BIOTECHNOLOGY IN THE PRODUCTION OF PHARMACEUTICAL INDUSTRY INGREDIENTS: AMINO ACIDS

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ABSTRACT

Amino acids play an important role in human nutrition and health maintenance. Nowadays amino acids are used as animal feed additives, flavor enhancers, ingredients in cosmetic and pharmaceutical products and as specialty nutrients in the medical field, and the production capacity requirements are constantly increasing. This paper reviews the manufacturing methods for the production of some amino acids used in the pharmaceutical industry and outlines the main achievements of biotechnology in this field. It also summarizes the weaknesses and strengths of the biotechnological methods used for the industrial production of the studied amino acids. Literature search was done through MEDLINE/PubMed, Scopus Database, Web of Knowledge search as well as an Internet-based search with predefined keywords. The present mini review is based on a total of 66 publications. Out of 15 amino acids included in our study, 14 are routinely manufactured applying biotechnology methods mostly from specially developed mutants of C. glutamicum or E. coli. Apart from the fact that some of the amino acids cannot be produced cost efficiently via microbiological methods, biotechnology plays a significant role in the production of proteinogenic amino acids and research is being conducted to improve the applied methods by modification of culture conditions, mutation, recombinant DNA technology etc.

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Introduction

Often called "the building blocks of life", amino acids have long played an important role in both human and animal nutrition and health maintenance (34, 38). They are used as animal feed additives (lysine, methionine, threonine), flavor enhancers (aspartic acid, monosodium glutamate, serine), ingredients in cosmetic and medicinal products and as specialty nutrients in the medical field (2, 6, 27, 45).

The major application of amino acids in human medicine is in transfusion, applied when oral consumption of proteinaceous food is not possible (36, 39, 46, 55, 56, 59). These mixtures account for a small percentage of the total volume of amino acids sold each year (the main consumers of amino acids are the food flavoring industry and animal feed industry), but the requirement that they be highly purified provides a value-added component (2). In addition, amino acids are used in the manufacture of artificial sweeteners such as aspartame. In the past only essential amino acids were used in transfusion but now non-essential amino acids are also added (42). Different amino acids are used for ammonia detoxification in blood in liver diseases, in the treatment of heart failure, peptic ulcer and male sterility. Some amino acids are used as intermediate precursors for the production of antibiotics (18, 22, 57). According to a survey by the Business Communication Company, the amino-acid market for synthesis application is growing at an annual rate of 7 % (34).

Amino acids are often used as ingredients for the production of various food supplements. There are mono products available on the market, but more often amino acids are combined. The most popular amino acid-containing products sold over the counter are arginine, tryptophan, tyrosine, glutamine, and lysine (50). Food supplements containing amino acids are recommended for body building, sleep aid, depression, premenstrual dysphoric disorder (PMDD), smoking cessation, bruxism, attention deficit-hyperactivity disorder (ADHD) etc.

With the exploitation of new uses and the growing markets of amino acids, amino acid production technology has made large progress during the latter half of the 20th century and biotechnology manufacturing methods have been developed and constantly upgraded (8, 27, 40, 49).

This paper reviews the manufacturing methods for the production of some amino acids used in the pharmaceutical industry and outlines the main achievements of biotechnology in this field. It also summarizes the weaknesses and strengths of the biotechnological methods used for the industrial production of the studied amino acids.

Materials and Methods

Literature search was done through MEDLINE/PubMed, Scopus Database, Web of Knowledge search as well as an Internet-based search with keywords "amino acids", "biotechnology", "manufacture", "synthesis", "enzymatic"

and "fermentation". The present mini review is based on a total of 62 publications satisfying the search criteria. Our study was limited to some amino acids used as active ingredients in the production of food supplements or in the pharmaceutical industry.

Results and Discussion

The most used amino acids in the pharmaceutical and food supplements' industry and their applications and preferred method for industrial production are shown in **Table 1**.

