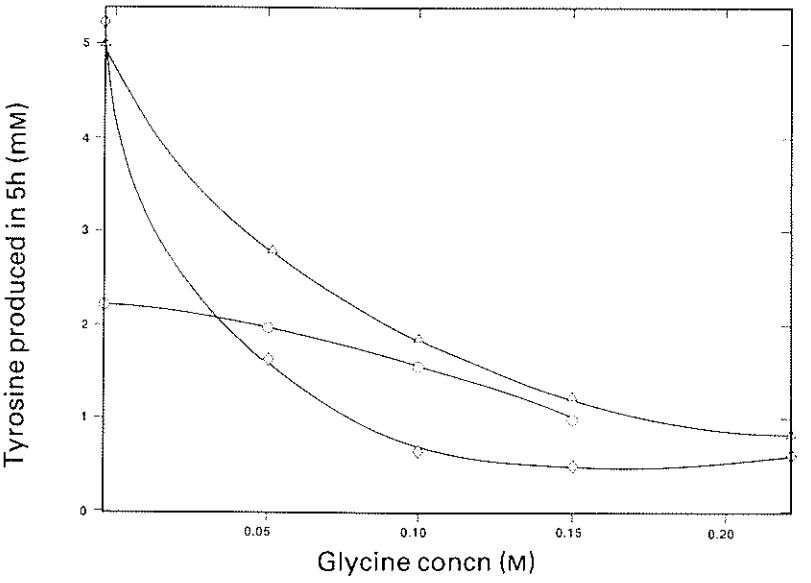


Figure 7. pH dependence of tyrosine synthesis in the presence of glycine. The reaction mixtures contained 0·5 M serine, 0·48% phenol, 0·5 mM PLP, 1 mM β-mercaptoethanol, 8 OD_{600nm} of *Erwinia herbicola* cell extract and glycine at concentrations ranging from 0 to 0·2 M. The volume was made up to 5 ml with 0·1 M potassium phosphate buffer and the pHs were adjusted to 6·5 (o-o), 7·0 (△-△) and 8·0 (◊-◊) for each series of reactions. At timed intervals, samples were withdrawn and the amount of tyrosine produced was quantitated by HPLC (Lee and Hsiao, 1986).

For this combined reaction to work, it was necessary to ensure that conditions for the two enzymatic reactions were compatible and that possible side reactions involving the toxic substrates (phenol and formaldehyde) were insignificant compared with the desired reactions.

As noted in a previous section, the successful synthesis of serine was heavily dependent on the regulation of the formaldehyde addition rate, and so the formaldehyde addition system already described was tested in the presence of phenol and tyrosine. It was demonstrated that phenol and tyrosine did not interfere with the reaction for serine synthesis. However, phenol and formaldehyde were quite reactive toward SHMT and TPL. SHMT was stable in the presence of 25 mM phenol but lost 35% and 65% of its original activity after prolonged incubation at 50 mM and 75 mM phenol, respectively. TPL was also sensitive to phenol.

As described earlier, the optimal pH for the SHMT serine synthesis is between 6·5 and 7·0. The optimal pH for the reaction catalysed by TPL is about 8·0 (Yamada and Kumagai, 1975). Glycine does not inhibit SHMT but it is a strong inhibitor of TPL (Lee and Hsiao, 1986). The problem of glycine inhibition was partially alleviated by manipulating the pH of the bioreaction. As shown in *Figure 7*, the optimal pH of TPL in the presence of glycine was changed to 7·0 due to a lesser degree of inhibition at this pH. Even at pH 7·0, the enzyme activity was still sensitive to glycine concentration.

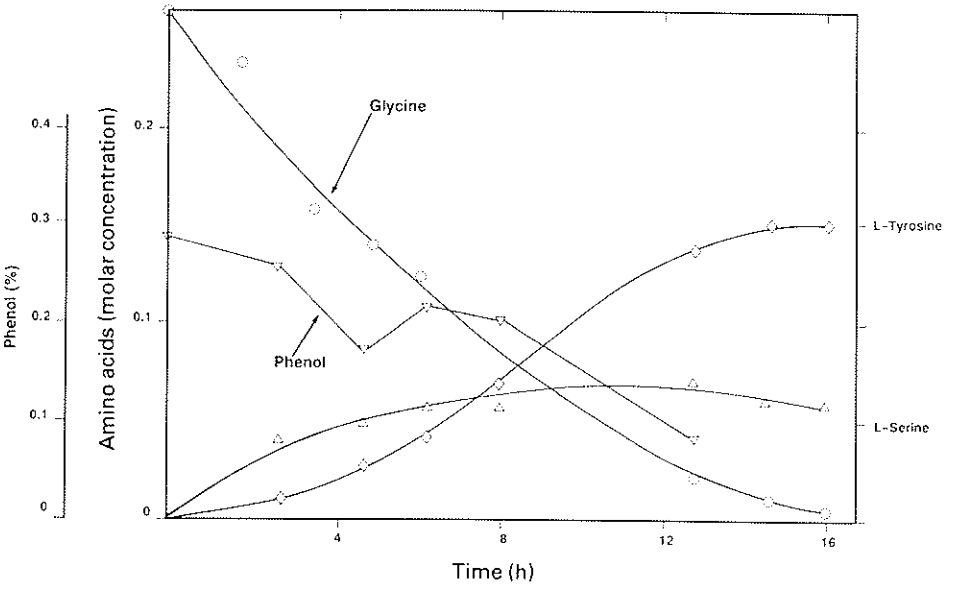


Figure 8. Time course of L-tyrosine synthesis from glycine, formaldehyde and phenol by the coupled reaction of SHMT and TPL. A reaction mixture containing 0·25 M glycine, 0·5 mM PLP, 130 ml processed *Klebsiella aerogenes* and cells of *Erwinia herbicola* from 2 litres of culture broth was made up to 500 ml with distilled water. The system was sealed, stirred and purged with nitrogen. After the pH was adjusted to 7·0, 2 ml β-mercaptoethanol was added followed by 20 ml 43·8 mM tetrahydrofolate. Phenol concentration was made up to 0·32%. The reaction was initiated by the infusion of 37% formaldehyde. The pH was maintained at 7·0 and the formaldehyde feeding rate was adjusted according to the fluctuation of pH. The rate ranged from 1·0 ml/h in the first 5 h to 0·4 ml/h in the later part of the reaction. Phenol was supplied to the reactor in 80% aqueous solution at an initial rate of 0·1 g/h. The rate was increased to 0·325 g/h at t=3 h and further to 0·64 g/h at t=6h. The quantitation of glycine (o-o), serine (△-△), tyrosine (◊-◊) and phenol (▽-▽) has been described by Lee and Hsiao (1986).

A stirred tank with a continuous feed of formaldehyde and phenol was used to produce tyrosine. The pH was maintained at 7·0 and temperature at 37°C. The initial glycine concentration was 0·25 M and formaldehyde was first fed into the bioreactor in the presence of SHMT, TPL and phenol. In the first 7 hours of reaction, the amount of serine and tyrosine accumulation did not account for the amount of glycine disappearance (*Figure 8*). During this period, pyruvate was detected (Lee and Hsiao, 1986). The result suggested that serine synthesized by SHMT was quickly degraded to pyruvate, ammonium and water by TPL. However, the production of pyruvate shifted the equilibrium of the SHMT reaction to near completion. Tyrosine synthesis was not significant in the first 7 h due to the strong glycine inhibition of TPL. At the end of reaction, 26·3 g/l tyrosine was produced at a 61·4% yield based on the initial glycine concentration used.

Case study 2—enzymatic routes to phenylalanine

L-phenylalanine is an essential amino acid that is used in parenteral and enteral therapy, but by far its largest use is in the synthesis of the dipeptide sweetener aspartame. The demand for L-phenylalanine has grown substantially in the 1980s and is expected to continue to grow (*Table 5*). Most L-phenylalanine is currently produced by fermentation, but recently several enzymatic routes to L-phenylalanine have been developed (*Table 6*).

Table 5. Projected demand for L-phenylalanine to make aspartame (adapted from Klausner, 1985)

Year	Metric Tons
1981	50
1982	250
1983	1250
1984	2000
1985	3125
1986	4400
1987	5700
1988	6800
1989	7500
1990	7900

Table 6. L-phenylalanine production by enzymatic synthesis

Transamination
Phenylalanine dehydrogenase/formate dehydrogenase
Phenylalanine dehydrogenase/hydroxyisocaproate dehydrogenase
Phenylalanine dehydrogenase/high pressure hydrogen
α-Acetamidocinnamic acylase/phenylalanine dehydrogenase/lactate dehydrogenase
Phenylalanine ammonia lyase
Hydantoinase

L-PHENYLALANINE PRODUCTION BY TRANSAMINATION

The production of L-phenylalanine by transamination was first explored about 30 years ago (Sakurai, 1956; Asai, Aida and Oishi, 1959). It was found that whole cells from a number of bacteria could catalyse the conversion of phenylpyruvic acid to L-phenylalanine if an amino acid such as aspartic acid or glutamic acid was used as the amine donor. Unfortunately, the equilibrium of this reaction is about unity so an equimolar mix of phenylpyruvic acid and amino acid will result in only about a 50% yield of L-phenylalanine. However, Rozzell (1985, 1987) found that if aspartic acid is used as the amino donor its keto acid analogue, oxaloacetic acid, can be decomposed to pyruvic acid and carbon dioxide by the enzyme oxaloacetic acid decarboxylase (EC 4.1.1.3). Thus, by coupling the transamination to oxaloacetate decarboxylation the transamination can be driven to achieve high yields of L-phenylalanine (*Table 7*). Alternatively, the decarboxylation of oxaloacetate can be catalysed by several metal salts (Krebs, 1942; Rozzell, 1987). The overall reaction sequence is presented in *Figure 9*.

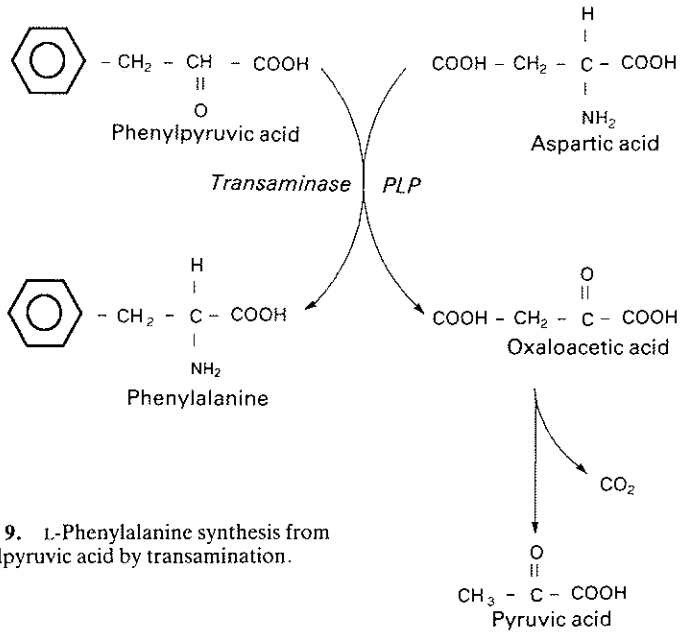


Figure 9. L-Phenylalanine synthesis from phenylpyruvic acid by transamination.

