

Survey Report on the Systematization of Amino Acid Fermentation Technology

Shigeru Nakamori

■ Abstract

Amino acids are the building blocks of proteins. There are 20 kinds of amino acids; these have been found to have various biological and chemical functions. They are used many useful applications, including seasoning, medicines, supplement, feed-additives, chemicals, and cosmetics. While there are three methods currently used to produce amino acids, namely hydrolysis from protein, chemical synthesis, and fermentation, the dominant method is fermentation. Amino acid fermentation, in which microbes are used to produce amino acids, originated and developed in Japan; this technology is now used to supply over half of the world's amino acid market. This report provides a systematic survey of amino acid fermentation technology, tracing its history and outlining the prospects for future technological developments.

Monosodium glutamate (MSG) was the first amino acid to be commercialized. In 1908, Kikunae Ikeda invented a method of producing this amino acid from gluten, wheat protein. It was released as a seasoning by Saburōsuke Suzuki under the brand name of "Aji-no-moto." While this enterprise succeeded despite much difficulty, further technological improvements were needed due to issues with securing raw materials and working with hot hydrochloric acid. These improvements were taken from two approaches: chemical synthesis and fermentation. Although a means of producing glutamic acid by chemical synthesis was established to the point of factory production, chemically synthesized glutamic acid was not well received by consumers. Meanwhile, researchers at Kyowa Hakko discovered a new glutamic-acid-producing bacterium, *Corynebacterium glutamicum*, resulting in the first successful fermentative production of glutamic acid. Amino acids produced by fermentation were characterized in that a natural form of amino acid could be produced from a low-cost raw material such as glucose through a reaction in mild conditions. Glutamic acid was followed by over 15 more amino acid products produced by fermentation, including lysine, aspartic acid, and threonine. Amino acid fermentation technology spread throughout the world, with an increase in amino acid production volume. MSG production reached 1.7 million tons worldwide in 2005, with an expected annual increase of 3-4 %.

While there are four elements to amino acid fermentation technology, namely breeding of producing strains, large-scale cultivation methods, separating/refining, and engineering, the fundamental element is the breeding producing strains. Microbe cells have a mechanism or metabolic regulation operating within them that governs the production of metabolites like amino acids. Accordingly, breeding amino-acid-producing strains involves applying science and technology from biochemistry, genetics and new genetic engineering techniques to overcome this metabolic regulation.

The following specific methods have been achieved to break down or bypass the metabolic regulation. Glutamic acid fermentation was accomplished by culturing *C. glutamicum* under the optimum conditions for its characteristics. Lysine and ornithine fermentation was developed by deriving auxotrophic mutants from *C. glutamicum* to reduce the concentration of threonine or arginine, which have a metabolic regulation effect. Many strains producing threonine, lysine, tryptophan, and other amino acids were developed by selecting amino acid analog resistant strains. Other methods developed include bioreactor and enzymatic production using microbes with enzymes that convert to amino acids from other cheaply available materials. The application of new genetic engineering technology has resulted in improvements in producing strains, for both fermentation and enzymatic approaches, and new fermentation production.

Large-scale cultivation for amino acid production was established based on cultivation methods for antibiotics production. Isolating and purifying the amino acids from the fermentation fluid is an important part of the process, not only because it determines the quality of the product, but also because the yield at this stage has a significant impact on the cost. Computer-controlled systems have been developed to handle the engineering of large-scale cultivation and large-scale purification.

Amino acid production plants using Japanese technology have spread throughout the world. The raw materials used include starch, raw sugar, or molasses. Therefore, amino acid production is strongly linked to the availability of raw materials. Most amino acids that are consumed overseas, such as glutamic acid, lysine, and threonine, are produced at overseas factories.

Amino acid fermentation processes are natural processes, such as those used at brewing plants for sake or soy sauce. Environmentally-friendly systems are being put in place. Grains and other livestock fodder are lacking in certain amino acids; adding these amino acids to livestock fodder is useful in not only improving the amino acid balance of foods, but also reducing ammonia emissions from animal waste and improving the environment.

The 1970s saw consumer action over the safety of amino acids, but it was proven scientifically that this was not an issue. Nevertheless, the "aftereffects" of this action still linger today, with deep-seated concern over the safety of amino acids.

Future developments can be expected in the field of amino acids, as there are no alternative products to them. A significant issue will be the rising price of raw materials; technology and price competition against overseas manufacturers with a secure supply of raw materials will be a major challenge for Japanese engineers, although they invented the technology. To meet this challenge, it will become all the more important to breed strains for improved production using genetic information and genetic engineering, to develop new raw materials and to cultivate strains that can use these raw materials, and to develop environmentally-focused processes. Technological developments can be expected in these areas.

Technology has recently been developed to synthesize peptides from amino acids using microbial enzymes. Use of this technology can be expected to extend to pharmaceuticals and other bioactive compounds.

■ Profile

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■ Contents

1	Introduction	2
2	About Amino Acids	3
3	Origins of Amino Acid Fermentation	8
4	Origins of Amino Acid Fermentation: Amino-Acid-Producing Bacteria Screening and Strain Breeding	11
5	Development of Cultivation Technology and Equipment	23
6	Development of Amino Acid Separation and Purification Technology	25
7	Amino Acid Standards	27
8	Main Raw Materials for Amino Acid Fermentation	28
9	Overseas Expansion of Amino Acid Fermentation Technology	29
10	Amino Acids and Environmental Issues	31
11	Safety of Amino Acids	32
12	Systematization of Amino Acid Fermentation Technology ..	34
13	Future Developments	36
14	Acknowledgements	38

1 Introduction

Amino acids are the building blocks of proteins. Even as simple substances, these have many useful functions and are widely used to benefit human welfare throughout the world, as flavor enhancers or as nutritional components in pharmaceutical products or animal feed. While amino acids have gained recent media attention and come into the awareness of the general public, it is not widely known that amino acid technology, particularly the fermentation process used at the core of the production technology, originated in Japan or that it was Japanese technology that grew it into a world-leading industry, accounting for over half of the world's production output. No other substance can replace the functions of amino acids. Accordingly, the industry can be expected to develop further, with further increase in demand inevitable. In light of the development that has taken place in the 50 years since amino acid fermentation began in Japan, it is very significant to anticipate the prospects of future

development.

Chapter 2 of this report discusses what kind of substance amino acids are, what functions they have, how they are used, the progress in production methods, and changes in production output, as well as providing an overview of amino acid fermentation technology, the main topic of this report. Chapter 3 outlines the factors leading up to the emergence of amino acid fermentation in Japan. Chapter 4 and onwards discusses the history and current state of the four basic technologies for amino acid fermentation, namely screening and breeding producing strains, large-scale cultivation, separation/purification, and engineering, as well as systematizing the technology and examining the future and prospects.

2 | About Amino Acids

2.1 What are Amino Acids?

Amino acid is a general term for chemical compounds with an amino group (-NH_2) and a carboxyl group (-COOH) in the same molecule. While there are different amino acids such as α , β , γ , etc., with the carbon in different positions in the amino and carboxyl group (Fig. 2.1), the α -amino acids are the most common and act as a structural component in the protein in all organisms. This report discusses α -amino acids (little mention is made of β -, γ - or other amino acids, as little substantive development has been made on them at this stage).

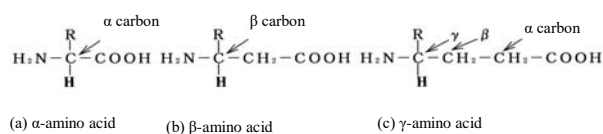


Fig. 2.1. α , β and γ -Amino Acid Structural Formulas
These structures differ according to the different amino group binding positions; R is a chemical side chain and represents an atomic group or hydrogen atom.

α -Amino acids are structural components in protein; their chemical formula can be represented as shown in Fig. 2.1 (a). There are 20 kinds of amino acids in ordinary protein, depending on differences in R. The names, abbreviations, single-letter designations, structural formulas, molecular formulas, and molecular weights are given in Table 2.1. Of these, isoleucine, threonine, tryptophan, methionine, phenylalanine, valine, lysine, and leucine cannot be synthesized *de novo* by humans, and so are called essential amino acids.

Amino acids rarely exist in an isolated state in an organism, instead existing as high molecular protein in which the amino group in one amino acid links to the carboxyl group in another amino acid through dehydrated and condensed peptide bonds. As is well-known, proteins are an essential component of biological functions, forming the muscles, blood, bones, skin, nails, and other tissue in an organism, as well as the hormones, or regulatory substances, and enzymes, which act as biocatalysts. If a protein is processed with hydrochloric acid or a proteolytic enzyme, the peptide bond hydrolyzes, resulting in amino acid (Fig. 2.2). In practice, proteins ingested as part of the diet are broken down through the action of pepsin, trypsin, chymotrypsin, and other proteolytic enzymes in the stomach, small intestine, and other parts of an animal's alimentary canal. The various amino acids produced as a result re-form into specific proteins needed by the animal.

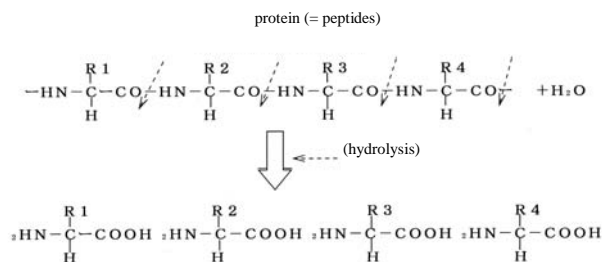


Fig. 2.2. Formation of Amino Acids due to Hydrolysis of Protein (= Peptides)

The position of the arrows represents the severing of the peptide bond.

Since there are theoretically 20 kinds of amino acids contained in the hydrolyzed solution obtained by processing proteins with hydrochloric acid or enzymes in a reaction vessel, each amino acid could be obtained by separating and purifying these. While these would appear difficult to differentiate from external appearance due to being in white crystal (or powder) form, the crystals have distinctive characteristics when viewed under a microscope. Glutamic acid crystals and lysine crystals are shown here as examples (Fig. 2.3).



Fig. 2.3 (1) Photograph of Glutamic Acid (α) Crystals (supplied by Ajinomoto Co., Inc.)

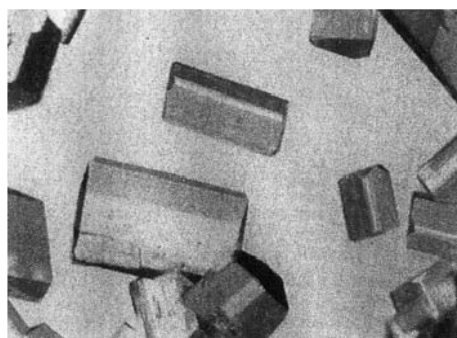


Fig. 2.3 (2) Photograph of Lysine Hydrochloride (supplied by Ajinomoto Co., Inc.)