Amino acids are produced by protein hydrolysis, chemical synthesis or microbiological (biotechnological) methods. The choice of industrial method depends on the available technology, costs of raw material, market characteristics, cost of running fermentation versus synthesis reactions, and environmental impact of the process itself (2).

The manufacture of amino acids has its roots in the food preparation practices in Japan. For centuries, seaweeds had been used as a flavoring ingredient in Japan as well as in other Asian countries. In 1908, the flavor enhancing molecule was isolated from the seaweed *konbu* (*Laminaria japonica*) by Kikunae Ikeda (Tokyo Imperial University), as crystals of monosodium glutamate (MSG). Soon after Ikeda's discovery, and recognizing the market potential of MSG, Ajinomoto Co. in Japan began extracting monosodium glutamate from acid-hydrolyzed wheat gluten or defatted soybean and selling it as a flavor enhancer (2, 42).

The production of monosodium glutamate via "fermentation" began in Japan after the end of World War II. Around 1957, Japanese researchers led by S. Kinoshita at Kyowa Hakko Kogyo Co. isolated soil bacteria that produced large amounts of glutamic acid, later identified as *Leuconostoc mesenteroides* (2).

Production of amino acids by protein hydrolysis

The original method for amino acid manufacture is protein hydrolysis (extraction method). Before 1950, most amino acids were produced by the denaturing and hydrolysis of various protein sources (3). Hair, keratin, blood meal and feathers are hydrolyzed using acid and the amino acids are extracted. This method is not very popular as it depends on the availability of limited raw materials. However, cysteine and cystine are still produced by isolating from chemically hydrolyzed keratin in hair and feathers, while proline and hydroxyproline are precipitated from gelatin hydrolysates (3, 8).

Some Chinese researchers studied the hydrolysis of fish proteins for the production of amino acids, as in the country which is one of the largest markets for fishery in the world there is a large amount of unused protein-rich biomass remaining from fishery processing (61). Yoshida et al. (61) studied the hydrolysis of fish for producing amino acids by using a set of stainless steel tubes with 5 mL capacity under argon protection. To reduce the cost of industrial production, Zhu et al. (62) studied the production of 18 amino acids from fish proteins in an atmosphere of air, nitrogen or carbon dioxide instead of argon.

The amino acids produced by protein hydrolysis sometimes also rely on other methods as well. For example, L-cysteine is manufactured by enzymatic synthesis in addition to the extraction method (42).

Protein hydrolysis as a method of obtaining L-amino acids is now of only limited importance; although still relevant for production of L-serine, L-proline, L-hydroxyproline, and L-tyrosine, for example, it is not suitable for large-scale production of amino acids (34).

Production of amino acids by chemical synthesis

Glycine and L-alanine are produced by chemical synthesis. In the series of chemical reactions known as Strecker amino acid synthesis, an amino acid is synthesized from an aldehyde (or ketone). The process includes condensation of the aldehyde with ammonium chloride in the presence of potassium cyanide, forming an α-aminonitrile, which is then hydrolyzed to obtain the desired amino acid. The original Strecker reaction combines acetaldehyde, ammonia, and hydrogen cyanide to give alanine after hydrolysis (13, 30, 54). In the traditional synthesis of Adolph Strecker from 1850, racemic α-amino nitriles are formed, but several protocols employing asymmetric catalysts or auxiliaries have been developed since (15, 26, 29).

An example present-day use of the Strecker synthesis is the industrial chemical synthesis of an L-valine derivative starting from 3-methyl-2-butanone, shown in **Fig. 1** (31).