Bulot and Cooney (1985) used growing cells of a mutant strain of *Corynebacterium glutamicum* to transform phenylpyruvate to L-phenylalanine at a titre of 7·5 g/l with a yield of 75%. Ziehr *et al.* (1987) used aspartate amino transferase (EC 2.6.1.1) purified from *Pseudomonas putida* in an enzyme membrane reactor for enzymatic synthesis of L-phenylalanine. This research achieved a maximum yield of 63% and a productivity of 1·7 g/l/h. Calton *et al.* (1986) used *E. coli* whole cells high in aspartate amino transferase activity immobilized by polyazetidine on IRA938 in a column reactor. These researchers reported an excellent projected half-life of over 8 months with a yield of over 90% and a productivity of 1·65 g/l/h. Evans *et al.* (1987a) used *Pseudomonas fluorescens* immobilized in calcium alginate beads to catalyse the transamination of phenylpyruvate with glutamic acid or aspartic acid as the amino donor. They found that aspartic acid was the preferred donor, and that the biocatalyst half-life was well over 60 days with an initial productivity of about 3 g/l/h. Walter (1986) used a genetically engineered *E. coli* strain that expressed high levels of aspartate amino transferase immobilized in gelatine beads in columns. This system achieved yields of over 90% with a productivity of over 6 g/l/h and a half-life of 65 days. These reports demonstrate that technology enabling commercial production of L-phenylalanine by transamination has been developed.

L-PHENYLALANINE PRODUCTION BY PHENYLALANINE DEHYDROGENASE AND FORMATE DEHYDROGENASE

Another synthetic route to L-phenylalanine that uses the precursor phenylpyruvic acid employs phenylalanine dehydrogenase and formate

Table 7. Bioreactor results for L-phenylalanine synthesis with transaminase

Biocatalyst	Phe concn (g/l)	Yield (%)	Productivity (g/l/h)	t-1/2 (days)	Reference	Organization
Free enzymes	31	90	1·7	—	Rozzell (1985)	GI
Free whole cells	7·5	75	0·15	—	Bulot and Cooney (1985)	MIT
Immobilized cells (Chitosan)	10	63	1·7	—	Ziehr *et al.* (1987)	German Research Institutes
Immobilized cells (Polyazetidin)	30	90	1·65	>100	Calton *et al.* (1986)	PEI
Immobilized cells (Alginate)	15	63	3·0	40	Evans *et al.* (1987a)	Allelix
Immobilized cells (Gelatin entrapped)	30	90	6·0	65	Walter (1986)	W.R. Grace

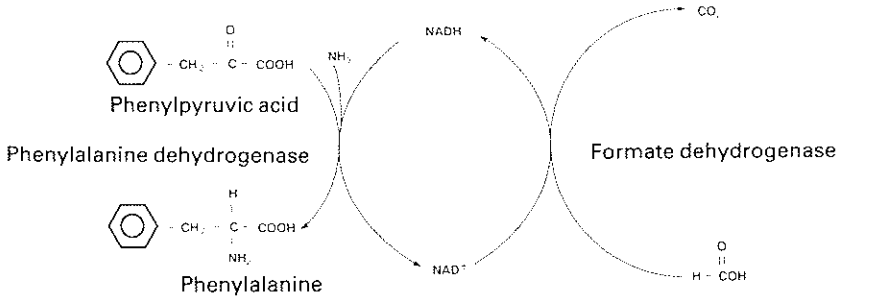


Figure 10. L-Phenylalanine synthesis by phenylalanine dehydrogenase with NADH regeneration by formate dehydrogenase.

dehydrogenase (EC 1.2.1.2). In this route, phenylalanine dehydrogenase catalyses the reaction of ammonia and phenylpyruvate to form phenylalanine and thus avoids the need for expensive amino acids as amino donors. However, phenylalanine dehydrogenase requires the cofactor NADH to complete this reaction. As NADH is expensive, it must be regenerated and recycled to make this process economical. Formate dehydrogenase is used to regenerate the NAD^+ to NADH through conversion of formate (*Figure 10*). However, efficient recycling of the NADH is not easy. Hummel *et al.* (1986) solved this problem by coupling NADH to PEG2000 and using an enzymatic membrane reactor to retain both of the enzymes and the cofactor. Using this system, these researchers obtained a yield of 93% and a volumetric productivity of 2 g/l/h. However, the reactor had a half-life of only about 10 days and attained a concentration of only 3 g/l of L-phenylalanine.

L-PHENYLALANINE PRODUCTION BY D, L-HYDROXYISOCAPROATE DEHYDROGENASE AND PHENYLALANINE DEHYDROGENASE

Another process to produce L-phenylalanine using an NAD^+ recycle has been developed with D,L-phenyllactate as the precursor. In this process, racemic D,L-phenyllactate is converted to the phenylpyruvic acid by two enzymes, D-2-hydroxyisocaproate dehydrogenase and L-2-hydroxyisocaproate dehydrogenase (*Figure 11*). This reaction requires the cofactor NAD^+, which must be recycled and regenerated. The phenylpyruvate and added ammonia is then converted to L-phenylalanine by phenylalanine dehydrogenase, simultaneously regenerating the NADH to NAD^+. Schmidt, Vasic'-Racki and Wandrey (1987) used NAD^+ attached to PEG2000 in a membrane reactor with the three purified enzymes and obtained a conversion of 65%, a productivity of 1·2 g/l/h and a half-life of over 30 days. This process offers both advantages and disadvantages in comparison to the two previous phenylpyruvate systems: the system uses a racemic aromatic substrate that is easy to synthesize chemically, and ammonia as the amine donor, but it requires three enzymes and a modified cofactor.

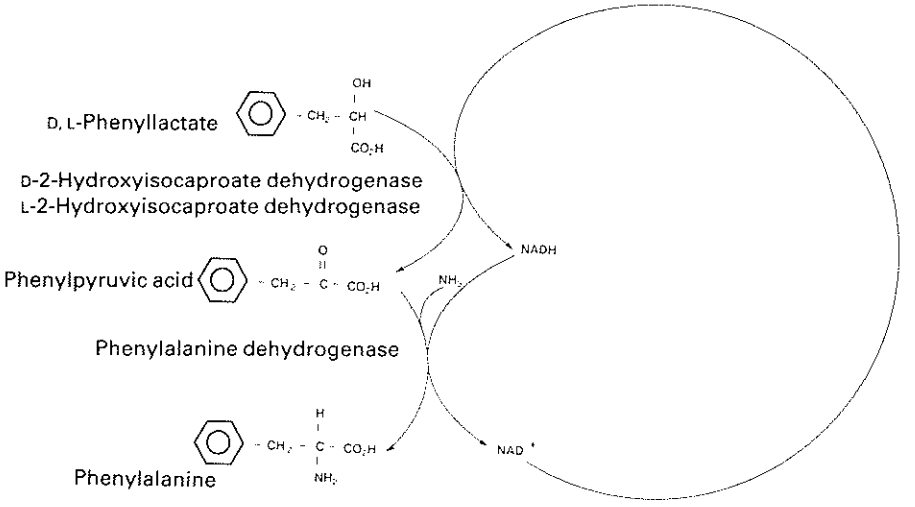


Figure 11. L-phenylalanine synthesis from D,L-phenyllactate by D- and L-2-hydroxyisocaproate dehydrogenases and phenylalanine dehydrogenase.

L-PHENYLALANINE PRODUCTION BY PHENYLALANINE DEHYDROGENASE UNDER HIGH HYDROGEN PRESSURE

Another variation on the use of phenylalanine dehydrogenase to produce L-phenylalanine was developed by Matsunaga *et al.* (1987), who used *Micrococcus luteus* cells immobilized in alginate and high hydrogen pressure to produce phenylalanine. In this system, the cofactor NAD^+ was partly regenerated by the high hydrogen pressure. However, the authors found it necessary also to include a catalyst containing alanine dehydrogenase (EC 1.4.1.1) and the substrates alanine and pyruvate to achieve high rates and yields. The authors reported a yield of more than 80% on phenylpyruvate when using the dual enzyme system. This dual enzyme system is very similar to one utilizing formate dehydrogenase to regenerate NAD^+ but requires the addition of the expensive substrate alanine and pyruvate. However, if hydrogen pressure alone could be used to regenerate NAD^+ in an industrial material cost. At the same time, the capital cost for handling a high pressure hydrogen system could be quite expensive.

PRODUCTION OF L-PHENYLALANINE BY α-ACETAMIDOCINNAMIC ACID ACYLASE, PHENYLALANINE DEHYDROGENASE AND LACTATE DEHYDROGENASE

Recently, Evans *et al* (1987g) have developed a whole-cell enzymatic system that used three enzymes, three substrates and a cofactor to produce phenylalanine. These researchers isolated a strain of *Corynebacterium equi*

that can convert the substrate α-acetamidocinnamate to L-phenylalanine through a multi-step process. First, acetamidocinnamic acid is deacylated to α-aminocinnamate which spontaneously deaminates to form phenylpyruvate, which is converted to L-phenylalanine by phenylalanine dehydrogenase by using ammonia and NADH. In order to regenerate the cofactor, lactate is also fed to the cells and is converted to pyruvate by lactate dehydrogenase.

The authors reported that this system can lead to titres of over 30 g/l L-phenylalanine with yields of over 95%. When the cells were immobilized in calcium alginate and utilized in a stirred tank reactor, the authors reported a volumetric productivity of about 1·5 g/l/h and useful lives of over 10 days. However, in light of the large number of substrates and enzymes involved, commercial practice of this process would be difficult.

L-PHENYLALANINE PRODUCTION BY PHENYLALANINE AMMONIA-LYASE

The enzymatic synthesis of L-phenylalanine by phenylalanine ammonia lyase (PAL: EC 4.3.1.5) was demonstrated about 20 years ago (Havir and Hanson, 1968). Under physiological conditions (e.g. low ammonia concentration), the equilibrium of the PAL reaction does not favour L-phenylalanine synthesis, but if the ammonia concentration is greatly increased, *trans*-cinnamic acid can be converted in high yield to L-phenylalanine (*Figure 12*).