Table 2.1 Classification of Proteinous Amino Acid, Notation, Chemical Structure, Molecular Formula, and Molecular Weight

Classification	Name	Symbol	Chemical Structure	Molecular Formula	Molecular Weight
Neutral amino acids	Glycine	Gly (G)	$\text{NH}_2\text{-CH}_2\text{-COOH}$	$\text{C}_2\text{H}_5\text{NO}_2$	75.07
	Alanine	Ala (A)	$\begin{array}{c} \text{CH}_3\text{-CH-COOH} \\ \\ \text{NH}_2 \end{array}$	$\text{C}_3\text{H}_7\text{NO}_2$	89.09
	Valine	Val (V)	$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3\text{-CH-CH-COOH} \\ \\ \text{NH}_2 \end{array}$	$\text{C}_5\text{H}_{11}\text{NO}_2$	117.15
	Leucine	Leu (L)	$\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3\text{-CH-CH}_2\text{-CH-COOH} \\ \\ \text{NH}_2 \end{array}$	$\text{C}_6\text{H}_{13}\text{NO}_2$	131.18
	Isoleucine	Ile (I)	$\begin{array}{c} \text{CH}_3 \\ \\ \text{C}_2\text{H}_5\text{-CH-CH-COOH} \\ \\ \text{NH}_2 \end{array}$	$\text{C}_6\text{H}_{13}\text{NO}_2$	131.18
	Serine	Ser (S)	$\begin{array}{c} \text{CH}_2\text{-CH-COOH} \\ \quad \\ \text{OH} \quad \text{NH}_2 \end{array}$	$\text{C}_3\text{H}_7\text{NO}_3$	105.09
	Threonine	Thr (T)	$\begin{array}{c} \text{CH}_3\text{-CH-CH-COOH} \\ \quad \\ \text{OH} \quad \text{NH}_2 \end{array}$	$\text{C}_4\text{H}_9\text{NO}_3$	119.12
	Cysteine	CysH (C)	$\begin{array}{c} \text{HS-CH}_2\text{-CH-COOH} \\ \\ \text{NH}_2 \end{array}$	$\text{C}_3\text{H}_7\text{NO}_2\text{S}$	121.15
	Cystine	Cys (CC)	$\begin{array}{c} \text{S-CH}_2\text{-CH-COOH} \\ \quad \\ \text{NH}_2 \quad \text{NH}_2 \\ \\ \text{S-CH}_2\text{-CH-COOH} \\ \\ \text{NH}_2 \end{array}$	$\text{C}_6\text{H}_{12}\text{N}_2\text{O}_4\text{S}_2$	240.30
	Methionine	Met (M)	$\begin{array}{c} \text{CH}_3\text{-S-CH}_2\text{-CH}_2\text{-CH-COOH} \\ \\ \text{NH}_2 \end{array}$	$\text{C}_5\text{H}_{11}\text{NO}_2\text{S}$	149.21
	Phenyl-alanine	Phe (F)	$\begin{array}{c} \text{C}_6\text{H}_5\text{-CH}_2\text{-CH-COOH} \\ \\ \text{NH}_2 \end{array}$	$\text{C}_9\text{H}_9\text{NO}_2$	165.19
	Tyrosine	Tyr (Y)	$\begin{array}{c} \text{HO-C}_6\text{H}_4\text{-CH}_2\text{-CH-COOH} \\ \\ \text{NH}_2 \end{array}$	$\text{C}_9\text{H}_9\text{NO}_3$	181.19
	Tryptophan	Trp (W)	$\begin{array}{c} \text{C}_8\text{H}_7\text{-CH}_2\text{-CH-COOH} \\ \\ \text{NH} \end{array}$	$\text{C}_{11}\text{H}_{12}\text{N}_2\text{O}_2$	204.23
	Proline	Pro (P)	$\begin{array}{c} \text{CH}_2\text{-CH}_2 \\ \quad \\ \text{CH}_2\text{-CH-COOH} \\ \\ \text{NH} \end{array}$	$\text{C}_5\text{H}_9\text{NO}_2$	115.13
	Hydroxy-proline	Hyp (-)	$\begin{array}{c} \text{HO-CH-CH}_2 \\ \quad \\ \text{CH}_2\text{-CH-COOH} \\ \\ \text{NH} \end{array}$	$\text{C}_5\text{H}_9\text{NO}_3$	131.13
	Asparagine	Asn (B)	$\begin{array}{c} \text{H}_2\text{NOC-CH}_2\text{-CH-COOH} \\ \\ \text{NH}_2 \end{array}$	$\text{C}_4\text{H}_8\text{N}_2\text{O}_3$	132.12
	Glutamine	Gln (Z)	$\begin{array}{c} \text{H}_2\text{NOC-CH}_2\text{-CH}_2\text{-CH-COOH} \\ \\ \text{NH}_2 \end{array}$	$\text{C}_5\text{H}_{10}\text{N}_2\text{O}_3$	146.15
Acidic amino acids	Aspartic acid	Asp (D)	$\begin{array}{c} \text{HOOC-CH}_2\text{-CH-COOH} \\ \\ \text{NH}_2 \end{array}$	$\text{C}_4\text{H}_7\text{NO}_4$	133.10
	Glutamic acid	Glu (E)	$\begin{array}{c} \text{HOOC-CH}_2\text{-CH}_2\text{-CH-COOH} \\ \\ \text{NH}_2 \end{array}$	$\text{C}_5\text{H}_9\text{NO}_4$	147.13
Basic amino acids	Lysine	Lys (K)	$\text{H}_2\text{N-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH-COOH}$ $ $ NH_2	$\text{C}_6\text{H}_{14}\text{N}_2\text{O}_2$	146.19
	Histidine	His (H)	$\begin{array}{c} \text{HC=C-CH}_2\text{-CH-COOH} \\ \quad \\ \text{N} \quad \text{NH} \quad \text{NH}_2 \\ \\ \text{CH} \end{array}$	$\text{C}_6\text{H}_9\text{N}_3\text{O}_2$	155.16
	Arginine	Arg (R)	$\begin{array}{c} \text{NH} \\ \\ \text{H}_2\text{N-C-NH-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH-COOH} \\ \\ \text{NH}_2 \end{array}$	$\text{C}_6\text{H}_{14}\text{N}_4\text{O}_2$	174.20

Carbon atoms that attach to four different atoms or atom groups, such as the amino group, the carboxyl group, and R (atom group or hydrogen atom) in the amino acid, are called asymmetric carbon atoms. When exposed to polarized light, chemical compounds with asymmetric carbons, such as amino acids, rotate the plane of polarized light at a constant angle to the left or to the right. Some compounds are L-compounds and others are D-compounds; all the natural amino acids in protein are L-compounds.

All amino acids produced by fermentation, the main topic of this report, are L-compounds. While this paper focuses on L-amino acids in principle, D-amino acids will sometimes be mentioned in a limited context. By contrast, amino acids obtained by chemical synthesis are DL-compounds (or racemic compounds) comprising an equal mixture of enantiomers D- and L-compounds. To obtain a biologically-active L-compound from a DL-compound requires an optical resolution process. While a small quantity of D-amino acid has recently been found in the brain and other organs, its purpose and function are still in the process of being identified, so it is not touched on in this report.

2.2 Functions and Uses of Amino Acids

While amino acids play an important role in organisms as the building blocks of proteins, what function do they serve? In other words, what utility value do they have? This utility value can be represented as the commodity value of amino acids.

The first amino acid to be developed into a commercial product was monosodium glutamate, or MSG. Kikunae Ikeda, a professor of the Tokyo Imperial University College of Science, discovered MSG as the flavor component of *kombu* and invented a method of producing it from wheat gluten.⁽¹⁾ It was released by Ajinomoto Co., Inc. under the name of “Ajinomoto” and continues to be a major product used around the world. The other amino acids produced as byproducts to glutamic acid initially received no particular attention, but their importance was later recognized when they were discovered to have various functions in nutrition and medicine as those fields advanced. They have continued to expand in use as the below applications for them have developed. To accommodate these applications, there has been a need to find a cheap production method for these amino acids as well as glutamic acid.

The uses for amino acids can be broadly divided into the following four categories according to function.⁽²⁾

1) Use as a Flavor Component

Amino acids each have a specific flavor. The most representative example of these is MSG, which is produced on a large scale as a flavor enhancer. Glycine

and alanine are sweet, while valine and isoleucine are bitter. Mixtures of amino acids are also used for seasoning. Aspartame, a dipeptide made up of aspartic acid and phenylalanine methyl ester, has also been developed as a low-calorie sweetener.

2) Use as a Nutritional Component

Amino acids are used as a nutritional component. Amino acid infusion and enteral nutrition involve dispensing a pharmaceutical product made up of a combination of effective amino acids to match the amino acid composition of a protein such as blood plasma, ovum, or milk to perioperative patients for nutritional support, fatigue recovery or to improve strength. Amino acids have also been used as nutritional fortifiers or food additives in health foods, sports nutrition products, and supplements, as well as in cosmetics. Many other applications have been developed, such as for improving the nutritional quality of the proteins in feed grain for pigs and chickens or as a medium component for culturing zooblasts or producing microbial enzymes. Combinations of effective amino acids are used in these applications rather than amino acids on their own.

3) Use in Pharmacological Functions

Amino acids also have particular pharmacological functions and are used in pharmaceutical products, including liver disease treatments (isoleucine, valine, leucine, arginine, etc.), digestive organ ulcer treatments (glutamine), Parkinson’s disease treatments (dioxiphenylalanine), and mucolytic agents (cysteine).

4) Uses for Amino Acid Reactivity

Amino acids also have a particular reactivity as chemical compounds and are used in the following applications: synthetic pharmaceutical materials, such as diabetes medicines (D-phenylalanine), anti-hypertensive agents (hydroxyproline, valine), antiviral agents (valine), and antibiotics (D-phenylglycine, D-*p*-hydroxyphenylglycine), as well as production materials, such as reaction flavors (cysteine), bread fermentation agents (cysteine), surfactants (N-acylglutamic acid), and perm solutions (cysteine). They have also been used in moisturizing products (proline). Amino acids also constitute the raw material for producing newly-developed peptides.

2.3 How are Amino Acids Produced?

The MSG production method invented by Kikunae Ikeda, mentioned in Section 2.2, was a protein hydrolysis method involving hydrolyzing wheat gluten or soy protein in hydrochloric acid and then separating and purifying the MSG from the resulting solution. While this method of production continued for around 50 years until about 1960, when the fermentation

method was established, it had a few technical areas that needed improvement, as shall be mentioned in Section 3.1. These obstacles were surmounted in a new chemical synthesis method that was developed, as well as in the fermentation method, which is the main topic of this report. While the fermentation method is the main method currently used to produce glutamic acid and many other amino acids, each method has its own advantages and disadvantages and all three methods are still in use today. The main factors for selecting a production method are cost and the image that method projects to consumers, since amino acids are mainly used in food and other internal applications. Amino acids produced by chemical synthesis include glycine, which has no enantiomers, and methionine, which has both D- and L-compounds and can be used in animal fodder, while tyrosine, leucine, cysteine, and asparagine are produced by protein hydrolysis. The main raw materials for protein hydrolysis are soy protein and casein; for chemical synthesis, the main raw materials are ethylene nitrile compounds and ammonia; the main raw materials used for the fermentation method are starch solutions (saccharified starch solutions), molasses, or raw sugar.