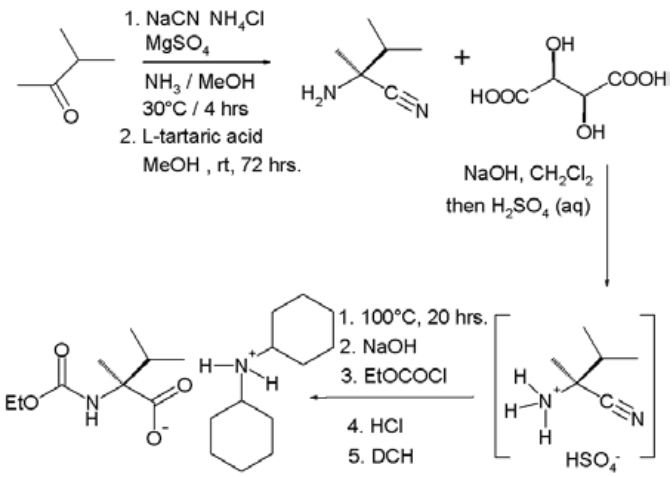

Fig. 1. Strecker synthesis application: valine (Kuethe, 2007) (31).

It is known that chemical synthesis can only produce racemic forms of amino acids and an additional step involving the use of an immobilized enzyme, aminoacylase, produced by *Aspergillus niger*, is required to obtain the biologically active L-form (38, 42). Due to the high production costs for this additional step, very few amino acids (glycine, methionine) are produced advantageously by chemical synthesis. Although chemical synthesis has made possible direct formation of L-isomers of amino acids by means of chiral catalysts, this method has not yet reached a commercially competitive

level. According to Ault (4), chemical synthesis is currently used only for the production of alanine, glycine, methionine, phenylalanine, threonine, tryptophan and valine.

Some authors consider that even though there is no commercially viable chemical process for the production of enantiomerically pure amino acids, the synthesis of racemic amino acids is still of respectable importance because racemates can be converted readily into enantiomerically pure compounds by a number of biocatalytic methods (8). On the other hand, chemical procedures that do not provide enantiomerically pure amino acids are used for the production of glycine (which is achiral) and the nonnatural enantiomer D-methionine (converted in adults and animals into the biologically active L-enantiomer through a transamination reaction).

Production of amino acids by biotechnology methods

Biotechnological production processes have been used for industrial production of amino acids for about 50 years now (1, 3, 4, 5, 9, 10, 12, 14, 17, 19, 20, 21, 24, 27, 28, 32, 33, 34, 35, 42, 43, 44, 52, 58).

Microbiological (biotechnology) methods for the industrial production of amino acids are of three types: use of **microbial enzymes or immobilized cells (enzymatic method)**, **semi-fermentation**, and **direct fermentation**.

Enzyme catalysis is well established in the chemical industry (19, 53). With the enzymatic method, an amino acid precursor is converted to the target amino acid using one or two enzymes (1). This method uses pure enzymes, rather than the enzyme systems of living microorganisms, as in the fermentation methods (4). The enzymatic method allows the conversion to a specific amino acid without microbial growth, thus eliminating the long process from glucose (1). Industrial use of enzymes for production of L-amino acids began about 40 years ago in Japan with the resolution of N-acetyl D,L-amino acids by immobilized acylase (12, 34). Significant production by enzymatic catalysis is currently in place for alanine, aspartic acid, cysteine, cystine, methionine, phenylalanine, serine, tryptophan and valine. For example, L-aspartic acid is manufactured mainly by enzyme-catalyzed addition of ammonia to fumaric acid and this is the preferred manufacturing method, as shown in (9). Since only the naturally occurring L-form is produced that way, resolution is not necessary, unlike for chemically synthesized amino acid racemic mixtures.

For the production of L-methionine, enzymatic resolution with acylase from *Aspergillus oryzae* in the enzyme membrane reactor is the method of choice (58). Several hundred tons of L-methionine and L-valine are produced each year using this technology (34).

L-cysteine is manufactured by industrially used enzymatic process in which the thiazoline derivative DL-2-amino-2-thiazoline-4-carboxylic acid is converted with the help of three enzymes (L-ATC hydrolase, S-carbamoyl-L-cysteine hydrolase, and ATC racemase) from *Pseudomonas thiazolinophilum* (46).

According to Leuchtenberger et al. (36) enzyme catalysis is a particularly elegant and popular method of producing D-amino acids and nonproteinogenic L-amino acids.