Several microbial sources of PAL and various bioreactor configurations have been reported in the literature (*Table 8*). Nelson (1976) used a batch reactor with immobilized whole cells of *Rhodotorula gracilis*. A 90% yield on *trans*-cinnamic acid was reported. The immobilized whole cells retained 77% of their original PAL activity after an 18 h batch reaction. Yamada *et al.* (1981) employed *R. glutinis* as the source of PAL. They used a free cell batch reactor and achieved a yield of 70% on *trans*-cinnamic acid with a titre of 18 g L-phenylalanine/litre, a productivity of 1·7 g/l/h and a cell half-life of about 40 h. Hamilton *et al.* (1985) used a genetically manipulated strain of *R. rubra* immobilized on vermiculite carrier to produce phenylalanine at about 60 g/l with a yield of about 90%. Anderson (1987) reported that this *R. rubra* process was run commercially in a fed-batch reactor mode. Evans *et al* (1987b) used immobilized whole cells of *R. rubra* in a recirculating column reactor, and concentrations of L-phenylalanine up to 50 g/l were observed representing a total yield of over 83%. Onishi *et al.* (1987) used *Endomyces lindneri* in a batch free cell reactor and achieved a yield of 71% with a productivity of 0·69 g/l/h. In addition, a number of papers and patents have been published on optimizing PAL processes (*Table 9*).

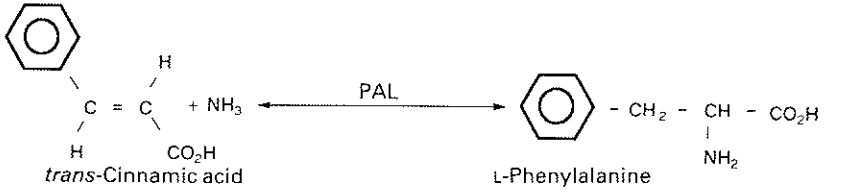


Figure 12. L-Phenylalanine synthesis from *trans*-cinnamic acid and ammonia by phenylalanine ammonia-lyase (PAL).

Table 8. Bioreactor results for L-phenylalanine synthesis with phenylalanine ammonia lyase

Reference	Organization	Bioreactor configuration	Micro-organism	Maximum L-Phe conc. (g/l)	Yield (%)	Productivity (g/l/h)	Biocatalyst half-life (h)
Nelson (1976)	Pfizer	Immobilized whole cells/ batch	*Rhodotorula gracilis*	52	90	2·9	about 40
Yamada *et al.* (1981)	Tanabe	Free cells/batch	*Rhodotorula glutinis*	18	70	1·7	about 40
Hamilton *et al.* (1985)	Genex	Immobilized whole cells/ continuous column	*Rhodotorula rubra*	59	90	not reported	not reported
Evans *et al.* (1987b)	Allelix	Immobilized whole cells/ recirculating column	*Rhodotorula rubra*	50	83	0·3	120 and longer under appropriate conditions
Onishi *et al.* (1987)	Ajinomoto	Free cells/batch	*Endomyces lindneri*	32	71	0·7	not reported

Table 9. Optimization of PAL processes

Feature		References	Firm
(1)	Immobilized whole cells	Nelson (1976)	Pfizer
		Swann (1984b, 1985a, b)	Genex
		Evans *et al.* (1987b)	Allelix
(2)	PAL stabilization during fermentation by amino acid addition	Yamada *et al.* (1981)	Tanabe
		Nakamichi *et al.* (1983)	Tanabe
(3)	Halide-free bioreactor feeds	Swann (1984a)	Genex
		Evans *et al.* (1987c, d)	Allelix
(4)	Bioreactor temperature optimization	Swann (1985a)	Genex
		Evans *et al.* (1987d)	Allelix
(5)	Use of PAL stabilizers in bioreactor	Kishore (1985)	Monsanto
		Evans *et al.* (1987c, e)	Allelix
(6)	Avoidance of substrate inhibition in bioreactor	Vollmer and Schruben (1986)	Genex
		Evans *et al.* (1987d)	Allelix
(7)	Oxygen exclusion at end of PAL fermentation	Finkelman and Yang (1986)	Genex
(8)	Use of reducing agents in bioreactor	Vollmer and Schruben (1986)	Genex
(9)	Oxygen exclusion from bioreactor	Vollmer *et al.* (1986)	Genex
		Evans *et al.* (1987c)	Allelix
(10)	High-activity PAL production strain-	McGuire (1986)	Genex
		Evans *et al.* (1987f)	Allelix

L-PHENYLALANINE PRODUCTION BY HYDANTOINASE

Another approach to enzymatically producing L-phenylalanine from a racemic substrate uses the enzyme L-phenylalanine hydantoin hydrolase. Yokozcki *et al.* (1976) showed that cells of *Flavobacterium aminogenes* can catalyse the conversion of D,L-phenylalanine hydantoin to L-phenylalanine. This process involves two steps: the enzymatic conversion of D,L-phenylalanine hydantoin to L-phenylalanine and the racemization of D-phenylalanine hydantoin to L-phenylalanine hydantoin (*Figure 13*). Although the first reaction is exclusively enzymatically catalysed, the racemization is at least partially spontaneous. In a batch free cell reactor, Yokozcki *et al.* (1976) achieved a 100% yield with a 20 g/l/h productivity, but the reactor had a half-life of only 12 days. This process offers the advantage of requiring only one substrate, and it generates by-products that are dissimilar to phenylalanine, thereby simplifying recovery.

Case study 3—enzymatic routes to cysteine

There are at least three enzyme systems for the synthesis of cysteine. Because of the easy oxidation of the free sulphydryl group in cysteine, the product of these enzyme reactions is either a mixture of cysteine and cystine or a cysteine derivative modified at the free sulphydryl group.

MICROBIAL TRANSFORMATION OF D,L-2-AMINO-Δ^2-THIAZOLINE-4-CARBOXYLATE TO CYSTEINE

Sano *et al.* (1977) first demonstrated that D,L-2-amino-Δ^2-thiazoline-4-carboxylate could be converted into L-cysteine by several bacterial strains. Among them, *Pseudomonas thiazolinophilum* was found to contain more

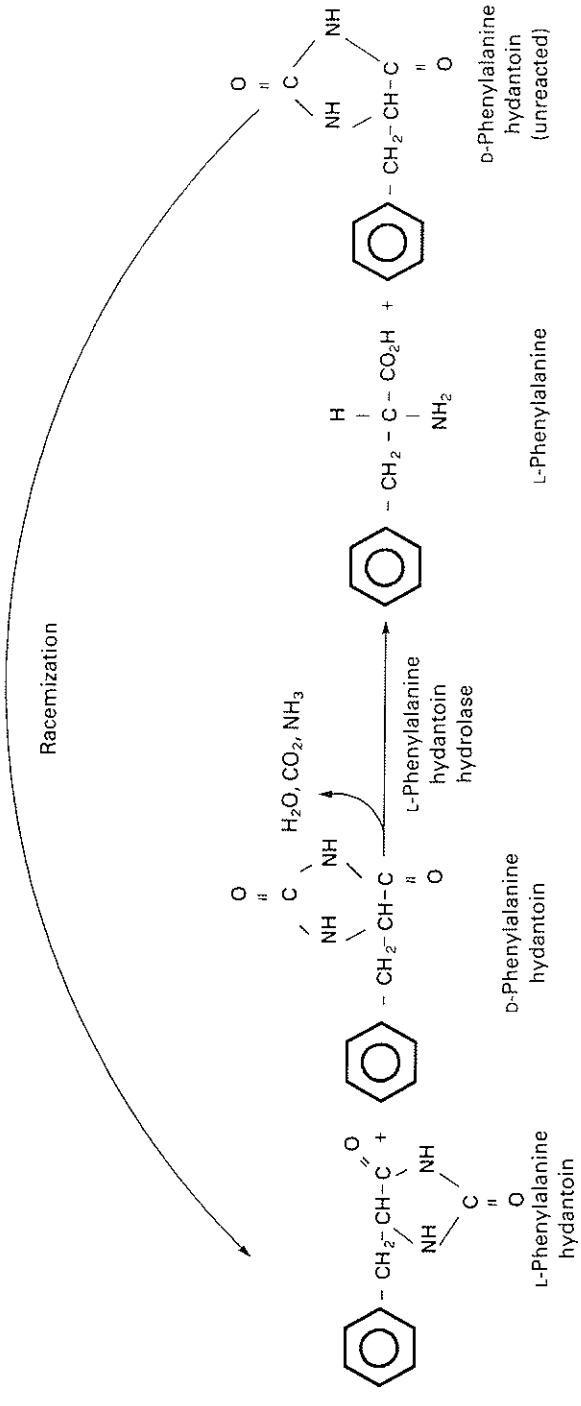


Figure 13. L-Phenylalanine synthesis from D,L-phenylalanine hydantoin by L-phenylalanine hydantoin hydrolase.

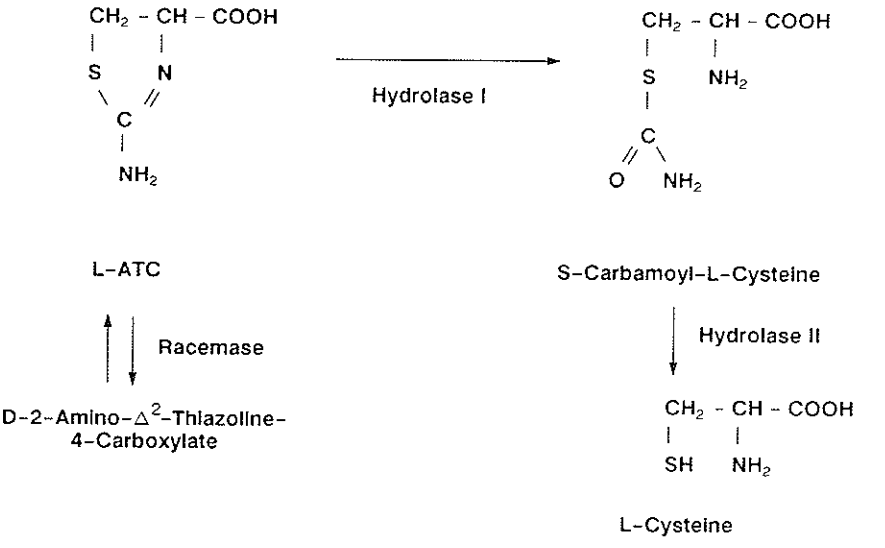


Figure 14. Proposed reaction scheme for enzymatic conversion of D,L-2-amino-Δ^2-thiazoline-4-carboxylate to L-cysteine (Nagasawa and Yamada, 1986).

enzyme activity than other strains. Since a yield close to 100% was obtained, a simultaneous racemization and hydrolysis of D,L-2-amino-Δ^2-thiazoline-4-carboxylic acid was proposed. The reaction scheme as shown in *Figure 14* involved three enzyme steps (Nagasawa and Yamada, 1986).