2.4 Amino Acid Production Quantities

The Japan Essential Amino Acids Association (now abolished) has published worldwide amino acid production quantity estimates every few years. Fig. 2.4⁽³⁾ shows the figures from this data for 1979 and 1996.

As indicated by the graphs, there is a clear significant increase in production quantities of all amino acids except for DL-alanine. Glutamic acid has a particularly notable increase in quantity, with 1 million tons in 1996 rising to 1.7 million tons by 2005, visibly increasing by a few per cent per annum. As a single chemical compound, it is an unprecedented product that has had continued growth since its inception 100 years before. While other epochal chemical compounds have emerged and been commercialized around the world, such as ammonium sulfate, urea, penicillin, and nylon, none of these have demonstrated such stable growth for such a long period of time. The significant value of glutamic acid is worth noting.

Other amino acids that have undergone significant growth in production quantity include lysine, used in animal fodder, with around 300,000 tons produced in 1996 rising to 800,000 tons in 2005, threonine, also used in animal fodder, which increased from only around 4,000 tons in 1996 to 80,000 tons in 2005, and tryptophan, which is estimated to have increased from 500 tons to 1,800 tons. Phenylalanine, used as a main ingredient in the low-calorie sweetener aspartame, and aspartic acid have also shown remarkable growth. Other amino acids have also increased in production quantities as pharmaceutical products and supplements.

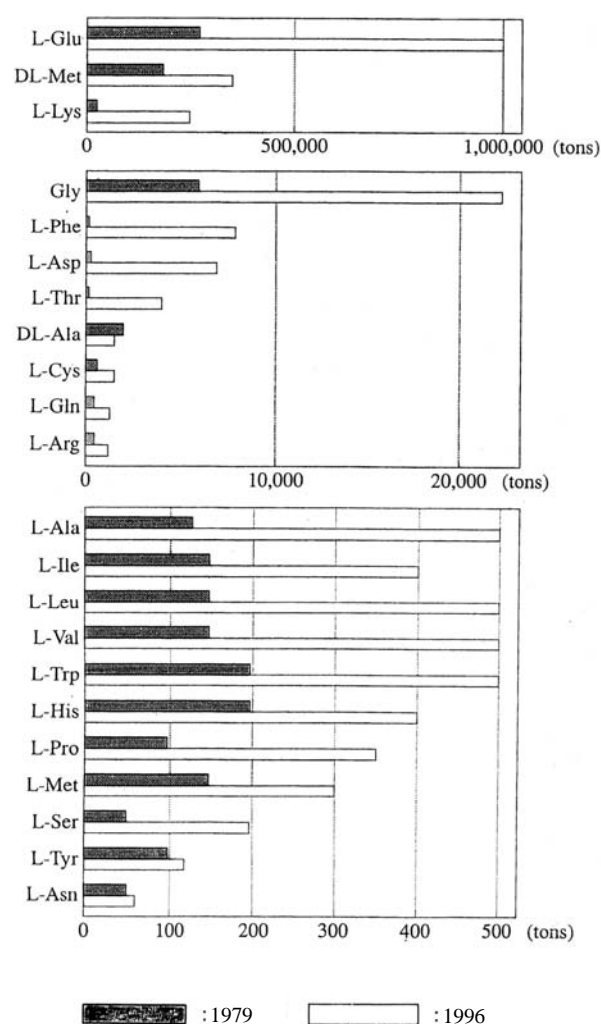


Fig. 2.4 Global Amino Acid Production Quantity Estimates⁽³⁾

2.5 Amino Acid Safety

While amino acids are the building blocks of proteins and were not considered to have any safety issues, in the 1970s there was a worldwide wave of concern over the safety of chemical compounds used in food and medicine and amino acids were caught up in these safety concerns as well. For example, there were reports of brain disorders in juvenile mice resulting from being force-fed MSG and other reports of the so-called “Chinese restaurant syndrome” occurring as a result of ingesting MSG added to Chinese cuisine. Nevertheless, earnest endeavors by mainly Japanese manufacturers established scientific evidence that these concerns were unfounded. Open and impartial discussions resulted in the USFDA and other agencies concluding that there were no safety issues and issuing a scientific “safety declaration.” However, the reality remains that there are many people around the world who do not believe that these are safe. In the 1980s, there were fatalities in the United States believed to be due to a tryptophan degradation product contained in

health foods, demonstrating the importance of checking the ingredients in an amino acid product.

2.6 What is the Amino Acid Fermentation Process?

The development of new applications and the decrease in costs due to improved production technology have made a significant contribution to the increase in amino acid production quantities shown in Section 2.4. While amino acids are essential substances, they are not a product that will always have a demand regardless of the price. Rather, the skillful combination of two factors – cost reduction due to improved production technology and the development of new applications – has resulted in increased demand.

Amino acid production technology includes the technology used for protein hydrolysis, fermentation, and chemical synthesis, mentioned in Section 2.3, as well as related technologies for separating, purifying, and engineering. Currently, the predominant production method is fermentation, the main topic of this report. Fig. 2.5 shows an overview of the amino acid fermentation process. The main technologies involved are for strain breeding, large-scale cultivation, separation/purification, and engineering. Of these, strain breeding is the fundamental technology that plays the greatest role in characterizing the amino acid fermentation.

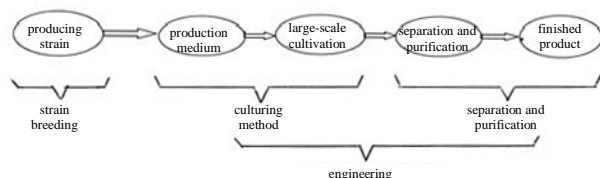


Fig. 2.5 Overview of Amino Acid Fermentation and Main Related Technologies

Strain breeding is the art of manipulating the cells involved in controlling the course of metabolism within a tiny cell, several micrometers in size, which takes the substrate (raw material) glucose and forms it into the resulting product amino acid, and secreting the amino acid outside of the cell. Specifically, this involves making repeated improvements to increase amino acid production by identifying the metabolic pathway for the amino acid and using cell mutation and gene manipulation to prevent metabolic regulation and thereby enable more effective production properties. There is usually no outwardly discernable difference between newly-obtained strains and parent strains. Large-scale cultivation technology involves placing these resulting microbes into the optimum conditions where they will multiply at a rate of 10^{16-17} and produce the maximum quantity of amino acid.

Separation/purification technology is the process of separating out the amino acid accumulated in the microbe culture fluid and refining it to crystals with a purity of almost 100 %. Engineering is the management of the processes involved in large-scale cultivation and large-scale purification, as well as managing the equipment. Computers are used for process control. Engineering also includes a lot of technological development challenges that can only be worked out at the stage of upscaling from a small-scale laboratory experiment.

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3 | Origins of Amino Acid Fermentation

Amino acid fermentation is a technology that originated in Japan around 50 years ago. An underlying factor leading up to the emergence of this technology is that as a humid rice-farming area situated in Asia's monsoon zone, Japan had long put microbes to use with its exceptional fermentation and brewing technology, typified by its sake, miso, and soy sauce. More directly, the Japanese already had MSG, the specific target of interest, and the scientific knowledge in biochemistry, microbiology, and molecular biology to enable its production by fermentation, as well as the necessary screening and cultivation technology used for antibiotic-producing strains.

3.1 The Success of “Ajinomoto” (MSG) and Technological Issues

The first target for amino acid fermentation was “Ajinomoto” or MSG. As mentioned in Section 2.2, MSG had been discovered as the flavor component of *kombu* by Kikunae Ikeda in 1908 and patented as a seasoning. Later, a method of producing it by hydrolyzing gluten, the protein found in wheat, or soy protein using hydrochloric acid was established and the famous “Ajinomoto” was developed through a joint venture between Prof. Ikeda and Saburōsuke Suzuki, founder of Suzuki Pharmaceutical Company, later Ajinomoto Co., Inc. Fig. 3.1 shows photographs of Kikunae Ikeda and Saburōsuke Suzuki II. Fig. 3.2 shows a photograph of the first glutamic acid sample (labelled “*glutami* acid”) successfully extracted from *kombu* and crystallized. This invention has been lauded as one of the three greatest inventions of the Meiji era, along with Kōkichi Mikimoto's cultured pearl and Sakichi Toyoda's automatic power loom.

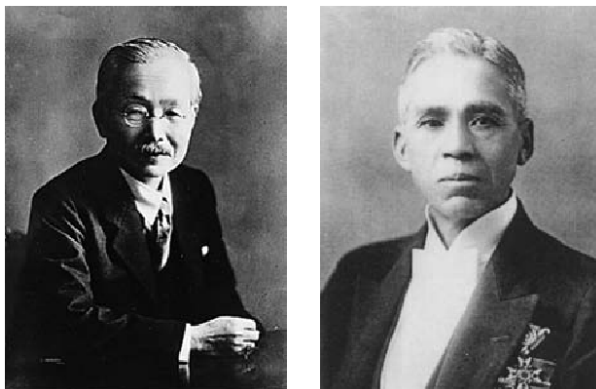


Fig. 3.1 Prof. Kikunae Ikeda (left) and Saburōsuke Suzuki II (right), Second President of Ajinomoto Co., Inc. (supplied by Ajinomoto Co., Inc.)



Fig. 3.2 Sample Bottle Containing Kikunae Ikeda's First Successful Extract of Glutamic Acid (supplied by Ajinomoto Co., Inc.)

Since “Ajinomoto” was a completely new product, there were many challenges in making it into a viable business, in terms of both technology and marketing; however, these were overcome and the business was successfully established.⁽¹⁾

Kikunae Ikeda majored in physical chemistry and so the invention of “Ajinomoto” was a successful achievement outside of his area of expertise. This success can be attributed to his strong entrepreneurial aptitude and his belief in advocating the idea that “taste promotes digestion” to the Japanese people, who had poor nutrition at the time. He had a strong desire to make food taste better and believed that he himself and all Japanese had more refined palates than people in the West. He sensed that a seasoning product would be accepted. Ten years after its founding, Ajinomoto Co., Inc. was performing stably and the business expanded its operations overseas.⁽¹⁾ Ikeda's patent lapsed in 1923, but a six-year extension was granted. When it expired in 1929, many companies started producing MSG. By 1930, these companies totaled 28, excluding Ajinomoto Co., Inc.⁽²⁾

However, a number of problems were identified with the production method that had been developed (proteolysis). One issue was with raw materials: as wheat and soy supplies were heavily reliant on imports, they were subject to quantitative limitations depending on circumstances overseas. There were also importing restrictions in place to protect domestic agriculture. Prices were high and it was difficult to keep costs down. Another issue was that hot hydrochloric acid was used for protein hydrolysis, which presented major problems with equipment corrosion. Various workarounds were devised, resulting in the development of a system using fine ceramic “*Dōmyōji-game*” pots as acid-proof containers. However this alone did not constitute any radical solution and there were still unresolved issues remaining, such as the difficulty of sequencing and automating operations and the need to consider workers' health and public pollution. A new method of

production was therefore needed to overcome these issues; investigations were carried out from two different approaches – chemical synthesis and fermentation. Ajinomoto Co., Inc. started working on this research around 1950.