The enzymatic production of L-tryptophan from precursors involves a single reaction step. It may be performed with isolated enzymes, either tryptophan synthase (TSase; EC 4.2.1.20) or tryptophanase (TPase; EC 4.1.99.1), or by a variety of microorganisms with these enzyme activities (23) such as *E. coli* (41, 53).

The enzymatic method has now been supplanted by a continuous microbiological process in which the reacting solution passes over a fixed bed of an immobilized microorganism (4).

With semi-fermentation, a metabolic intermediate in the amino acid biosynthesis or its precursor is converted to the amino acid during fermentation.

The fermentation method is being applied to industrial production of most L-amino acids. This method utilizes the phenomenon that microorganisms convert nutrients to various vital components they need. With the fermentation method, raw materials such as syrups are added to microorganism culture media, and the proliferating microorganisms are allowed to produce amino acids. Enzymes play an important role in the production of amino acids by fermentation. Consecutive reactions by 10 to 30 types of enzymes are involved in the process of fermentation, and various amino acids are produced as a result of these reactions (1). The fermentation may take place on a culture medium composed of grains, sugar, molasses, yeast, or other biological material, e.g. petrochemicals, such as paraffin, and synthetic nutrients such as ammonium chloride, ammonium nitrate, and potassium phosphate. Extraction is achieved by physical and mechanical means, such as heat or maceration, as well as by chemical methods such as petroleum solvents, ammonia, strong acids, strong bases, and/or ion exchange. The final product is obtained as crystalline powder (3, 33).

Once a suitable microorganism has been selected for the fermentation method, it is necessary to enhance its potential in order to take full advantage of the potential of the organism. Generally, microorganisms produce the 20 kinds of amino acids only in the amounts they need. They have a mechanism for regulating the quantities and qualities of enzymes to yield amino acids only in the amounts necessary for themselves. Releasing this regulatory mechanism allows manufacturing of the target amino acid in large amounts (1). The yield of an amino acid depends on the quantities and qualities of the enzymes. The yield increases if the enzymes involved in the production of the target amino acid are present in large quantities under workable conditions, whereas, if the enzymes are present in small quantities, it decreases. Strains are improved using various techniques (1).

TABLE 1

Amino acids used in pharmaceutical industry and food supplement production

Amino acids	Manufacturing process	Production organisms	Use related to pharmaceutical industry
Glycine (achiral)	Chemical	-	Buffering agent in antacids, analgesics, antiperspirants and cosmetics.
L-Alanine	Chemical, Enzymatic	*Pseudomonas dacunhae* (1982)	Ingredient for food supplements for body building and improvement of physical performance.
L-Arginine	Fermentation	*C. glutamicum* (*C. acetoacidophilum*); *Brevibacterium flavum*; *E. coli*	Ingredient in dental products (e.g. toothpastes) as provides effective relief from sensitive teeth by depositing a dentin-like mineral. Ingredient in food supplements.
L-Aspartic acid	Enzymatic	*E. coli*	Ingredient in food supplements, sweetener aspartame.
L-Cysteine	Extraction, Enzymatic	*E. coli*; *Pseudomonas thiazolinophilum*	Precursor in pharmaceutical and personal care industry. Ingredient in food supplements for bodybuilders.
L-Glutamine	Fermentation	*C. glutamicum*	Ingredient in food supplements for muscle growth in bodybuilding. Glutamine parenteral nutrition reduces healing time after operation. Important in brain metabolism hence various analogues of glutamic acid are used in treating various neuropathic diseases.
L-Histidine	Fermentation	*Brevibacterium flavum*	Ingredient for food supplements for premenstrual pain, antispasmodic, anti-inflammatory etc.
L-Isoleucine	Fermentation	*C. glutamicum*; *E. coli* H-8461	Ingredient for food supplements for athletes and bodybuilders for boosting energy levels and muscles recovering from strenuous exercise and other physical activities.
L-Leucine	Fermentation, Extraction	*Brevibacterium flavum*; *E.coli*	Ingredient for food supplements for muscle growth and muscular insurance. The most important amino acid for muscle building
L-Lysine	Fermentation	*C. glutamicum*	Ingredient for food supplements for ensuring adequate absorption of calcium and formation of collagen for bone, cartilage and connective tissue. Cold sore treatment.
L-Proline	Protein hydrolysis, Fermentation	*Brevibacterium flavum*; *E. coli*	Used as osmoprotectant in pharmaceutical industry. Stabilizer in many intravenous immunoglobulin pharmaceutical products. Ingredient for food supplements with muscle-sparing effect, recommended for use during prolonged bouts of endurance exercise. Sport drinks for athletes and bodybuilders.
L-Serine	Protein hydrolysis, Fermentation	*Methylobacterium sp.*	Ingredient for food supplements for treatment of chronic fatigue syndrome, enhancement and improvement of mental health. Ingredient for medicines (amino acids transfusions).
L-Tryptophan	Fermentation, Enzymatic	*E. coli* (enzymatic and fermentation); *C. glutamicum*; *Bacillus* sp. (fermentation)	Ingredient for food supplements for sleep aid, depression, premenstrual syndrome called premenstrual dysphoric disorder (PMDD), smoking cessation, bruxism, attention deficit-hyperactivity disorder (ADHD), etc.
L-Tyrosine	Protein hydrolysis, Fermentation	*E. coli*	Used in protein supplements to treat phenylketonuria, attention deficit-hyperactivity disorder (ADHD), narcolepsy, chronic fatigue syndrome, etc. Precursor of melanin. Used for the production of L-DOPA.
L-Valine	Fermentation, Enzymatic	*E. coli*; *C. glutamicum*; *Brevibacterium flavum*	Ingredient for food supplements for bodybuilders. Supplemental dosages are often used to correct deficiencies in alcoholics and drug addicts.