The equilibrium is driven by the two hydrolase reactions to allow a complete racemization of D-2-amino-Δ^2-thiazoline-4-carboxylate to L-2-amino-Δ^2-thiazoline-4-carboxylate for the synthesis of L-cysteine.

The induction of these three enzymes has been studied (Sano and Mitsugi, 1978). D,L-2-Amino-Δ^2-thiazoline-4-carboxylate was an effective inducer and a good level of enzyme activity was obtained by feeding the inducer at mid-logarithmic phase. Since the whole cells were used as catalyst, surfactants such as cetyltrimethylammonium chloride and sodium laurylsulphate were introduced to lyse the cell membrane partially and to increase the effective enzyme activity. A mutant of *P. thiazolinophilum* which was deprived of cysteine degradation activity was deployed to produce 31·4 g/l of L-cysteine with a molar yield of 95% (Sano and Mitsugi, 1978).

CYSTEINE DESULPHYDRASE

Cysteine desulphydrase (L-CySH hydrogen sulphide lyase, EC 4.4.1.1), a pyridoxal-5′-phosphate-containing enzyme, normally catalyses cysteine degradation to pyruvate, ammonium and hydrogen sulphide (Kredich, Keenan and Foote, 1972). It also degrades serine to pyruvate, ammonium and water. In addition to α,β-elimination reactions, cysteine desulphydrase catalyses β-replacement reactions as shown in *Figure 15*.

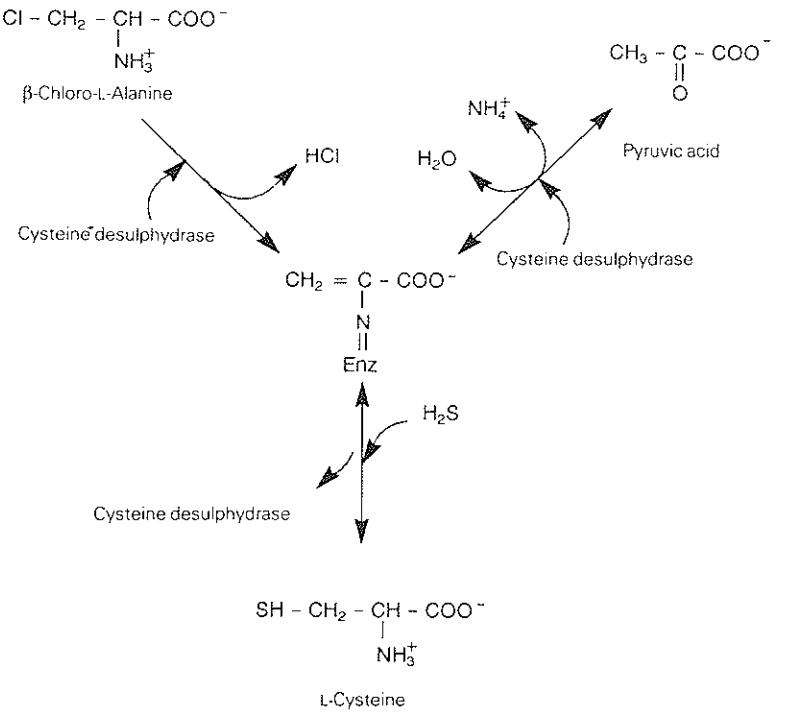


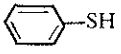
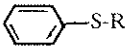
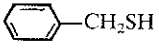
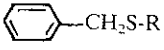
Figure 15. α,β-Elimination and β-replacement reactions catalysed by L-cysteine desulphydrase.

Although the enzyme accepts several different kinds of free thio compounds as substrates (*see Table 10*), hydrogen sulphide is the best nucleophile for the β-replacement reaction (Kumagai *et al.*, 1977; Ohkishi *et al.*, 1981). Cysteine desulphydrase can be found in different species of bacteria such as *Aerobacter aerogenes*, *Salmonella typhimurium* and *Enterobacter cloacae*. A high titre of enzyme production in *Enterobacter cloacae* was established by using L-cysteine as inducer (Kumagai, Choi and Yamada, 1975).

Yamada and Kumagai (1978) successfully demonstrated the possibility of using a β-replacement reaction catalysed by cysteine desulphydrase to produce L-cysteine from 3-chloro-alanine and hydrogen sulphide. In this reaction, acetone has to be incorporated in the reaction mixture in order to obtain a high yield and high titre.

It is believed that acetone trapped cysteine by forming a thiazolidine compound and, therefore, avoided the formation of a dead-end adduct between cysteine and an α-aminoacrylate enzyme intermediate. In the presence of a suitable amount of acetone, cysteine as high as 50 g/l was produced with an 80% yield (Yamada and Kumagai, 1978).

Table 10. Synthesis of L-cysteine-related amino acids from β-chloro-L-alanine and mercaptans by cysteine desulphydrase (Yamada and Kumagai, 1978)

Thiol compound	L-Amino acid* synthesized	Rel. rate
H_2S	HS-R	100
CH_3SH	CH_3S-R	0·6
CH_3CH_2SH	CH_3CH_2S-R	2·0
$CH_3(CH_2)_2SH$	$CH_3(CH_2)_2S$-R	0·49
$CH_3{=}CH\text{-}CH_2SH$	$CH_2{=}CH\text{-}CH_2S$-R	7·2
$CH_3(CH_2)_3SH$	$CH_3(CH_2)_3S$-R	0·13
H_3C, H_3C >CHCH$_2$SH	H_3C, H_3C >CHCH$_2$S-R	0·09
CH_3H_2C, H_3C >CHSH	H_3CH_2C, H_3C >CHS-R	0·05
H_3C, H_3C, H_3C >C-SH	H_3C, H_3C, H_3C >C-S-R	0·04
		0·02
		0·17

* R represents the L-alanyl moiety

O-ACETYLSERINE SULPHYDRYLASE

O-Acetylserine sulphydrylase (EC 4.2.99.8), a pyridoxal-5′-phosphate-containing enzyme, is widely found in enteric bacteria such as *E. coli* and *Salmonella typhimurium*. Kredich and Tomkins (1969) demonstrated that this enzyme, together with serine-*O*-transacetylase (EC 2.3.1.30), was responsible for the biosynthesis of L-cysteine in enteric bacteria. The reaction scheme is shown below:

$$\text{L-Serine} + \text{acetyl-CoA} \xrightarrow{\text{serine-}O\text{-transacetylase}} O\text{-acetylserine} + \text{CoA}$$

$$O\text{-Acetylserine} + H_2S \xrightarrow{O\text{-acetylserine sulphydrylase}} \text{L-cysteine} + \text{acetate}$$

O-Acetylserine sulphydrylase from *Salmonella typhimurium* has been purified and well studied (Becker, Kredich and Tomkins, 1969; Cook and Wedding, 1976). There are two types of *O*-acetylserine sulphydrylase. The first, approximately 5% of total activity, is complexed with serine-*O*-transacetylase and is called *O*-acetylserine sulphydrase-STA. The second type is not associated with serine-*O*-transacetylase and is called *O*-acetylserine sulphydrylase A (Becker, Kredich and Tomkins, 1969). Both types are identical as far as physical and kinetic properties are concerned.

O-Acetylserine sulphydrylase only catalyses the β-replacement reaction and

is highly specific for *O*-acetylserine. However, either methyl mercaptan or cyanide can replace sulphide as substrate (Becker, Kredich and Tomkins, 1969). *O*-Acetylserine sulphydrylase A is a very active enzyme. Its specific activity is 8300 units/mg and it has a complicated kinetic pattern. *O*-Acetylserine sulphydrylase catalyses a Bi Bi Ping Pong reaction between *O*-acetylserine and sulphide.

Both substrates exhibit strong competitive inhibition. The K_m for O-acetylserine and sulphide is 0·149 mM and 0·066 mM, respectively. The K_i for *O*-acetylserine and sulphide is 46·91 mM and 0·013 mM, respectively. Cysteine exerts product inhibition on the enzyme at two places along the reaction sequence with K_i of 1·048 mM and 11·4 mM, respectively. Acetate is not a strong inhibitor (Cook and Wedding, 1976).

The structural gene for *O*-acetylserine sulphydrylase A in *S. typhimurium* was identified to be *cysK* (Hulanicka, Kredich and Treiman, 1974; Byrne *et al.*, 1988). The synthesis of *O*-acetylserine sulphydrylase A is subject to derepression by sulphur starvation and to repression by sulphide and cysteine (Kredich, 1971). When *S. typhimurium* was grown in a medium with L-djenkolic acid as a sole sulphur source, about 2% of the soluble protein in the crude extract was *O*-acetylserine sulphydrylase (Becker, Kredich and Tomkins, 1969).

O-Acetylserine sulphydrylase was used to synthesize L-cysteine (Hsiao and Wei, 1987). The reaction is irreversible. *O*-Acetylserine was prepared in high yield by reacting L-serine with acetic anhydride under acidic conditions (Nagai and Flavin, 1967). In an undesirable side reaction, *O*-acetylserine was readily transacylated to *N*-acetylserine with the production of a proton (Flavin and Slaughter, 1965). The rate of this isomerization reaction was shown to increase with higher pH, temperature and *O*-acetylserine concentration. However, the second substrate, either sodium sulphide or sodium hydrosulphide, was not stable at a pH lower than 10. The pH optimum of the reaction rate for *O*-acetylserine sulphydrylase is 8·0 but the loss of *O*-acetylserine at this pH is higher than that at pH 7·0. This result indicates that the isomerization side reaction is very significant at an alkaline pH. Although *O*-acetylserine sulphydrylase has very complicated kinetic properties, the expressed activity increased with *O*-acetylserine concentration.