Several methods of chemical synthesis were devised and trialed. A method was perfected using a Strecker reaction to combine carbon monoxide and hydrogen cyanide with acrylonitrile and Ajinomoto Co., Inc. started production at its plant in Tokai. This technology was lauded as epoch-making technology that could produce glutamic acid, a foodstuff, from petroleum products, initially thought to be inexhaustible. It was awarded the Chemical Society of Japan Award for Technology in 1964 and the Okouchi Memorial Production Award in 1965. However, consumers had a huge change of heart soon afterwards and the concept of “making food by chemosynthesis” was no longer accepted. Progress had also been made with fermentation, which had begun around the same time, and so the MSG chemical synthesizing plant closed in 1973.⁽³⁾

Meanwhile, the fermentation method had certain scientific and technological prerequisites, such as biochemical expertise on microbial amino acid metabolism, microbial genetics expertise on applied microbiology screening methods, mutation and harvesting mutants, and methods for culturing microbes. One key point of success was that these requirements happened to coincide with a sudden boom in this science and technology, which was quickly applied to the establishment of amino acid fermentation technology.

3.2 Advances in Biochemistry and Microbiology

From the 1930s to the 1950s, significant progress had been made in research on metabolisms within organisms, including the discovery of the glycolytic pathway, which breaks down glucose, the tri-carboxylic acid (TCA) cycle, and other major reaction pathways and identifying the biochemical significance of these. Naturally, this also meant an increase of knowledge about amino acid metabolism, which is closely linked to these areas. Research from the 1960s onwards clarified the regulation of enzyme synthesis, typified in Jacob & Monod’s operon theory on the metabolic control of amino acids and other substances, and the existence and mechanisms of negative feedback control to inhibit feedback. In other words, amino acid synthesis in an organism is strongly linked to metabolic regulation, in which the amino acid itself regulates the production and activity of the enzymes that catalyze the synthesis reaction (see Section 4.3). Accordingly, it became clear that overproduction or fermentation of amino acid could not be achieved unless the control effect in the amino acid itself could be eliminated. This

knowledge was a prerequisite strategy in making amino acid fermentation possible.

3.3 Advances in Molecular Biology and Microbial Genetics

The transformation of *Pneumococcus* by O. Avery et al. demonstrated that DNA is the main substance in genes. In 1953, Watson & Crick clarified the mechanisms in the central dogma, in which DNA has a double helix structure and that the genetic information in DNA is transcribed to RNA and then translated to protein. This opened up a new major research field, molecular biology. Major research avenues were opened relating to the collection and utilization of mutant strains, including the idea that mutation occurs due to changes in the base sequence of the DNA and the idea that mutations occur in greater frequency when treated with ultra-violet rays, X-rays, or chemical substances. These discoveries provided the tools required to develop amino acid fermentation technology using mutant microbe strains. The use of mutant strains was not directly related to glutamic acid fermentation, but it was used in lysine fermentation and subsequent compounds.

3.4 Microbe Screening Technology / Discovery of Antibiotics and Development of Production Processes

In 1929, A. Fleming discovered that blue mold produces penicillin. Around 1940, H. W. Florey et al. improved penicillin production to practical implementation level and it proved to be remarkably effective in treating infections contracted by wounded soldiers in the Second World War. This was followed by the discoveries of a number of antibiotics, such as streptomycin by S. A. Waksman, chloramphenicol and aureomycin. The golden age of antibiotics continued from around 1940 to around 1980. These discoveries were the major dream of microbe researchers. Professor Kinichiro Sakaguchi of the University of Tokyo, an authority on applied microbiology with a long record of achievements, is quoted as saying, “I put my hope in microbes and have never been let down.” There are a great variety of microbes and countless numbers of them; it is certain that there were researchers that believed in the existence of microbes that produce amino acids. There was a lot to be learnt about microbe screening from the discovery of antibiotics.

3.5 Advances in Large-Scale Cultivation Technology

As discussed in Section 3.4, antibiotics were being produced using blue mold and actinomycetes. Jar fermenters and fermentation tanks were developed for aeration-agitation cultivating. Many companies in Japan started working on developing technology for penicillin fermentation in the late 1940s; this experience proved to be a major advantage in establishing amino acid fermentation.

<References>

- (1) Ajinomoto Co., Inc.: *Aji o Tagayasu: Ajinomoto Hachijūnen-shi [Cultivating Taste: 80-Year History of Ajinomoto]*, pp. 48-102, July 1990.
- (2) S. Ota: *Umami Chōmiryō no Chishiki [Knowledge of Flavor Seasoning]*, p. 4, Saiwai Shobo, June 1992.
- (3) Ajinomoto Co., Inc.: *Kenkyūjo 50-nen-shi: 'Ashita no Moto' o Motomete [A 50-Year History of the Institute: Searching for the 'Substance of Tomorrow']*, p. 100, September 2006.

4 Origins of Amino Acid Fermentation: Amino-Acid-Producing Bacteria Screening and Strain Breeding

4.1 Glutamic Acid Production through Two-Step Fermentation

Glutamic acid was known to be produced by introducing an amino group to 2-oxo-glutaric acid (2OG, α -ketoglutarate) produced by a reaction in the TCA cycle (Fig. 4.1) in an organism. It was also known that 2OG could be produced by fermentation. It was thus believed that glutamic acid production was “almost within reach” from the perspective of biochemical metabolic pathways and appears to have come to the attention of many researchers. Initial studies by companies including Ajinomoto Co., Inc. and Kyowa Hakko Kogyo Co., Ltd. investigated two-stage fermentation to increase and aminate the 2OG output. The need for Ajinomoto Co., Inc. to develop technology as the prior specialist manufacturer is mentioned above. The fact that Kyowa Hakko was a newcomer to glutamic acid did not mean that it had any trouble producing MSG. It already had fermentation technology, as it had intended to use it in a new research project producing protein to address the nutrition problems in postwar Japan. Instead, it changed its aim from protein to glutamic acid and started its research.⁽¹⁾

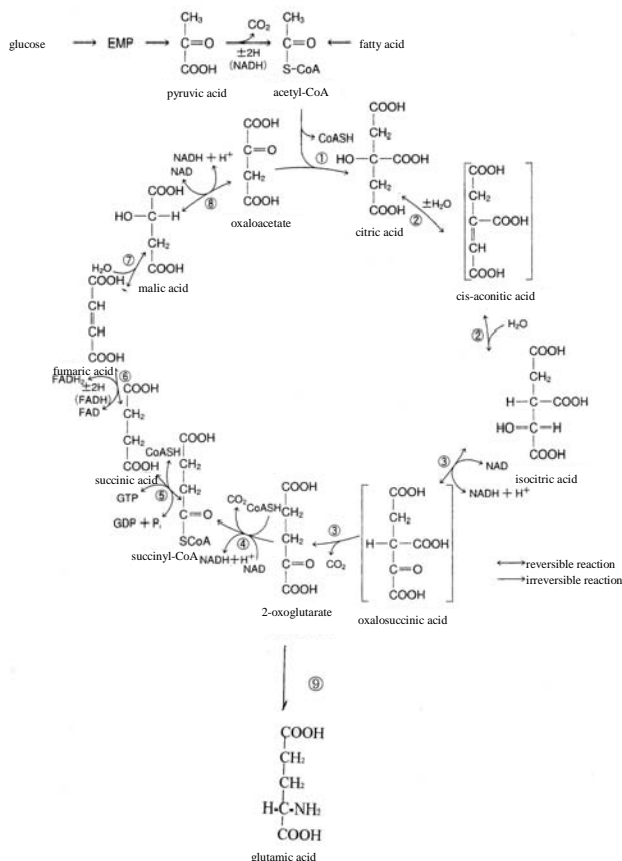


Fig. 4.1 Positional Relationship of Glutamic Acid in the TCA Cycle
 (1) citrate synthase; (2) aconitate hydratase; (3) isocitrate dehydrogenase;
 (4) 2-oxoglutarate dehydrogenase complex; (5) succinyl-CoA synthetase;

(6) succinate dehydrogenase complex; (7) fumarate hydratase;
 (8) malate dehydrogenase; (9) glutamate dehydrogenase

While the production quantities of 2OG increased considerably, there are no reports of its amination. This is probably because the direct fermentation method proved to be successful in the meantime, as shall be discussed next, and this method was discontinued.

4.2 Glutamic Acid Production through Direct Fermentation

4.2.1 Discovery of Glutamic-Acid-Producing Microbes

As well as working on the two-stage fermentation process mentioned above, Kyowa Hakko also successfully screened a strain of bacterium that directly produced glutamic acid and succeeded in establishing a new fermentation method, namely glutamic acid fermentation.⁽²⁾

It was not common knowledge at the time amino acids such as glutamic acid could be produced directly from glucose. Accordingly, although this experiment literally defied common sense, perhaps some researchers believed that they could achieve a glutamic acid microbe. The keys to this success were in the accurate method of strain screening and in the clever application of a bioassay method to quantify the amino acid. In other words, some lactic acid bacteria require amino acids for growth; the bioassay determined the quantity of the amino acid using the proportional relationship between the quantity of the required amino acid and the growth amount. Specifically, the lactic acid bacteria that require several types of amino acids for growth were pre-coated onto nutrient agar with the glutamic acid removed and the naturally-selected microbes were then cultivated. This system made it possible to easily detect the microbes that produced glutamic acid, as they had an observable ring of lactic acid bacteria growth around them. Within a short time, the ones with a ring of growth around them were collected and confirmed to be producing glutamic acid in a liquid culture.⁽³⁾

Microscope observation revealed that this strain exhibited a spherical shape. It was categorized as a new strain under the name *Micrococcus glutamicus*; this name was later changed to *Corynebacterium glutamicum* as it was observed to change in shape under different cultivation conditions.

Cultivation was carried out using several combinations of culture mediums to alter the amount of required nutrients. The amount of added biotin, a type of vitamin, was found to be a significant factor. In other words, while biotin had to be added to the culture

medium because the microbe required biotin to grow, the concentration was extremely important. Anything over 10 µg/L resulted in no glutamic acid production at all, while cultivation at 3 µg/L resulted in 40-50 g of glutamic acid per liter from 100 g of glucose.⁽⁴⁾ Once Kyowa Hakko had reproduced this experiment at its pilot plant at flask level, it issued a press release on September 21, 1956 announcing its successful glutamic acid fermentation and its intention to enter the MSG industry. This successful glutamic acid fermentation is regarded as the start of amino acid fermentation. The discovery of a glutamic acid microbe had huge significance in that it opened up an entirely new technological field, that of producing not only glutamic acid but also many other amino acids by fermentation. This announcement by Kyowa Hakko shocked Ajinomoto Co., Inc. and other existing MSG manufacturers. Having taken over the patent rights to a “Method for Cultivating α -Ketoglutaric-acid-producing strains in a culture medium containing a large amount of nitrogen” (the “Tada patent”) granted to Yasuji Tada and Oki Nakayama in 1954, Ajinomoto Co., Inc. and Sanraku Shuzo handled the matter at patent level. At the same time, the various companies and universities actively engaged in developing new microbe strains. This resulted in a succession of strain discoveries, including *Brevibacterium flavum* and *Brevibacterium lactofermentum* by Ajinomoto Co., Inc. and Sanraku Shuzo, *Microbacterium ammoniaphilum* by Asahi-Kasei, *Brevibacterium thiogenitalis* by Takeda Pharmaceutical Co., Ltd., and *Brevibacterium divaricatum* by the University of Tokyo. These strains shared similar taxonomical characteristics, such as (1) producing glutamic acid under aerobic conditions; (2) requiring biotin for growth; (3) showing positive in a Gram stain test, a classification indicator for bacteria; (4) forming no spores; (5) having no motility; (6) being short-rod-shaped or spherical. Due to these factors, a recommendation was made in the 1980s to unite them under the name *Corynebacterium glutamicum*; this name is now popularly used.