The first report on the introduction of fermentation in the industrial production of amino acids was that of Kinoshita et al. in 1957 (42). They highlighted the discovery of *Corynebacterium glutamicum*: a soil bacteria with the unique ability to produce considerable amounts of L-glutamine from sugar and ammonia. A few years later, a homoserine-auxotrophic mutant of *C. glutamicum* produced a large amount of L-lysine by fermentation. (11, 18, 31, 32). Other amino acids produced by Corynebacteria include L-valine, L-isoleucine, L-threonine, L-aspartic acid and L-alanine (17, 21, 24, 28, 52). The amino acids L-phenylalanine, L-threoine and L-cysteine can be obtained by fermentation with *E. coli* strains (14, 27).

Almost all proteinogenic amino acids, with a few exceptions (i.e. methionine), can be produced industrially by specially developed mutants of *C. glutamicum* (24) or *E. coli* (11, 34, 51), as shown in **Table 1**. Other species that are used in amino acid manufacture are *Brevibacterium spp.* (*B. divaricartum*; *B. alanicum*), *Microbacterium spp.* (*M. flavum* var. *glutamicum*), *Arthrobacter spp.* (*A. globiformis*; *A. aminofaciens*) etc.

Coryneform bacteria have played a principal role in the advances of amino acid fermentation industry (31, 47, 60). *Corynebacterium crenatum* and *Corynebacterim pekinese*, as well as *Corynebacterium glutamicum*, are used in amino acid production process in China. Strain improvement has mainly been carried out through an iterative process of mutagenesis and screening. By these methods, however, it is difficult to increase the production yield further, which is why genetic and metabolic engineering are employed for strain improvement (5).

The advantages and disadvantages of the presented amino acid manufacturing methods are shown in **Table 2**. The advantage of chemical synthesis is that it can be carried out on a very large scale, and often in a continuous way (4). Its major disadvantage is that it typically gives a racemic mixture of L- and D-forms of amino acids.

Extraction of amino acids from protein hydrolysate as a method of obtaining L-amino acids is now of only limited importance. Although still relevant for production of L-serine, L-proline, L-hydroxyproline, and L-tyrosine, for example, it is not suitable for large-scale production of amino acids. The extraction method for obtaining L-glutamate was superseded nearly 50 years ago by fermentation, following a sharp increase in the demand for the flavor enhancer MSG (34).