With all these factors in mind, a stirred tank reactor with controlled substrate feeds was designed to produce cysteine (Hsiao and Wei, 1987). The pH was maintained at 7 and the temperature was controlled at 25°C. These conditions were chosen because the decrease in the rate of cysteine formation from pH 8 to 7 and 37°C to 25°C is much smaller than that of the rate of isomerization of *O*-acetylserine. An automatic control system for substrate addition was developed to control the *O*-acetylserine and sulphide concentrations in the reactor. This was done by connecting a pH electrode to a pH controller. A two-channel pump which delivered two substrate solutions at the same rate was connected to the pH controller. Both substrates were stored under their respectively stable conditions. Sodium hydrosulphide was dissolved in water to give a concentration between 2·1 M and 2·3 M, which has a pH around 11·5. A saturated solution of *O*-acetylserine (2·05 M) was prepared and the pH was

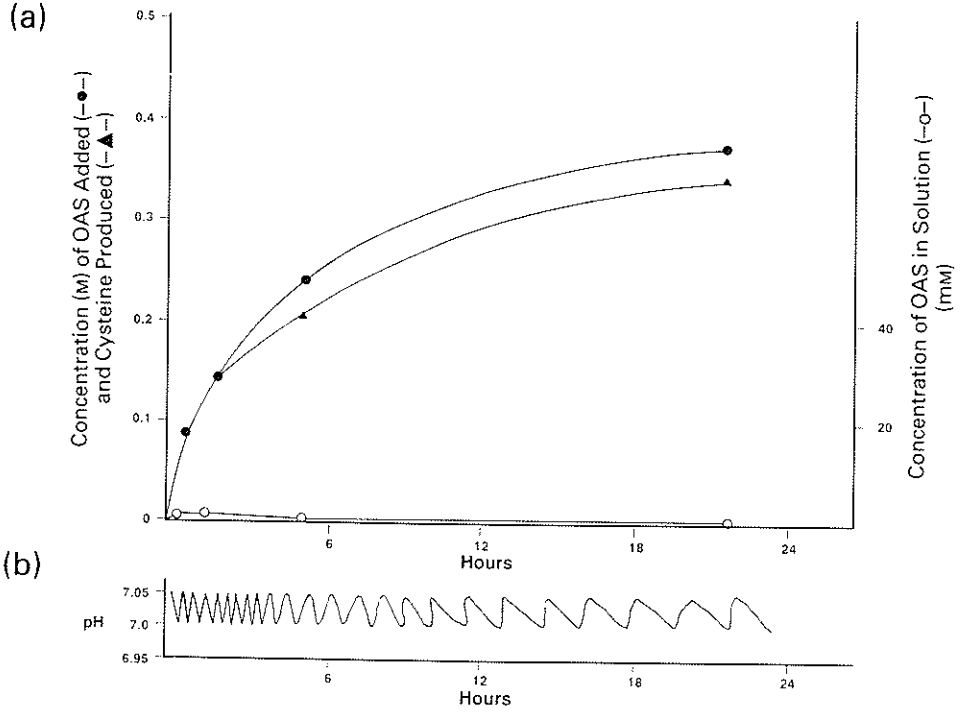


Figure 16. L-Cysteine production by *O*-acetylserine sulphydrylase (OASS) with 2·3 M sodium hydrosulphide as substrate. (a) (●) Cumulative amount of *O*-acetylserine (OAS) added. (▲) Cysteine and cystine produced. (o) OAS actually present in reaction mixture. (b) pH profile of bioconversion (Hsaio and Wei, 1987).

adjusted to 5. The rate of the O->N acyl transfer reaction at pH 5 and 25°C was 0·43 mM/h, which was tolerable. The reason for storing *O*-acetylserine at pH 5, rather than a lower pH, was that the two substrates, upon mixing in the bioreactor, should behave as an alkaline solution to titrate the protons released by the cysteine synthesis reaction. The alkalinity of the substrate solution was controlled by the concentration of sulphide solution, and consequently it determined the concentration of *O*-acetylserine in the reactor.

An example was given to illustrate how the control system functioned. Crude enzyme extract (13 ml) containing 0·5 mM pyridoxal-5′-phosphate, 5 mM EDTA and 5 mM sodium sulphide was introduced into a 50 ml bioreactor at 25°C and pH was adjusted to 6·9. A two-channel pump which delivered two substrate solutions at the same rate was connected to the base position of the pH controller. The setpoint of the controller was 7·0. The sodium hydrosulphide concentration was 2·3 M. The reaction was initiated by turning on the controller and the pump. Each substrate was fed at 1·2 ml/h. The pH went up to 7·03 and the controller turned the pump off. As the synthesis reaction and, to a lesser degree, the side reaction progressed, the pH dropped to 6·97 and automatically triggered the delivery of the two substrates. The cycle of pH fluctuation continued until the reactor was full. The results are shown in *Figure 16*. The final product was a mixture of cysteine and cystine with a molar

ratio of 4 to 1 in favour of cysteine. The yield based on *O*-acetylserine was 94% and the concentration of *O*-acetylserine in the bioreactor remained between 1·6 and 2·8 mM. The productivity was 0·69 g/h/gDCW. When sodium hydrosulphide concentration was lowered to 2·1 M and the same feed rate was used, the productivity increased significantly (from 0·69 to 4·1 g/h/gDCW) with a yield of about 78%. When 2·15 M sodium hydrosulphide was used with a feed rate between 4·2 and 6·2 ml/h, the yield was 94% and productivity was 3·6 g/h/gDCW. The final cysteine titre was 83 g/l.

Recently, *O*-acetylserine sulphydrylase was isolated from a 3-chloro-alanine resistant strain, *Bacillus sphaericus* L-118, and the purified enzyme was specific for both *O*-acetylserine and 3-chloro-alanine (Dhillon, Nagasawa and Yamada, 1984). The enzyme only catalyses the β-replacement reaction of *O*-acetylserine or 3-chloro-alanine to form cysteine. The *Bacillus* enzyme has a higher K_m for *O*-acetylserine (7·8 mM *vs* 0·149 mM) and is less active (240 units/mg *vs* 8300 units/mg) than *O*-acetylserine sulphydrylase from *S. typhimurium*. The specific activity for 3-chloro-alanine is 38 units/mg. 3-Chloro-alanine was shown to enhance the synthesis of *O*-acetylserine sulphydrylase in *B. sphaericus* L-118 and L-cysteine markedly repressed the enzyme synthesis (Nagasawa *et al.*, 1985). However, 3-chloroalanine at various concentrations (0–0·35%) did not enhance the synthesis of *O*-acetylserine sulphydrylase in *S. typhimurium*.

Resting cells of *B. sphaericus* were used to produce L-cysteine from 3-chloro-alanine and sulphide (Nagasawa *et al.*, 1985). Acetone was included to prevent enzyme inactivation and the highest yield was obtained when sulphide concentration was five times higher than that of 3-chloro-alanine. Under optimal conditions, an 80% yield with 70 g/l of L-cysteine was achieved and the productivity was high (20 g/h/gDCW).

Case study 4—enzymatic routes to tryptophan

Both tryptophan synthase (L-serine hydro-lyase (adding indole), EC 4.2.1.20) and tryptophanase (L-tryptophan indole-lyase) have been used to synthesize L-tryptophan (Nakazawa *et al.*, 1972a, b; Asai, Shimada and Soda, 1982; Bang *et al.*, 1983; Takao, Yokota and Tanida, 1984; Hamilton *et al.*, 1985; Yukawa *et al.*, 1988). Both enzymes from *E. coli* have been purified and well characterized (Miles, 1979; Snell, 1975).

Tryptophan synthase from *E. coli* was shown to be a hetero-tetrameric complex ($\alpha_2\beta_2$) and it was readily dissociated into two α subunits and a β_2 subunit (Crawford and Yanofsky, 1958; Goldberg *et al.*, 1966). The enzyme carries out the last step of the biosynthesis of tryptophan as shown below (Crawford and Yanofsky, 1958):

$$\text{Indole-3-glycerol phosphate} + \text{serine} \xrightarrow[\text{synthase}]{\text{tryptophan}} \text{tryptophan} + \text{glyceraldehyde-3-phosphate}$$

Table 11. Reactions catalysed by *Escherichia coli* tryptophan synthase and its subunits (Adachi and Miles, 1974)

Reaction	Catalysed by
1. Indole + L-serine $\xrightarrow{PLP}$ L-tryptophan + H_2O	$\alpha_2\beta_2$ $\beta_2 + NH_4^+$
2. Indole-3-glycerol phosphate ⟶ indole + D-glyceraldehyde-3-phosphate	$\alpha_2\beta_2$ α
3. Indole-3-glycerol phosphate + L-serine $\xrightarrow{PLP}$ L-tryptophan + D-glyceraldehyde-3-phosphate + H_2O	$\alpha_2\beta_2$
4. L-Serine $\xrightarrow{PLP}$ pyruvate + ammonia	β_2
5. β-Mercaptoethanol + L-serine $\xrightarrow{PLP}$ *S*-hydroxyethyl-L-cysteine + H_2O	$\alpha_2\beta_2$ β_2
6. β-Mercaptoethanol + L-serine + PLP → *S*-hydroxyethyl mercaptopyruvate + PMP	β_2

Abbreviations: PLP, pyridoxal-5′-phosphate; PMP, pyridoxamine-5′-phosphate.

However, the native enzyme as well as its subunits also catalyses several α, β-elimination and β-addition reactions as shown in *Table 11* (Adachi and Miles, 1974; Miles, Hatanaka and Crawford, 1968). The first reaction in *Table 11* has been used to produce tryptophan enzymatically (Hamilton *et al.*, 1985).

The development of strains for the production of tryptophan synthase, either through genetic engineering or more traditional genetic techniques, is greatly aided by extensive basic research that has been conducted with the tryptophan operon in *E. coli* (Platt, 1980) and other species. The relatively low turnover number of tryptophan synthase, as in several other cases discussed, suggests that high-level producing strains would be necessary for success. The relative instability of trypotophan synthase in the presence of indole also makes very efficient enzyme production advantageous for its use in a tryptophan producing process.

The tryptophan operon genes (including the *trp*A and *trp*B genes that code for the two subunits of tryptophan synthase) obtained from a specialized transducing phage were among the very first genes cloned by the recombinant DNA method (Hershfield *et al.*, 1974). The DNA sequence of the entire *trp* operon has been determined (Crawford, Nichols and Yanofsky, 1980), including the *trp*A gene (Nichols and Yanofsky, 1979). Plasmids with the *trp* genes as genetic markers were designed (Enger-Valk *et al.*, 1980). It is not surprising that plasmids such as these, or plasmids with further refinements, can be used to produce high levels of tryptophan synthase. The *trp* promoter is very strong, but regulatable, and has been used extensively for the production of foreign proteins in *E. coli*, as suggested in 1980 (Hallewell and Emtage, 1980).