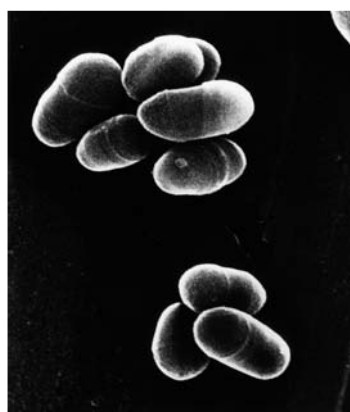


Fig. 4.2 (1) Electron Micrograph of *Corynebacterium glutamicum* (supplied by Kyowa Hakko Kogyo Co., Ltd.)

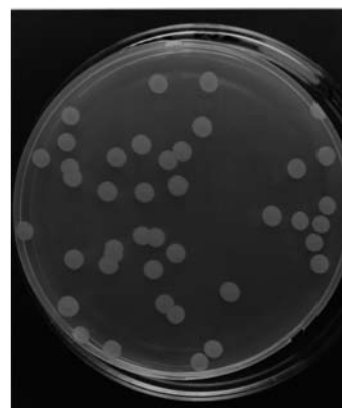


Fig. 4.2 (2) Photograph of *Corynebacterium glutamicum* Colony (supplied by Kyowa Hakko Kogyo Co., Ltd.)

These strains were not only used to produce glutamic acid, but also as the parent strains for mutations producing many amino acids, including lysine, threonine, and phenylalanine, as shall be discussed in Sections 4.5 and 4.7.

Kyowa Hakko received the Okouchi Memorial Award in 1958 and the Japan Science and Technology Agency Director-General's Award in 1960 for the invention of glutamic acid fermentation, as well as the Japan Academy Prize in 1966 for the establishment of amino acid fermentation including ornithine and lysine fermentation, as shall be discussed in Section 4.5.

In 1957, an agreement was made that all glutamic acid produced using Kyowa Hakko's fermentation method was to be supplied to Ajinomoto Co., Inc. for the next five years. However, there was trouble with the purification process and the product was not supplied as planned. In 1961, Ajinomoto Co., Inc. cancelled the agreement, having developed a method that did not infringe on Kyowa Hakko's patent. Kyowa Hakko later supplied MSG to Takeda Pharmaceutical for its nucleic acid seasoning “Inoichiban,” but Takeda Pharmaceutical discontinued the supply in 1966, having also started producing MSG from strains it had developed on its own.⁽⁵⁾ These developments were fraught with microbe-strain-related patent disputes.

4.2.2 Glutamic-Acid-Producing Strain Cultivation Technology

Large-scale cultivation methods for producing glutamic acid involved using flasks and jar fermenters developed for antibiotic fermentation. It was clear that the key to achieving the maximum production quantity was limiting the biotin concentration to around 3 µg/L,⁽⁴⁾ as mentioned in Section 4.2.1, but there was a major issue in that molasses, a cheap raw material, contains high concentrations of biotin and could not be used to produce glutamic acid. Faced with this practical problem, Merck, which had adopted Kyowa Hakko's technology, successfully developed the technology that enabled the use of molasses, having proven that

glutamic acid could be produced from molasses with a high biotin concentration by adding a low concentration of penicillin to the molasses culture.⁽⁶⁾ Meanwhile, Ajinomoto Co., Inc. developed a method involving the adding of a surfactant.⁽⁷⁾

4.2.3 Glutamic Acid Production Mechanism

There was a lot of practical and academic interest as to why such high concentrations of glutamic acid accumulated and much research was devoted to clarify the matter. As mentioned in Section 4.2.2, there were two main cultivation methods for producing large quantities of glutamic acid: (1) suppressing the concentration of biotin; (2) adding penicillin or a surfactant to high concentrations of biotin. Later research demonstrated the following.

1) Wild-type strains inhibit enzyme activity related to glutamic acid biosynthesis or repress the formation of those enzymes. In other words, they have a metabolism controlling function, mentioned in Section 4.3, as seen in *B. flavum*.

2) Under glutamic-acid-producing conditions, as the glutamic acid produced within the microbe is secreted, the metabolic flow predominantly progresses towards glutamic acid.

In the second point above, since biotin is a factor in the synthesis of the fatty acids that form the cellular membrane, limiting the biotin means limiting the synthesis of fatty acids and limiting the synthesis of the cellular membrane. Adding penicillin or a surfactant has a similar effect. While the cellular membrane thus plays an important role,⁽¹²⁾ a recent joint study between Ajinomoto Co., Inc. and Tokyo Institute of Technology on the secretion function of the cellular membrane has found that when glutamic acid is produced, mutations occur in the genes believed to correspond to mechanosensitive channels. Mechanosensitive channels are channels (pathways) that have the function of sensing changes in the osmotic pressure on the microbe's cellular membrane and secreting a corresponding solute (in this case glutamic acid) outside of the cell. This has been explained as the channel always being "on" in this mutation, while in wild-type strains the channel only turns "on" and secretes glutamic acid in conditions that induce the production of glutamic acid ((1) and (2) above).⁽⁸⁾

4.2.4 Academic Research on Glutamic-Acid-Producing Bacteria

As mentioned in Section 4.2.1, glutamic acid bacteria such as *C. glutamicum* and *B. flavum* were just used for producing glutamic acid. In later studies, these extremely important strains served as the parent stock for the mutant strains that produced lysine and many other amino acids. Extensive academic research was carried out on these microbes, mainly in Japan,

covering not only the breeding of amino-acid-producing strains, but also taxonomy, amino acid metabolism, gene manipulation systems, and genome analyses. The results of these studies are summarized in the literature.^{(9), (10), (11)}

4.2.5 Overseas Expansion of Glutamic Acid Fermentation Technology

Kyowa Hakko's technology is said to have been licensed to Merck, a major American chemical company, and to companies in Taiwan. Meanwhile, Ajinomoto Co., Inc. started production at its factories in Japan and as early as 1960 started actively expanding its operations overseas in the form of MSG plants in Thailand, Malaysia, the Philippines, Italy, and other places. Since the price of MSG heavily depends on the raw materials, there are hardly any MSG factories in Japan any more, having spread instead to Southeast Asia, Brazil, the United States, Taiwan, and China that produce molasses and starch. As mentioned in Section 2.4, glutamic acid fermentation developed into a major global industry, with an estimated MSG production of 1.7 million tons in 2005.

4.2.6 Industry-University Collaboration on Amino Acid Fermentation Research

Given the success of glutamic acid fermentation, Kinichiro Sakaguchi of the University of Tokyo proposed that Japan develop the technology for amino acid production by fermentation into a uniquely Japanese industry. In 1958, the Ministry of Education, Culture, Sports, Science and Technology started an amino acid research group that included the leading universities across the country. The "Amino Acid Group" was launched as a forum for presenting research and many members of the industry actively took part as well, carrying out vigorous research and discussions. Although the group later changed its name to the "Amino Acid/Nucleic Acid Group" and now exists as the "Technical Committee on Fermentation and Metabolism" within the Japan Bioindustry Association, it still continues its activities as the main driving force behind amino acid fermentation research in Japan.

4.3 What is Metabolic Regulation to Prevent Overproduction of Amino Acid?

Since amino acid production is closely connected to metabolic regulation (Section 3.2), let us provide an outline of that here.

Microbes have a regulating mechanism to prevent excessive production of amino acids and other metabolites necessary to their biological activity. This is metabolic regulation.

Alcohol fermentation is similar to glutamic fermentation in that it produces a substance that is useful to humans through the fermentation process. While the reaction from the action of the yeast produces alcohol as well as carbon dioxide gas from glucose, the alcohol is produced as a byproduct of the yeast growth reaction. Consequently, no metabolic regulation operates in this reaction. By contrast, glutamic acid is an essential substance as protein material, so it would be irrational for the microbe to produce it in excess and secrete it. The mechanism preventing this from happening is metabolic regulation. Thus, while alcohol fermentation and amino acid fermentation are similar in that they both use microbes to produce a useful substance, they differ in the details. Due to this metabolic regulation effect, ordinary wild-type microbes cultivated through normal methods do not over-produce amino acid (an exception is wild-type *C. glutamicum*, which sometimes stores up small amounts of alanine and valine with a low metabolic regulation effect).

An overview of the amino acid biosynthesis pathways in microbes and the regulation of these was determined in 1955-1960s (Fig. 4.3), with research in molecular biology also shedding light on these mechanisms. The regulating functions mentioned here are feedback inhibition and repression of enzyme formation by end product, or amino acid. For example, in a metabolic pathway that synthesizes end product E from start substance A, as shown in Fig. 4.4, if the concentration of E increases due to over-production, feedback inhibition inhibits the direct binding to the A→E reaction-catalyzing enzyme (a) while repression suppresses the production of the enzymes (a-d) that catalyze the reactions from A to E. Thus, if there is a high concentration of E, the A→E reaction stops, but once consumption of E eventually reduces the concentration, the control is lifted and synthesis resumes.

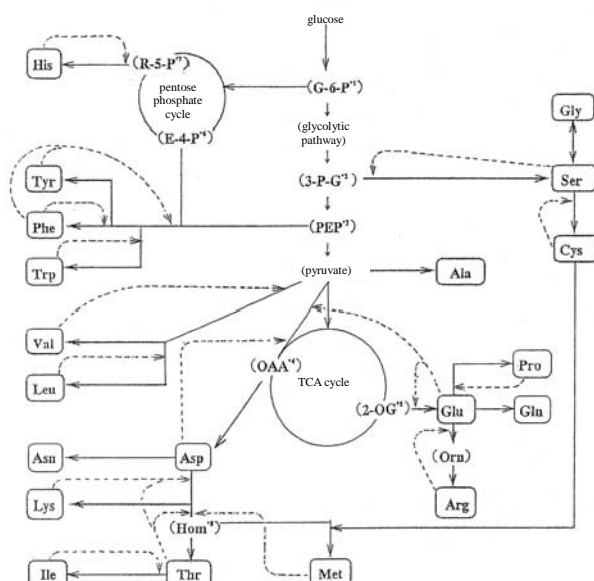


Fig. 4.3 Schematic Representation of Amino Acid Biosynthesis and Regulation in Microbes

□ : amino acid () : intermediate → : biosynthesis pathway
 - - - - - : Main Feedback Control

* 1: glucose-6-phosphate, * 2: 3-phosphoglycerate, * 3: phosphoenolpyruvate, * 4: oxaloacetate, * 5: 2-oxoglutarate, * 6: erythrose-4-phosphate, * 7: ribose-5-phosphate, * 8: homoserine

Generally, enzyme (a), the starting enzyme involved in the A→E reaction is called a key enzyme. This enzyme has an active site, where A binds, and a control site, where end product E binds. If E binds, the spatial structure of the enzyme protein changes and A cannot bind, thereby inhibiting the reaction. These enzymes are called allosteric enzymes. While feedback inhibition directly binds E to the enzyme, repression is more indirect, suppressing the production of enzymes through mRNA transcription. *E. coli* and other gram-negative bacteria also have an amino-acid-decomposition reaction; this is also metabolic regulation.