Biotechnology methods (fermentation and enzymatic methods) have economic and ecological advantages. The enzymatic method has an advantage of producing optically pure amino acids in higher concentrations with less by-products. The fact that the method uses specific substrates that are converted to amino acids limits the competitiveness of the method which is primarily dependent on the costs for manufacturing the substrate. In the industrial production of L-amino acids, the enzymatic method is not as popular as the fermentation method. The fermentation method is applied for the industrial production of most L-amino acids, except for a few kinds of amino acids for which high production yields have not been achieved by fermentation. According to Ikeda (27), the economy of this method depends mainly on the cost of the carbon source, fermentation yield, purification yield, and productivity in the overall process. Some problems in the fermentation processes can occur such as contamination of the culture with other microorganisms, bad fermentation reproducibility due to differences in the raw materials, back mutation or loss of genetic material in the production strain, infection of the culture etc. (27).

Improvements in biotechnology production of amino acids

The genome of the production organism is altered at random, and more efficient mutants are identified in a subsequent selection process. The mutants are usually characterized by increased membrane permeability, regulation defects, or biosynthesis enzymes with altered kinetic characteristics (7, 16, 25, 48). Fundamental understanding of the microbial amino acid metabolism is necessary for further increases in productivity. Detailed analysis allows quantification of the flow of metabolites in a fermentation process as a function of time (metabolic flux analysis). In this way the addition of nutrients can be optimized, and yields can be increased as a result. Metabolic flux analysis also makes it possible to model the metabolism of a given production strain (metabolic modeling).

Another important requirement for the targeted improvement of amino acid production strains is knowledge about the genome of the microorganism concerned. The genome of *C. glutamicum* has already been sequenced (8).

Not only fermentation processes, but also enzymatic processes are undergoing continual improvement, especially concerning the identification of novel enzymes. Unlike the classical screening, modern technologies access the genetic information directly (49). Specific characteristics of known

TABLE 2

Amino acids manufacturing methods: weaknesses and strengths

Manufacturing method	Weaknesses	Strengths
Protein hydrolysis	Limited raw materials (hair, feather, soybean, etc.). Not suitable for large scale production.	Unused protein-rich biomass remaining from different processes can be used as raw materials.
Chemical synthesis	Produced amino acids are D,L-forms and additional optical resolution step is needed in order to obtain bioactive L-isomers. High costs associated with the additional step.	Very large-scale production.
Fermentation	Not all amino acids can be produced cost efficiently – i.e. L-methionine.	Simple process. Economic and ecological advantages. High production capacity. Use of cheap and renewable carbon sources (sucrose, glucose, molasses, etc.)
Enzymatic (biotransformation)	Specific substrates used as starting materials.	Manufacture of optically pure amino acids. Economic and ecological advantages.

enzyme systems can also be improved through incorporation of mutations into the genes of the biocatalyst (37, 40). The optimized biocatalysts can also be transferred into production strains to avoid bottle necks in amino acid biosynthesis.

Conclusions

Biotechnology methods are widely established in the production of proteinogenic amino acids. Out of 15 amino acids included in our study, 14 are routinely manufactured applying biotechnology methods mostly from specially developed mutants of *C. glutamicum* or *E. coli*. Despite the fact that some of the amino acids cannot be produced cost effectively via biotechnology, biotechnology plays a significant role in the production of proteinogenic amino acids.

Modern biotechnology is applied to increase the production yield: genetic engineering and metabolic engineering are used for strain improvement, a fact that will additionally strengthen the microbial amino acid production. Enzyme catalysis will remain the preferred production method for nonproteinogenic amino acids and amino acid derivatives (2).

Both the fermentation and enzyme-based production methods play a central role in the production of L-amino acids used as ingredients in the pharmaceutical industry and provide the constant growth of the amino acid market. Research is being conducted to improve the biotechnology methods applied in the production of these biologically important compounds by modification of culture conditions, mutation, recombinant DNA technology, etc.

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