Table 12. Reactions catalysed by tryptophanase (Newton, Morina and Snell, 1965)

1.	L-Tryptophan + H_2O ⟶ pyruvate + NH_3 + indole
2.	L-Serine ⟶ pyruvate + NH_3
3.	L-Cysteine + H_2O ⟶ pyruvate + NH_3 + H_2S
4.	*S*-Methyl-L-cysteine + H_2O ⟶ pyruvate + NH_3 + CH_3SH
5.	L-Serine + indole ⟶ L-tryptophan + H_2O
6.	L-Cysteine + indole ⟶ L-tryptophan + H_2S
7.	*S*-Methyl-L-cysteine + indole ⟶ L-tryptophan + CH_3SH

Tryptophanase in *E. coli* catalyses α, β-elimination reactions (reactions 1–4, *Table 12*) and β-replacement reactions (reactions 5–7, *Table 12*; Newton *et al.*, 1965). Watanabe and Snell (1972) also demonstrated that degradation of tryptophan catalysed by tryptophanase is readily reversible at high concentrations of pyruvate and ammonia. The low solubility of tryptophan in water helps to shift the equilibrium toward the synthesis of tryptophan.

Several different microbial species have been screened and selected for ability to produce tryptophanase (Enei *et al.*, 1974; Konosuke and Mitsugi, 1975; Asai, Shimada and Soda, 1982) without any genetic manipulation. An *E. coli* B strain that constitutively produces tryptophanase about fivefold over the wild type induced level (Snell and Newton, 1962) has been deposited at the American Type Culture Collection (ATCC 27553).

Tryptophanase, like SHMT and tryptophan synthase, has a relatively low turnover number. Therefore, the most efficient use of this enzyme as biocatalyst for the production of tryptophan requires the development of a high-level-producing strain. Otherwise, the addition of a relatively large amount of biomass as whole cells or crude enzyme extract is required to obtain sufficient rates. The large biomass in turn creates the problem of high viscosity in the bioreactor and additional purification requirements.

The tryptophanase gene from *E. coli* has been extensively studied. The gene has been amplified in several specialized lambda transducing phages (Miki *et al.*, 1978; Hansen and Meyenburg, 1979; Hansen *et al.*, 1984). The gene also has been cloned *in vivo* using Mu-mediated transposition on to a conjugative plasmid and the DNA sequence of the structural gene was determined after subcloning (Deeley and Yanofsky, 1981). The selection methods as well as the gene clones developed in these studies are useful for the development of tryptophanase over-producing biocatalyst strains.

The *E. coli* tryptophanase gene from plasmid pMD6 (Deeley and Yanofsky, 1981) was utilized in the construction of plasmid pGX2308 (Anderson, 1987). This plasmid, in conjunction with host strain GX1734, was used for the production of tryptophanase in small fermentors at a level of about 1·3 g/litre (Anderson, 1987). Similar to the plasmid and host strain designed for the production of SHMT, as described in an earlier section, plasmid pGX2308 was effectively stabilized by placing the *trp*ED genes on the plasmid and deleting the corresponding genes from the chromosome of the host. Plasmid pGX2308 also carries the lambda lysis genes *RRz* similar to previous uses of phage lysis genes (e.g. Garretty *et al.*, 1981), which allows gentle cell lysis on demand and

Table 13. A comparison of chemical and physical properties of tryptophanase (Snell, 1975) and tryptophan synthase (Miles, 1979)

	Tryptophanase	Tryptophan synthase
K_m (serine)	160 mM	77 μM
K_m (indole)	0·019 mM	3·7 μM
Inhibition by glycine	Strong	No
Inhibition by indole	Weak	Strong
Serine degradation	K_m = 160 mM	No
Tryptophan degradation	K_m = 0·33mM	No
Specific activity	10–12 units	17 units
Stability toward indole	Good	Poor

improves the recovery of cell free tryptophanase.

The tryptophanase gene from *Alcaligenes fecalis* was recently cloned (Shibatani, Omori and Tosa, 1987) and used to increase the production of tryptophanase by *A. fecalis* fourfold in preliminary experiments.

As mentioned above, both tryptophan synthase and tryptophanase can be used to synthesize tryptophan from L-serine and indole. Scientists at Mitsui used a mixture of tryptophan synthase and serine racemase (EC 5.1.1.10) to convert D, L-serine to L-tryptophan completely (Asai, Shimada and Soda, 1982). As indole not only inhibited tryptophan synthase but also inactivated the enzyme, indole was first dissolved in toluene or detergent and slowly fed into the reactor. When a crude serine solution (90% serine and 10% glycine) prepared from the SHMT reaction as described earlier was used for tryptophan production, both enzymes (tryptophan synthase and tryptophanase) were tested. In *Table 13*, properties of these two enzymes are listed.

Although tryptophan synthase was a better enzyme in terms of kinetic properties, tryptophanase was chosen to run the reaction simply because of its high stability toward indole. When a reactor containing 1 M crude serine solution was used, solid indole as high as 110 g/l was directly introduced. Tryptophanase successfully converted more than 90% of serine and indole to tryptophan at a concentration of 200 g/l (Hamilton *et al.*, 1985).

A view towards the future

Probably the biggest question about industrial application of the enzymatic synthesis of amino acids is whether or not use of this technology will grow in the future. *Table 1* shows that the list of successful enzymatic processes for amino acid manufacture is not long, even though the first such process was commercialized over 15 years ago. The enzymatic method usually relies upon substrates that are substantially more expensive than fermentation feeds such as glucose, and also requires the development of cheap biocatalysts, either in whole cell or purified enzyme form. The challenges of reducing high substrate and biocatalyst costs have led to extensive research, some of which has been reviewed in this report. Such research efforts can be expected to continue in the future.

References

ADACHI, O. AND MILES, E.W. (1974). A rapid method of preparing crystalline β_2 subunit of tryptophan synthetase of *Escherichia coli* in high yield. *Journal of Biological Chemistry* **249**, 5430–5434.

ALEXANDER, N. AND GREENBERG, D.M. (1956). Studies on the purification and properties of the serine-forming enzyme system. *Journal of Biological Chemistry* **220**, 775–785.

ANDERSON, D.M. (1987). Enzymatic production of L-amino acids. *Biotec* **1**, 41–59.

ANDERSON, D.M., YOUNG, D. AND KALK, E.S. (1986). *Method for the Selection and Stabilization of Producing Cell Lines and Producing Cell Lines so Stabilized.* European Patent Application EP 178764 A1.

ANDERSON, D., HSIAO, H.Y., YANG, H.H. AND BHATIA, D. (1984). Production of SHMT in *Klebsiella aerogenes.* 188th American Chemical Society National Meeting, Philadelphia, Pennsylvania.

ASAI, T., AIDA, K. AND OISHI, K. (1959). On the enzymatic preparation of L-phenylalanine. *Journal of General and Applied Microbiology* **5**, 150–152.

ASAI, Y., SHIMADA, M. AND SODA, K. (1982). *Process for Preparation of L-Tryptophan by Enzyme.* US Patent 4,335,209.

BANG, W., LANG, S., SAHM, H. AND WAGNER, F. (1983). Production of L-tryptophan by *Escherichia coli* cells. *Biotechnology and Bioengineering* **25**, 999–1011.

BECKER, M.A., KREDICH, N.M. AND TOMKINS, G.M. (1969). The purification and characterization of *O*-acetylserine sulfhydrylase-A from *Salmonella typhimurium. Journal of Biological Chemistry* **244**, 2418–2427.

BIENKOWSKU-SZEWCZYK, K. AND TAYLOR, A. (1980). Murein transglycosylase from phage lysate. Purification and properties. *Biochemica et biophysica acta* **615**, 489–496.

BLAKLEY, R.L. (1960a). A spectrophotometric study of the reaction catalyzed by serine transhydroxymethylase. *Biochemical Journal* **77**, 459–465.

BLAKLEY, R.L. (1960b). Crystalline dihydropterolglutamic acid. *Nature* **188**, 231–232.

BULOT, E. AND COONEY, C.L. (1985). Selective production of phenylalanine from phenylpyruvate using growing cells of *Corynebacterium glutamicum. Biotechnology Letters* **7**, 93–98.

BYRNE, C., MONROE, R.S., WARD, K.A. AND KREDICH, N.M. (1988). DNA sequences of cysk regions of *Salmonella typhimurium* and *Escherichia coli* and linkage of the cysk regions to ptsH. *Journal of Bacteriology* **170**, 3150–3157.

CALTON, G.J., WOOD, L.L., UPDIKE, M.H., LANTZ II, L. AND HAMMAN, J.P. (1986). The production of L-phenylalanine by polyazetidine immobilized microbes. *Biotechnology* **4**, 317–320.

CHIBATA, I. AND TOSA, T. (1976). Industrial applications of immobilized enzymes and immobilized microbial cells. In *Applied Biochemistry and Bioengineering: Immobilized Enzyme Principles* (L.B. Wingard, Jr, E. Katchalski-Katzir, L. Goldstein, Eds), volume 1, pp. 329–357. Academic Press, New York.

CHIBATA, I., TOSA, T. AND KAKIMOTO, T. (1986). Alanine. In *Progress in Industrial Microbiology: Biotechnology of Amino Acid Production* (K. Aida, I. Chibata, K. Nakayama, K. Takinami, and H. Yamada, Eds), volume 24, pp. 224–232. Elsevier, New York.

CHIBATA, I., TOSA, T. AND SATO, T. (1986). Aspartic acid. In *Progress in Industrial Microbiology: Biotechnology of Amino Acid Production* (K. Aida, I. Chibata, K. Nakayama, K. Takinami, and H. Yamada, Eds), volume 24, pp. 144–151. Elsevier, New York.

COOK, P. AND WEDDING, R.T. (1976). A reaction mechanism from steady state kinetic studies for *O*-acetylserine sulfhydrylase from *Salmonella typhimurium. Journal of Biological Chemistry* **251**, 2023–2029.

CRABTREE, S. AND CRONAN, J.E. (1984). Facile and gentle method for quantitative lysis of *Escherichia coli* and *Salmonella typhimurium. Journal of Bacteriology* **158**, 351–356.

CRAWFORD, I.P. AND YANOFSKY, C. (1958). On the separation of the tryptophan synthetase of *Escherichia coli* into two protein components. *Proceedings of the National Academy of Sciences, USA* **44**, 1161–1170.

CRAWFORD, I.P., NICHOLS, B.P. AND YANOFSKY, C. (1980). Nucleotide sequence of the *trp*B gene in *Escherichia coli* and *Salmonella typhimurium*. *Journal of Molecular Biology* **142**, 489–502.