Thus, breaking down or bypassing metabolic regulation is a necessary condition for the over-production of amino acid. The following methods can be used to bypass the specific types of metabolic regulation discussed thus far.

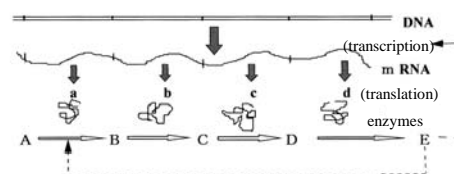


Fig. 4.4 Schematic Representation of Feedback Inhibition and Repression (Suppression of Enzyme Production)

Feedback inhibition is the phenomenon of inhibiting the enzyme a that catalyzes the A→B reaction in E in the series of biosynthetic reactions that turn starting product A into end product E (amino acid). Repression is the phenomenon of suppressing the production of proteins (= enzymes) a-d, acting at the stage in which E transcribes the genetic information in the DNA to mRNA.

→ : biosynthetic reaction - - - - - : Feedback inhibition - - - - - : repression

4.4 Overcoming Metabolic Regulation (1) Promoting Glutamic Acid Secretion

As discussed in Section 4.2.3, increasing the secretion of glutamic acid, in other words, increasing the permeability of the cell membrane of the glutamic acid microbe has been shown to cause it to produce large quantities of glutamic acid even with the metabolic regulation found in the glutamic acid biosynthesis, as mentioned in Section 4.3.⁽¹²⁾ A molecular-level analysis of glutamic acid secretion suggests the existence of secretion channels.⁽⁸⁾

4.5 Overcoming Metabolic Regulation (2) Production Using Auxotrophic Mutants

While mutants are strains with hereditary characteristics that differ from those of the parent stock, auxotrophic mutants are mutants that genetically lose the enzymes related to synthesizing amino acids or other metabolites. Since they cannot biosynthesize these metabolites, they require them as nutritive materials in order to grow. These mutants have been induced in order to block specific reactions. The method discussed here uses auxotrophic mutants to eliminate feedback inhibition by reducing the concentration of amino acid, the feedback inhibitory factor, thereby producing an excess of amino acid.

4.5.1 Ornithine

Having achieved the capability to discover glutamic-acid-producing microbes, Kyowa Hakko attempted to screen other amino-acid-producing microbes from soil and other natural elements, but it appears that these attempts were not successful. As mentioned in Section 4.3, there were no further reports of wild-type microbes producing an excess of amino acids other than glutamic acid, alanine, and valine. Given the knowledge of metabolic regulation mentioned above, the obvious conclusion is that there are no producing strains for these other amino acids. Meanwhile, there have been reports of auxotrophic mutants of *E. coli* with the amino acid biosynthetic pathway blocked secreting an intermediate before the blocking reaction. Kyowa Hakko used these clues to select various auxotrophic mutants of *C. glutamicum* and examining what they produce. Strains requiring arginine were found to produce ornithine; strains requiring homoserine were found to produce lysine; strains requiring threonine were found to produce homoserine; strains requiring tyrosine were found to produce phenylalanine. Of these, the highest quantities produced were of ornithine⁽¹³⁾ and lysine.⁽¹⁴⁾ These were put to practical use, resulting in the creation of ornithine and lysine fermentation. Rather than simply blocking biosynthesis and storing up the intermediate, over-production was achieved by eliminating the feedback inhibition involved in amino acid biosynthesis.

As shown in Fig. 4.5, biosynthesis of wild-type arginine is achieved by the starting enzyme N-acetylglutamate synthase being subject to feedback inhibition from the arginine, as well as regulation by repressing the synthesis of a series of enzymes related to biosynthesis. Arginine-requiring strains lacking ornithine carbamoyl transferase (OCT), which catalyzes the next arginine reaction, are cultivated with a limited low concentration of arginine, which eliminates feedback inhibition and repression. While this also results in a relative increase in N-acetylglutamate synthase activity, the excessively-produced ornithine is stored due to the lack of OCT. The quantity of ornithine produced using arginine-requiring strains indicated a 36 % higher

molar yield than glucose.⁽¹³⁾

While ornithine is an amino acid not found in protein, it is used as an ingredient in amino acid transfusions, as well as in a salt form with aspartic acid as an agent to improve liver function.

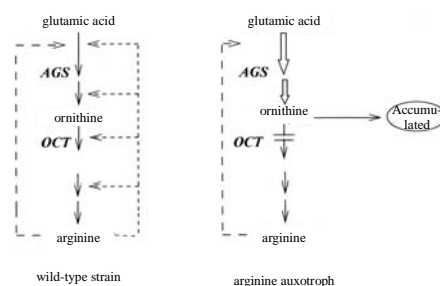


Fig. 4.5 Regulation of Arginine Biosynthesis in *C. glutamicum* and Ornithine Production in Arginine Auxotrophs⁽¹⁰⁾

→ : biosynthetic reaction - - - : feedback inhibition
 - - - - - : regulation of enzyme synthesis, AK: aspartokinase, HD: homoserine dehydrogenase
 OCT : ornithine carbamoyl transferase

Since arginine auxotrophs lack OCT (represented by \dashrightarrow) and can be cultivated with a limited low concentration of arginine, ACS feedback inhibition and the production of a series of enzymes can be regulated. Enzyme activity increases (represented by \rightleftharpoons) and excessively-produced ornithine is accumulated.

4.5.2 Lysine

As shown in Fig. 4.6, there is concerted feedback inhibition in wild-type *C. glutamicum* when aspartokinase, the starting enzyme for lysine, threonine, and methionine synthesis, coexists with both lysine and threonine. If homoserine auxotrophs lacking homoserine dehydrogenase are cultivated with a low concentration of homoserine (or threonine plus methionine), there will be no feedback inhibition of aspartokinase due to the low threonine concentration and an excess of the intermediate aspartate-semialdehyde will be produced. Since there is a lack of homoserine dehydrogenase, the intermediate will not convert to threonine, but flow into lysine, causing an excess to be produced and accumulated⁽¹⁴⁾.

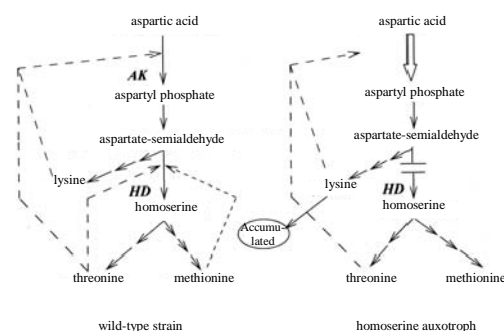


Fig. 4.6 Regulation of Lysine, Threonine, and Methionine Biosynthesis in *C. glutamicum* and Lysine Production in Homoserine Auxotrophs⁽¹¹⁾

→ : biosynthetic reaction - - - : feedback inhibition
 - - - - - : regulation of enzyme synthesis, AK: aspartokinase; HD: homoserine dehydrogenase
 Homoserine auxotrophs lack HD (represented by \dashrightarrow); if they are cultivated with a limited low concentration of threonine and methionine, there is no AK feedback inhibition and AK activity increases (represented by \rightleftharpoons). Excessively-produced lysine is accumulated via aspartate-semialdehyde.

4.5.3 Other Amino Acids

Based on these successes, various other auxotrophic mutants have been selected, including proline-producing strains derived from isoleucine auxotrophs⁽¹⁵⁾ and citrulline-producing strains from arginine auxotrophs.⁽¹⁶⁾

Production using these auxotrophic mutants has been adopted as a historically-significant discovery. However, when using these methods of using auxotrophic mutants it is difficult to control the optimum concentration of the auxotrophic substance to obtain the maximum production quantity. There are also inherent limitations; in other words, amino acid auxotrophy cannot be achieved in these producing strains because they block amino acid synthesis. The use of analog-resistant strains, as shown in Fig. 4.7, solved these issues all at once.

4.6 Overcoming Metabolic Regulation (3) Production by Converting Intermediate Metabolites

Amino acid metabolic regulation is mainly achieved through inhibition of the starting enzyme in the synthesis reaction (Section 4.3). Accordingly, since there is essentially no inhibition of reactions from the starting enzyme onwards or of formation reactions from side substances, it was believed possible to convert amino acid precursors or intermediates in this position into amino acids. Examples include turning threonine precursor homoserine into threonine, tryptophan precursors anthranilic acid or indole into tryptophan, fumaric acid into aspartic acid, glycine into serine, and chemically-synthesized α -aminobutyrate into isoleucine. The method involves screening strains capable of converting to amino acids using these precursors and intermediates as the substrate (raw material), thereby proceeding with the reaction while ingeniously avoiding the key enzymes subject to metabolic regulation. Although, such methods made it possible to achieve the target strain, many such methods were never implemented. The reasons for this were that the precursors and intermediates used as raw material were expensive and that a means of directly fermenting amino acids using analog-resistant strains, mentioned in Section 4.7, was established not long afterwards. Methods that successfully made it to practical implementation were production methods for included those that used relatively inexpensive raw materials such as fumaric acid to produce aspartic acid and alanine and those that could not be adopted using analog-resistant strains, such as D-*p*-hydroxyphenylglycine and cysteine. These were implemented as fermentation production methods, with more details given in Section 4.8. Studies on analog-resistant strains of serine made slow progress, and it was produced from glycine for a while.

4.7 Overcoming Metabolic Regulation (4) Production using Analog-Resistant Strains

Analogues are substances with a similar structure to natural amino acids or nucleic acid bases and are mainly used as ingredients for pharmaceutical products or metabolism research. Some example analogs for amino acids include ethionine for methionine, α -amino- β -hydroxyvaleric acid (AHV) for threonine, and S-(2-aminoethylcysteine) (AEC) for lysine. Fig. 4.7 shows a comparison of structural formulas between these and their natural amino acid counterparts.

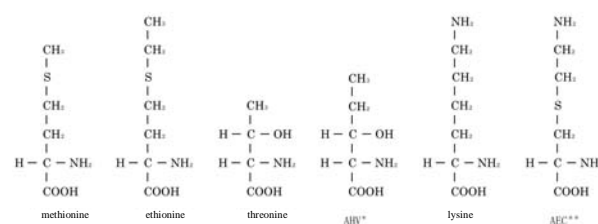


Fig. 4.7 Example Amino Acid Analog Structural Formulas

*AHV: α -amino- β -hydroxyvaleric acid
** AEC: S-(2-aminoethyl) cysteine

While wild-type microbes cannot grow in minimal media (media containing only the minimum components necessary for the growth of the microorganism) with the analog added, they can be revived if the corresponding amino acid is supplemented. Since these analogs are “counterfeits” of the real amino acids, they can antagonistically interfere with the real amino acids in reactions that capture amino acids to build them into protein (aminoacyl tRNA synthetase) and reactions for key enzyme feedback inhibition. Accordingly, an analog-resistant strain that can grow in a medium that includes an analog is a mutant strain thought to be able to withstand interference and produce an excess of real amino acid. Thus, in the early 1960s there were reports of *E. coli* and other amino acid analog-resistant strains overproducing and secreting various types of amino acids. These secretions were literally miniscule amounts and these reports did not include any quantitative descriptions. Examples include secretions of methionine from ethionine-resistant strains, threonine from AHV-resistant strains, and tryptophan from 5-methyl-tryptophan-resistant strains (Later, when the author visited Dr. G. N. Cohen at the Pasteur Institute, who had reported the first threonine secretion from an AHV-resistant strain of *E. coli*, Dr. Cohen said that he “never dreamed that such research would have any potential commercial interest”).