DEELEY, M.C. AND YANOFSKY, C. (1981). Nucleotide sequence of the structural gene for tryptophanase of *Escherichia coli* K-12. *Journal of Bacteriology* **147**, 787–796.

DEV, I.K. AND HARVEY, R.J. (1984a). Regulation of synthesis of serine hydroxymethyltransferase in chemostat cultures of *Escherichia coli*. *Journal of Biological Chemistry* **259**, 8394–8401.

DEV, I.K. AND HARVEY, R.J. (1984b). Role of methionine in the regulation of the synthesis of serine hydroxymethyltransferase in *Escherichia coli*. *Journal of Biological Chemistry* **259**, 8402–8406.

DHILLON, G.S., NAGASAWA, T. AND YAMADA, H. (1984). L-Cysteine-synthesizing enzyme from 3-chloro-L-alanine resistant *Bacillus sphaericus* L-118. *Journal of Biotechnology* **1**, 47–56.

ENEI, M., MATSUI, H., OKUMURA, S. AND YAMADA, H. (1971). Enzymatic preparation of L-tyrosine and 3,4-dihydroxylphenyl-L-alanine. *Biochemical and Biophysical Research Communications* **43**, 1345–1349.

ENEI, Z.H., NAKAZAWA, K.H., MATSUI, Y.H., OKUMURA, V.S. AND YAMADA, H. (1974). *Production of L-Tryptophan and Derivatives*. US Patent 3,808,101.

ENGER-VALK, B.E., HEYNEKER, H.L., OOSTERBAAN, R.A. AND POUWELS, P.H. (1980). Construction of new cloning vehicles with genes of the tryptophan operon of *Escherichia coli* as genetic markers. *Gene* **9**, 69–85.

EVANS, C.T., PETERSON, W., CHOMA, C. AND MISAWA, M. (1987a). Biotransformation of phenylpyruvic acid to L-phenylalanine using a strain of *Pseudomonas fluorescens* ATCC 11250 with high transaminase activity. *Applied Microbiology and Biotechnology* **26**, 305–312.

EVANS, C.T., CHOMA, C., PETERSON, W. AND MISAWA, M. (1987b). Bioconversion of *trans*-cinnamic acid to L-phenylalanine in an immobilized whole cell reactor. *Biotechnology and Bioengineering* **30**, 1067–1972.

EVANS, C.T., CONRAD, D., HANNA, K., PETERSON, W., CHOMA, C. AND MISAWA, M. (1987c). Novel stabilization of phenylalanine ammonia-lyase catalyst during bioconversion of *trans*-cinnamic acid to L-phenylalanine. *Applied Microbiology and Biotechnology* **25**, 399–405.

EVANS, C.T., HANNA, K., PAYNE, C., CONRAD, D. AND MISAWA, M. (1987d). Biotransformation of *trans*-cinnamic acid to L-phenylalanine: optimization of reaction conditions using whole yeast cells. *Enzyme and Microbial Technology* **9**, 417–421.

EVANS, C.T., CHOMA, C., PETERSON, W. AND MISAWA, M. (1987e). Effect of glycerol, polyethylene glycol and glutaraldehyde on stability of phenylalanine ammonia-lyase activity in yeast. *Journal of Industrial Microbiology* **2**, 53–58.

EVANS, C.T., HANNA, K., CONRAD, D., PETERSON, W. AND MISAWA, M. (1987f). Production of phenylalanine ammonia-lyase (PAL): isolation and evaluation of yeast strains suitable for commercial production of L-phenylalanine. *Applied Microbiology and Biotechnology* **25**, 406–414.

EVANS, C.T., BELLAMY, W., CLEESON, M., AOKI, H., HANNA, K., PETERSON, W., CONRAD, D. AND MISAWA, M. (1987g). A novel, efficient biotransformation for the production of L-phenylalanine. *Biotechnology* **5**, 818–823.

FELDMAN, L.I. AND GUNSALUS, I.C. (1950). The occurrence of a wide variety of transaminases in bacteria. *Journal of Biological Chemistry* **187**, 821–830.

FINKELMAN, M.A. AND YANG, H.-H. (1986). *Method for the Production of Phenylalanine Ammonia-Lyase by Fermentation*. US Patent 4,584,273.

FLAVIN, M. AND SLAUGHTER, C. (1965). Synthesis of the succinic ester of homoserine, a new intermediate in the bacterial biosynthesis of methionine. *Biochemistry* **4**, 1370–1375.

GARRETTY, J., FUSSELMAN, R., HISE, J., CHIOU, L., SMITH-GRILLO, D., SCHULZ, J., AND YOUNG, R.Y. (1981). Cell lysis by induction of cloned lambda lysis genes. *Molecular and General Genetics* **182**, 326–331.

GOLDBERG, M.E., CREIGHTON, T.E., BALDWIN, R.L. AND YANOFSKY, C. (1966). Subunit structure of the tryptophan synthetase of *Escherichia coli*. *Journal of Molecular Biology* **21**, 71–82.

HALLEWELL, R.A. AND EMTAGE, S. (1980). Plasmid vectors containing the tryptophan operon promoter suitable for efficient regulated expression of foreign genes. *Gene* **9**, 27–47.

HAMILTON, B.K. AND JACKSON, D.A. (1985). Amino acid production: recent advances. In *Proceedings: Bio-Expo '85*, pp. 295–304. Cahners Exposition Group, Stamford, Connecticut.

HAMILTON, B.K., HSIAO, H.Y., SWANN, W.E., ANDERSON, D.M. AND DELENTE, J.J. (1985). Manufacture of L-amino acids with bioreactors. *Trends in Biotechnology* **3**, 64–68.

HANSEN, E.B., ATLUNG, T., HANSEN, F.G., SKOVGAARD, O. AND VON MEYENBURG, K. (1984). Fine structure genetic map and complementation analysis of mutations in the *dnaA* gene of *Escherichia coli*. *Molecular and General Genetics* **196**, 387–396.

HANSEN, F.G. AND MEYENBURG, K. VON (1979). Characterization of the *dnaA*, *gyrB* and other genes in the *dnaA* region of the *Escherichia coli* chromosome on specialized transducing phages lambda-*tna*. *Molecular and General Genetics* **175**, 135–144.

HAVIR, E.A. AND HANSON, K.R. (1968). L-phenylalanine ammonia-lyase. II. Mechanism and kinetic properties of the enzyme from potato tubers. *Biochemistry* **7**, 1904–1914.

HERSHFIELD, V., BOYER, H.W., YANOFSKY, C., LOVETT, M.A. AND HELINSKI, R. (1974). Plasmid *ColE1* as a molecular vehicle for cloning and amplification of DNA. *Proceedings of the National Academy of Sciences, USA* **71**, 3455–3459.

HSIAO, H.Y. AND WEI, T. (1986). Enzymatic production of L-serine with a feedback control system for formaldehyde addition. *Biotechnology and Bioengineering* **28**, 1510–1518.

HSIAO, H.Y. AND WEI, T. (1987). Enzymatic synthesis of L-cysteine. *Biotechnology and Bioengineering* **30**, 875–881.

HSIAO, H.Y., WEI, T. AND CAMPBELL, K. (1986). Enzymatic production of L-serine. *Biotechnology and Bioengineering* **28**, 857–867.

HULANICKA, M.D., KREDICH, N.M. AND TREIMAN, D.M. (1974). The structural gene for *O*-acetylserine sulfhydrylase A in *Salmonella typhimurium*. *Journal of Biological Chemistry* **249**, 867–872.

HUMMEL, W., SCHMIDT, E., WANDREY, C. AND KULA, M.-R. (1986). L-phenylalanine dehydrogenase from *Brevibacterium* sp. for production of L-phenylalanine by reductive amination of phenylpyruvate. *Applied Microbiology and Biotechnology* **25**, 175–185.

KALLEN, R.G. AND JENCKS, W.P. (1966). The mechanism of the condensation of formaldehyde with tetrahydrofolic acid. *Journal of Biological Chemistry* **241**, 5851–5863.

KISHORE, G.M. (1985). *Stabilization of L-Phenylalanine Ammonia-Lyase Enzyme*. US Patent 4,562,151.

KLAUSNER, A. (1985). Building for success in phenylalanine. *Bio/technology* **3**, 301–307.

KONOSUKE, M.S. AND MITSUGI, Y.K. (1975). *L-Tryptophan Production*. US Patent 3,929,573.

KREBS, H.A., (1942). The decarboxylation of oxaloacetic acid. *Biochemical Journal* **36**, 303–309.

KREDICH, N.M. (1971). Regulation of L-cysteine biosynthesis in *Salmonella typhimurium*. *Journal of Biological Chemistry* **246**, 3474–3484.

KREDICH, N.M. AND TOMKINS, G.M. (1969). The enzymatic synthesis of L-cysteine in

Escherichia coli and *Salmonella typhimurium*. *Journal of Biological Chemistry* **241**, 4955–4965.

KREDICH, N.M., KEENAN, B.S. AND FOOTE, L.J. (1972). The purification and subunit structure of cysteine desulfhydrase from *Salmonella typhimurium*. *Journal of Biological Chemistry* **247**, 7157–7162.

KUMAGAI, H., CHOI, Y. AND YAMADA, H. (1975). Formation of cysteine desulfhydrase by bacteria. *Agricultural and Biological Chemistry* **39**, 387–392.

KUMAGAI, H., MATSUI, H. AND YAMADA, H. (1970). Formation of tyrosine phenol-lyase by bacteria. *Agricultural and Biological Chemistry* **34**, 1259–1261.

KUMAGAI, H., TANAKA, H., SEJIMA, S. AND YAMADA, H. (1977). Elimination and replacement reactions of β-chloro-L-alanine by cysteine desulfhydrase from *Aerobacter aerogenes*. *Agricultural and Biological Chemistry* **41**, 2071–2075.

LEE, T.K. AND HSIAO, H.Y. (1986). Synthesis of L-tyrosine by a coupled reaction of serine hydroxymethyltransferase and β-tyrosinase. *Enzyme and Microbial Technology* **9**, 523–526.

MCGUIRE, J.C. (1986). *Phenylalanine Ammonia Lyase-Producing Microbial Cells*. US Patent 4,598,047.

MCKENNEY, K., SHIMATABE, H., COURT, D. AND ROSENBERG, M. (1980). A system to study transcription regulatory signals in single and multiple copy. *Federation Proceedings* **39**.