Ajinomoto Co., Inc. induced an AHV-resistant mutant strain from *Brevibacterium flavum* (*C. glutamicum*) parent stock and achieved a strain that could produce large quantities of threonine. This was far from the “secretions” previously reported on. The company immediately industrialized threonine fermentation using this producing strain. This was the first successful industrialization of amino acid fermentation using an analog-resistant strain. The reason this mutant strain produced threonine was that it genetically excluded feedback inhibition of homoserine dehydrogenase through threonine, as shown in Fig. 4.6, and that its sensitivity to inhibition was around 1,300 times lower than the parent stock, showing a clear correlation between production and enzyme activity. Later research showed that this mutation occurred due to permutation of an amino acid residue in the homoserine dehydrogenase control area amino acid sequence. A threonine-producing strain derived at the same time from *E. coli* K-12 was used for improvement through genetic engineering 15 years later.⁽¹⁷⁾

As the pioneers in successful threonine fermentation, Ajinomoto Co., Inc., Kyowa Hakko, and Tanabe Pharmaceuticals Co., Ltd. (now Mitsubishi Tanabe Pharma Corporation) reported production of various amino acids from analog-resistant strains. While these reports are many in number, the main products thought to have immediately gone on to industrialization are given in Table 4.1.

Thus, the use of analog-resistant strains resulted in the achievement of a number of amino acids being produced by fermentation. It is increasingly evident that for many of these the activity of the starting enzyme for amino acid synthesis was excluded through amino acid feedback inhibition or regulating enzyme

production, such as using lysine + threonine to inhibit the feedback of aspartokinase, the key enzyme in lysine-producing strains from *B. flavum*, or using histidine to eliminate the inhibition of ATP-phosphoribo-syltransferase, likewise the key enzyme in histidine-producing strains from *C. glutamicum*. The research findings by Tanabe Pharmaceuticals are characterized by the use of enteric bacteria *Serratia marcescens* as a parent stock. This strain resembles *E. coli* and is distinctive in that it can transduce by phage. Some mutant strains have been created by using this distinctive trait to inhibit feedback and eliminate repression of enzyme production.

4.8 Enzymatic Method and Production using Bioreactors

The enzymatic method is a technology developed through application of the methods outlined in Section 4.6, producing amino acids by converting raw materials other than glucose, such as intermediates on the metabolic pathway or chemically synthesized intermediates, using microbial enzymes or microbes that contain enzymes as the catalyst. This differs slightly from the fermentation method in that while the fermentation method produces amino acids as the microbes multiply, the enzymatic method uses the multiplying enzymes as the catalyst. In this report, the enzymatic method is treated as a fermentation method in the broader sense. The main research findings using this method are as follows.

Table 4.1 Amino-Acid-Producing Strains Obtained through Analog-Resistant Mutations

Amino Acid	Analog Used	Strain	Literature
threonine	α -amino- β -hydroxyvaleric acid	<i>E. coli</i> <i>B. flavum</i> <i>C. glutamicum</i> <i>S. marcescens</i> *	I. Shioo and S. Nakamori ⁽¹⁸⁾ I. Shioo and S. Nakamori ⁽¹⁹⁾ H. Kase et al. ⁽²⁰⁾ S. Komatsubara, et al. ⁽²¹⁾
lysine	S-(2-aminoethylcysteine)	<i>B. flavum</i> <i>B. lactofermentum</i>	K. Sano and I. Shioo ⁽²²⁾ O. Tosaka et al. ⁽²³⁾
isoleucine	AHV+ α -methyl threonine AHV+ethionine isoleucine hydroxamate	<i>B. flavum</i> <i>B. flavum</i> <i>S. marcescens</i> *	I. Shioo et al. ⁽²⁴⁾ S. Ikeda et al. ⁽²⁵⁾ M. Kisumi, et al. ⁽²⁶⁾
histidine	1,2,4-triazole alanine 2-thiazole alanine	<i>C. glutamicum</i> <i>B. flavum</i>	K. Araki et al. ⁽²⁷⁾ H. Kamiyo et al. ⁽²⁸⁾
tryptophan	5-fluoro tryptophan, etc. 5-fluoro tryptophan, etc.	<i>B. subtilis</i> * <i>B. flavum</i>	I. Shioo et al. ⁽²⁹⁾ I. Shioo et al. ⁽³⁰⁾
phenylalanine	<i>p</i> -fluorophenylalanine, etc.	<i>B. lactofermentum</i>	T. Tsuchida et al. ⁽³¹⁾
proline	3,4-dehydropoline	<i>B. flavum</i>	S. Nakamori et al. ⁽³²⁾
arginine	D-Ser, D-Arg, etc. 2-thiazole alanine	<i>C. glutamicum</i> <i>B. flavum</i>	N. Nakayama et al. ⁽³³⁾ K. Kubota et al. ⁽³⁴⁾
glutamine	sulfur guanidine	<i>B. flavum</i>	T. Tsuchida et al. ⁽³⁵⁾
valine	α -amino butyric acid 2-thiazole alanine	<i>S. marcescens</i> * <i>B. lactofermentum</i>	M. Kisumi et al. ⁽³⁶⁾ T. Tsuchida et al. ⁽³⁷⁾
leucine	α -amino butyric acid	<i>S. marcescens</i> *	M. Kisumi et al. ⁽³⁸⁾
serine	azaserine	<i>B. flavum</i>	W. Hibino et al. ⁽³⁹⁾

* Thought to be no longer used

While *B. flavum* and *B. lactofermentum* are now recommended to be designated as *C. glutamicum*, the former designations are used here in accordance with the original report.

4.8.1 Production of L-Amino Acids from DL-Amino Acids with Aminoacylase

Before the regularization of amino acid production by fermentation, the method of production that had made the most progress in research was chemical synthesis. The amino acids produced using this method were DL-compounds (racemic compounds). While it is relatively simple to get to the DL-compound stage, the optical resolution to convert a DL-compound into a natural L-compound is quite a difficult challenge and hardly ever made it to industrialization. A method developed by Tanabe Pharmaceuticals achieved this through asymmetric hydrolysis using aminoacylase, an enzyme that derives N-acyl-DL-amino acid, easily synthesized from DL-amino acid, from *Aspergillus oryzae*. This enzyme catalyzes a reaction that detaches the acyl group from the N-acyl-DL-amino acid and is characterized in that it only acts on L-compounds, not on D-compounds. The remaining D-compounds that have not decomposed are racemized by heating to form DL-compounds. Repeating this reaction eventually converts everything into L-compounds (Fig. 4.8). Examples of early L-amino acids successfully produced by the enzymatic method include L-methionine, phenylalanine and valine, mainly used in amino acid infusion requiring L-compounds.

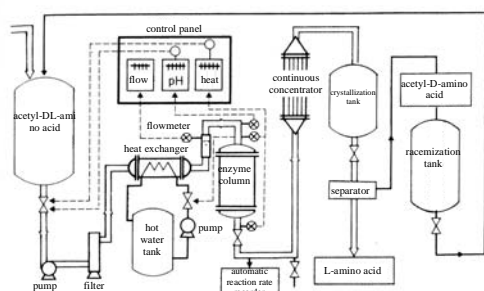
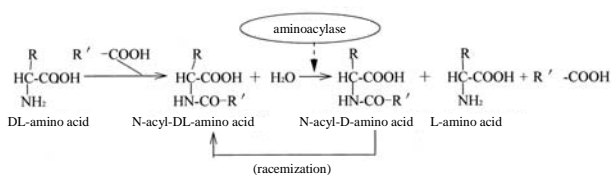


Fig. 4.8 Reaction Formula for Producing L-Amino Acids from DL-Amino Acids using Aminoacylase and Flow Diagram of Production Equipment⁽⁴⁰⁾

A point of difficulty lies in that since the enzymes are used in an aqueous solution state, they have low stability and cannot be used for repeated continuous use like solid catalysts. Enzyme immobilization technology solved this issue by making it possible to treat these enzymes like solid catalysts. Reactions using these immobilized enzymes are called bioreactor. Enzyme immobilization is achieved by binding the enzyme to a high-molecular carrier such as an ion-exchange resin, linking the enzyme using glutaraldehyde or other binding agent or entrapping the enzyme in a bilayer membrane such as a liposome or a high polymer such as carrageenan or polyacrylamide.

The entrapping method targets not only the enzyme but also the microbe containing the enzyme.

In 1969, Tanabe Pharmaceuticals succeeded in the continuous production of L-amino acid using aminoacylase with an immobilized bioreactor reaction, having immobilized the enzymes by ionically bonding them to a DEAE-Sephadex ion-exchange resin.⁽⁴⁰⁾

4.8.2 Aspartic Acid and Alanine

Aspartic acid is used as a component in amino acid infusions and in oral, enteral, and other clinical nutritional supplements, as well as in pharmaceutical applications in salt form together with arginine and ornithine for fatigue recovery and improving liver function. It is also used as a seasoning to boost sourness and heartiness and is in high demand as the raw material used in the sweetener aspartame. Alanine is used in amino acid infusions and in oral, enteral, and other clinical nutritional supplements.

In 1959, both Tanabe Pharmaceuticals and Kakuo Kitahara et al. of Tokyo University reported a method of producing aspartic acid from fumaric acid using *E. coli*. The enzyme catalyzing this reaction was aspartase.

In 1973, Tanabe Pharmaceuticals successfully industrialized the continuous production of aspartic acid using immobilized aspartase⁽⁴¹⁾ (Fig. 4.9).

Tanabe Pharmaceuticals also discovered *Pseudomonas dacunhae*, which produces aspartic acid decarboxylase, that converts aspartic acid into alanine, and successfully developed a process for producing alanine from aspartic acid⁽⁴²⁾ (Fig. 4.9).

Since production of aspartic acid from glucose by fermentation offers low yields and production of alanine results in a DL-compound, both aspartic acid and alanine are produced industrially using the enzymatic method.

Tanabe Pharmaceuticals immobilized microbes containing aspartase and aspartic acid decarboxylase by entrapping them in polyacrylamide (and later in κ-carrageenan) and succeeded in long-term, stable, continuous production of aspartic acid and alanine from fumaric acid.⁽⁴³⁾ This reaction system is shown in Fig. 4.9.