MATSUNAGA, T., HIGASHIJIMA, M., NAKATSUGAWA, H., NISHIMURA, S., TSUJI, M. AND KAWAGUCHI, T. (1987). Conversion of phenylphyruvate to L-phenylalanine by immobilized *Clostridium butyricum*–alanine dehydrogenase – *Micrococcus luteus* under hydrogen pressure. *Applied Microbiology and Biotechnology* **27**, 11–14.

MIKI, T., HIRAGA, S., NAGATA, T., AND YURA, T. (1978). Bacteriophage lambda carrying the *Escherichia coli* chromosomal region of the replication origin. *Proceedings of the National Academy of Sciences, USA* **75**, 5099–5103.

MILES, E.W. (1979). Tryptophan synthase: structure, function, and subunit interaction. In *Advances in Enzymology* (A. Meister, Ed.), volume 49, pp. 127–186. Wiley, New York.

MILES, E.W., HATANAKA, M. AND CRAWFORD, I.P. (1968). A new thiodependent transamination reaction catalyzed by the B protein of *Escherichia coli* tryptophan synthetase. *Biochemistry* **7**, 2742–2753.

NAGAI, S. AND FLAVIN, M. (1967). Acetylhomoserine, an intermediate in the fungal biosynthesis of methionine. *Journal of Biological Chemistry* **242**, 3884–3895.

NAGASAWA, T. AND YAMADA, H. (1986). Cysteine. In *Progress in Industrial Microbiology: Biotechnology of Amino Acid Production* (K. Aida, I. Chibata, K. Nakayama, K. Takinami and H. Yamada, Eds), volume 24, pp. 217–223. Elsevier, New York.

NAGASAWA, T., DHILLON, S., ISHII, T. AND YAMADA, H. (1985). Enzymatic synthesis of L-cysteine by *O*-acetylserine sulfhydrylase of 3-chloro-L-alanine resistant *Bacillus sphaericus* L-118. *Journal of Biotechnology* **2**, 365–377.

NAKAMICHI, K., NABE, K., YAMADA, S. AND CHIBATA, I. (1983). Induction and stabilization of L-phenylalanine ammonia-lyase activity in *Rhodotorola glutinis*. *European Journal of Applied Microbiology and Biotechnology* **18**, 158–162.

NAKAZAWA, H., ENEI, H., OKUMURA, S., YOSHIDA, H. AND YAMADA, H. (1972a). Enzymatic preparation of L-tryptophan and *S*-hydroxy-L-tryptophan. *FEBS Letters* **25**, 43–45.

NAKAZAWA, H., ENEI, H., OKOMURA, S., YOSHIDA, H. AND YAMADA, H. (1972b). Synthesis of L-tryptophan from pyruvate, ammonia and indole. *Agricultural and Biological Chemistry* **36**, 2523–2528.

NAKAZAWA, K.H., ENEI, Z.H., KUBOTA, K.K. AND OKUMURA, S. (1975). *Biological Method of Producing Serine and Serinol Derivatives*. US Patent 3,871,958.

NELSON, R.P. (1976). *Immobilized Microbial Cells*. US Patent 3,957,580.

NEWTON, W.A., MORINA, Y. AND SNELL, E. (1965). Properties of crystalline

tryptophanase. *Journal of Biological Chemistry* **240**, 1211–1213.

NICHOLS, B.P. AND YANOFSKY, C. (1979). Nucleotide sequences of *trp*A of *Salmonella typhimurium* and *Escherichia coli*: an evolutionary comparison. *Proceedings of the National Academy of Sciences, USA* **76**, 5244–5248.

OKHISHI, H., NISHIKAWA, D., KUMAGAI, H. AND YAMADA, H. (1981). Synthesis of L-cysteine and its analogues by intact cells containing cysteine desulfhydrase. *Agricultural and Biological Chemistry* **45**, 259–263.

ONISHI, N., YOKOZEKI, K., HIROSE, Y. AND KUSOTA, K. (1987). Enzymatic production of L-phenylalanine from *trans*-cinnamic acid by *Endomyces lindneri*. *Agricultural and Biological Chemistry* **51**, 291–292.

PLATT, T. (1980). Regulation of gene expression in the tryptophan operon of *Escherichia coli*. In *The Operon* (J.H. Miller and W.S. Reznikoff, Eds), 2nd edn, pp. 263–302. Cold Spring Harbor, New York.

ROZZELL, J.D. (1985). *Production of L-Amino Acids by Transamination*. US Patent 4,518,692.

ROZZELL, J.D. (1987). Immobilized aminotransferases for amino acid production. *Methods in Enzymology* **136**, 479–497.

SAKURAI, S. (1956). Enzymatic preparation of optically active essential amino acids. I. The preparation of L-phenylalanine. *Journal of Biochemistry* **43**, 851–855.

SANO, K. AND MITSUGI, K. (1978). Enzymatic production of L-cysteine from D,L-2-amino-δ^2-thiazoline-4-carboxylic acid by *Pseudomonas thiazolinophilum*: Optimal conditions for the enzyme formation and enzymatic reaction. *Agricultural and Biological Chemistry* **42**, 2315–2321.

SANO, K., YOKOZEKI, K., TAMURA, F., YASUDA, N., NODA, I. AND MITSUGI, K. (1977). Microbial conversion of D,L-2-amino-δ^2-thiazoline-4-carboxylic acid to L-cysteine: Screening of microorganisms and identification of products. *Applied and Environmental Microbiology* **34**, 806–810.

SCHIRCH, L. (1982). Serine hydroxymethyltransferase. In *Advances in Enzymology* (A. Meister, Ed.), volume 53, pp. 83–111. Wiley, New York.

SCHMIDT, E., VASIC'-RACKI, D. AND WANDREY, C. (1987). Enzymatic production of L-phenylalanine from the racemic mixture of D,L-phenylacetate. *Applied Microbiology and Biotechnology* **26**, 42–48.

SHIBATANI, T., OMORI, K. AND TOSA, T. (1987). Cloning of tryptophanase gene of *Alcaligenes fecalis* for effective production of L-tryptophan. *American Society Microbiology Annual Meeting, March 1–8, 1987*, Poster presentation.

SNELL, E.E. (1975). Tryptophanase. In *Advances in Enzymology* (A. Meister, Ed.), volume 42, pp. 287–333. Wiley, New York.

SNELL, E.E. AND NEWTON, W.A. (1962). An inducible tryptophan synthetase in tryptophan auxotrophs of *Escherichia coli*. *Proceedings of the National Academy of Sciences, USA* **48**, 1431–1439.

STAUFFER, G.V., PLAMANN, M.D. AND STAUFFER, L.T. (1981). Construction and expression of hybrid plasmids containing the *Escherichia coli gly*A gene. *Gene* **14**, 63–72.

SWANN, W.E. (1984a). *L-Phenylalanine Production*. UK Patent Application GB 2,127,821A.

SWANN, W.E. (1984b). *Immobilization of Biological Materials in Condensed Polyalkyleneimine Polymers*. US Patent 4,434,228.

SWANN, W.E. (1985a). *Production of L-Phenylalanine*. European Patent Application 853041283.

SWANN, W.E. (1985b). *Vermiculite as a Carrier Support for Immobilized Biological Materials*. US Patent 4,504,582.

TAKAO, S., YOKOTA, A. AND TANIDA, M. (1984). Enzymatic production of tryptophan coupled to pyruvic acid fermentation. *Journal of Fermentation Technology* **62**, 329–334.

TAYLOR, A. (1971). Endopeptidase activity of phage λ-endolysin. *Nature New Biology* **233**, 144–145.

TOSAKA, O., ENEI, H. AND HIROSE, Y. (1983). The production of L-lysine by fermentation. *Trends in Biotechnology* **1**, 70–74.

ULEVITCH, R.J. AND KALLEN, R.G. (1977). Purification and characterization of pyridoxal 5′-phosphate dependent serine hydroxylmethylase from lamb liver and its action upon β-phenylserines. *Biochemistry* **16**, 5342–5350.

VINING, L.C. AND MAGASANIK, B. (1981). Serine utilization by *Klebsiella aerogenes*. *Journal of Bacteriology* **146**, 647–655.

VOLLMER, P.J. AND SCHRUBEN, J.J. (1986). *Stabilization of Phenylalanine Ammonia-Lyase in a Bioreactor Using Reducing Agents*. US Patent 4,574,117.

VOLLMER, P.J., SCHRUBEN, J.J., MONTGOMERY, J.P. AND YANG, H.-H. (1986). *Method of Stabilizing the Enzymatic Activity of Phenylalanine Ammonia-Lyase During L-Phenylalanine Production*. US Patent 4,584,269.

WALTER, J.F. (1986). The use of gelatine immobilized enzyme in amino acid production. *ASM Annual Meeting, Washington, DC*.

WALTER, J.F. AND BULL, C. (1987). Phenylserine production by serine hydroxymethyltransferase. *Society of Industrial Microbiology Annual Meeting, Baltimore, MD*.

WATANABE, T. AND SNELL, E.E. (1972). Reversibility of the tryptophanase reaction: Synthesis of tryptophan from indole, pyruvate, and ammonia. *Proceedings of the National Academy of Sciences, USA* **69**, 1086–1090.

WOOD, L.L. AND CALTON, G.J. (1987). *Process for Preparing Phenylalanine*. US Patent 4,710,467.

YAMADA, H. AND KUMAGAI, H. (1975). Synthesis of L-tyrosine related amino acids by β-tyrosinase. *Advances in Applied Microbiology* **19**, 249–288.

YAMADA, H. AND KUMAGAI, H. (1978). Microbial and enzymatic processes for amino acid production. *Pure and Applied Chemistry* **50**, 1117–1127.

YAMADA, S., NABE, K., ISUO, N., NAKAMICHI, K. AND CHIBATA, I. (1981). Production of L-phenylalanine from *trans*-cinnamic acid with *Rhodotorula glutinis* containing L-phenylalanine ammonia-lyase activity. *Applied and Environmental Microbiology* **42**, 773–778.

YOKOZCKI, K., SANO, K., EQUCHI, T., YASUDA, N., NODA, I. AND MITSUGI, K. (1976). Production of amino acids. *Proceedings of the Annual Meeting of the Agricultural Chemistry Society of Japan*, 238–239.

YUKAWA, H., KURUSU, Y., SHIMAZU, M., YAMAGATA, H. AND TERASAWA, M. (1988). Stabilization of an *E. coli* plasmid by a mini-F fragment of DNA. *Journal of Industrial Microbiology* **2**, 323–328.

ZIEHR, H., KULA, M.-R., SCHMIDT, E., WANDREY, C. AND KLEIN, J. (1987). Continuous production of L-phenylalanine by transamination. *Biotechnology and Bioengineering* **29**, 482–487.