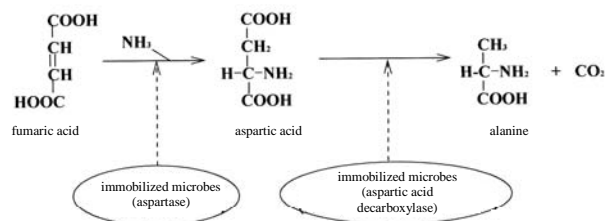


Fig. 4.9 Production of Aspartic Acid and Alanine from Fumaric Acid using Immobilized Microbes containing Enzymes

4.8.3 Dioxyphenylalanine

Dioxyphenylalanine (DOPA) is a precursor amino acid of a series of chemical compounds such as dopamine, which governs the neural functions in the brain. It is a pharmaceutical product shown to be very effective in treating Parkinson's disease and other cranial nerve disorders in the elderly. Since the amino acid is unrelated to microbial biological activity, it cannot be produced through direct fermentation, but a method has been developed to synthesize it using microbial enzymes. Hideaki Yamada et al. of Kyoto University discovered that microbes such as *Citrobacter intermedius* and *Erwinia herbicola* produce β -tyrosinase, the enzyme that catalyzes the reactions below which decompose tyrosine at β position⁽⁴⁴⁾ (Fig. 4.10). While the reverse reaction to this reaction shows that tyrosine is composed of phenol, pyruvate, and ammonia within the same group, the enzyme has broad substrate specificity and DOPA can be synthesized by replacing phenol with pyrocatechol as the raw material (Fig. 4.11). This process was industrialized by Ajinomoto Co., Inc. in 1992.⁽⁴⁵⁾

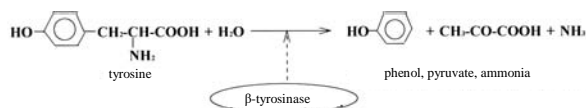


Fig. 4.10 Tyrosine Decomposition Reaction with β -Tyrosinase

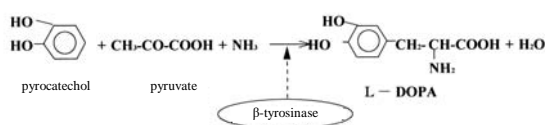


Fig. 4.11 Synthesis of DOPA using β -Tyrosinase Reverse Reaction

4.8.4 Cysteine

Cysteine has many uses as a raw material for infusions, liver function improvement agents, pigmentation improvement agents, and bread fermentation agent, with its acetyl derivative used as a raw material for expectorants. Cysteine is produced by hydrolyzing hair or keratin with a high cysteine content in hydrochloric acid. As yet there has been no method established for producing it by fermentation. Ajinomoto Co., Inc. developed a method of obtaining L-cysteine by asymmetrically hydrolyzing DL-2-aminothiazoline-4-carboxylic acid (DL-ATC), an intermediate in cysteine chemical synthesis, using microbial enzymes.⁽⁴⁶⁾ The microbe *Pseudomonas thiazolinophilum* has been screened as one that can be cultivated using DL-ATC as the sole nitrogen source and is presumed to contain ATC racemase and hydrolase, enzymes involved in subsequent reactions (Fig. 4.12). This production method was industrialized by Ajinomoto Co., Inc. in 1982.

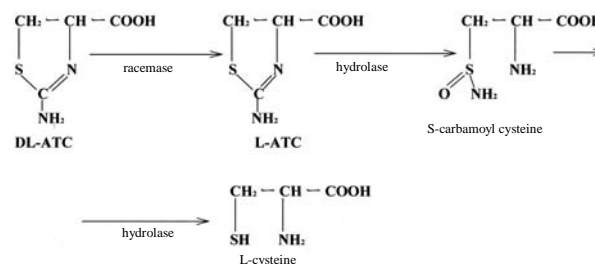


Fig. 4.12 Enzymatic Production of L-Cysteine from DL-ATC
ATC: 2-aminothiazoline-4-carboxylic acid

4.8.5 D-*p*-Hydroxyphenylglycine

D-*p*-hydroxyphenylglycine (D-HPG) is a D-amino acid developed as a component in semi-synthetic penicillin amoxicillin, rather than a natural amino acid. Amoxicillin is synthesized by introducing D-HPG to the amino group at position 6 in 6-aminopenicillanic acid, the penicillin structure. While there have been several reports of methods for obtaining L-amino acids by enzymatic asymmetric hydrolysis of 5-substituted hydantoin, intermediates in amino acid chemical synthesis, Hideaki Yamada et al. of Kyoto University screened microbes that produce hydantoinase, the enzyme that specifically produces D-amino acids.⁽⁴⁷⁾ Of these, *Pseudomonas putida* was selected as being the most active in converting DL-5-(*p*-hydroxyphenyl) hydantoin into D-N-carbamoyl-*p*-hydroxyphenylglycine. The decarbamoyl reaction to convert D-N-carbamoyl-*p*-hydroxyphenylglycine into D-HPG was performed using a method of nitrite treatment under acidic conditions. Kaneka achieved industrial production of D-HPG and runs the manufacturing process mentioned as follows at its Singapore plant (Fig 4.13).

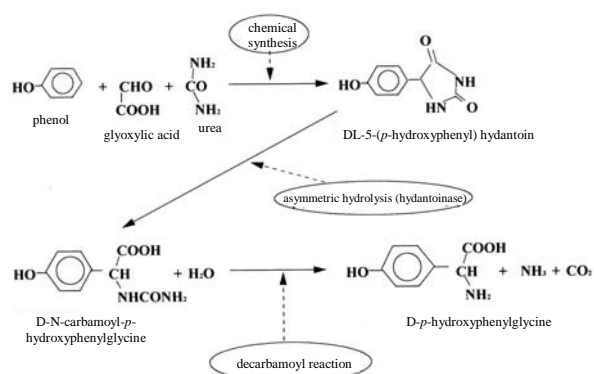


Fig. 4.13 Synthesis Reaction of D-*p*-Hydroxyphenylglycine

The decarbamoyl reaction at the final stage in this process was initially carried out by nitrite treatment under acidic conditions, as mentioned above. However, this method produced a low yield and a lot of byproduct. It also had significant environmental issues due to heavy use of alkalis. Kaneka Corporation screened *Agrobacterium* sp., a microbe containing the D-carbamylase enzyme that catalyzes this reaction and successfully cloned and expressed the D-carbamylase gene in *E. coli*. This made it possible to establish a production method using a bioreactor system and immobilizing hydantoinase-containing *Pseudomonas putida* and carbamylase-containing *E. coli*.⁽⁴⁸⁾

4.8.6 Lysine

Toray Industries, Inc. developed a system of producing L-lysine from DL- α -amino- ϵ -caprolactam (ACL), the raw material for nylon, using the enzymatic method.⁽⁴⁹⁾ As shown in Fig. 4.14, the reaction involves selecting *Achromobacter obae*, a microbe with an ACL racemase activity, and *Cryptococcus laurentii*, a yeast containing a hydrolyase that hydrolyzes ACL, combining these and converting the DL-ACL almost quantitatively to L-lysine. Although this method was temporarily industrialized in partnership with Takara Shuzo, it is not practiced today.

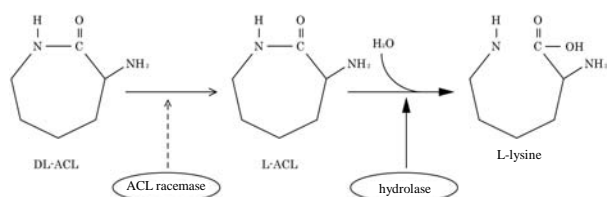


Fig. 4.14 Production of L-Lysine from DL- α -Amino- ϵ -Caprolactam using the Enzymatic Method
ACL: α -amino- ϵ -caprolactam

4.9 Improving Producing Strains by Applying Genetic Engineering (Recombinant DNA) Technology

Genetic engineering technology, established around the 1970s-1980s, has enabled various applications, such as the isolating of specific genes and analyzing, modifying or amplifying these genes. Since overproduction of amino acids requires eliminating the metabolic regulation for each amino acid and increasing the enzyme activity related to amino acid biosynthesis as mentioned in Section 3.3, the ability to alter enzymes by gene-specific cloning or to increase enzyme production meant increased amino acid biosynthesis. This technology has an extremely wide range of applications in breeding and improving strains.

When genetic engineering technology was first established, there were concerns about the safety of organisms with heterologous genes and the impact on the ecosystem. Experimentation guidelines were enacted for gene recombination experiments and then legislated in 2004 as the *Act on the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms* (commonly known to the Japanese as the “Cartagena Protocol Hypothecation Act”). Since there are no issues with safety where the origin of the microbe or gene is clear, appropriate experimentation is permitted and progress has been made on practical implementation. The *E. coli* K-12 strain has been demonstrated to be safe and is widely used throughout the world. The wealth of information on relevant materials and methods make it the easiest strain to apply gene manipulation technology. Improvements have been made using this strain and it has even been used to produce new amino acids.

By contrast, genome analysis on *C. glutamicum*, the main strain used in amino acid fermentation, has come to an end. Although vector construction, gene transfer methods, and other gene manipulation systems have been developed for it and *Brevibacterium* and these have been shown to increase production of threonine and phenylalanine, they have not reached the point of practical implementation.

4.9.1 Threonine

Ajinomoto Co., Inc. obtained threonine-producing strains from *E. coli* K-12 as an analog AHV-resistant mutant.⁽¹⁸⁾ As mentioned in Section 4.7, homoserine dehydrogenase, the key enzyme for threonine synthesis, was desensitized in this mutation to make it insensitive to threonine feedback inhibition. Based on this information, Genetika, the Russian State Research Institute for Genetics and Selection of Industrial Microorganisms, selected its own threonine-producing strains and applied gene manipulation technology to amplify the desensitized homoserine dehydrogenase gene to increase threonine production to three times that of the parent stock, reporting a yield of 20 g/L by cultivating it in a medium containing 6.2 % glucose.⁽⁵⁰⁾ Ajinomoto Co., Inc. likewise increased threonine production by three times that of the parent stock by amplified the *E. coli* K-12 threonine-producing gene and went on to achieve a yield of 55 g/L by cultivating it in a jar fermenter.⁽⁵¹⁾

Ajinomoto Co., Inc. later improved this by introducing microbes from Genetika. In other words, the HD gene was inserted into the plasmid used as a vector, but the plasmid was often unstable and the threonine production titer would decrease. This was successfully stabilized by cloning by chromosomal recombination using Mu phage.⁽⁵²⁾ Fig. 4.15 shows a schematic outline of strain breeding. This strain was industrialized in 1991 by French

company Eurolysine, a joint venture of Ajinomoto Co., Inc., with the approval of the French government, having been presented with the data on its safety. This is thought to be the first instance of industrialization of a microbe strain achieved by gene manipulation.

4.9.2 Lysine and Tryptophan

While Ajinomoto Co., Inc. made no specific external announcements regarding this, the company is believed to have worked on improving lysine- and tryptophan-producing strains by applying gene manipulation technology to the *E. coli* K-12 strain with the approval of the Ministry of Agriculture, Forestry and Fisheries Agricultural Materials Council Feed Committee and confirmation of adherence to Ministry of Economy, Trade and Industry guidelines.⁽⁵³⁾

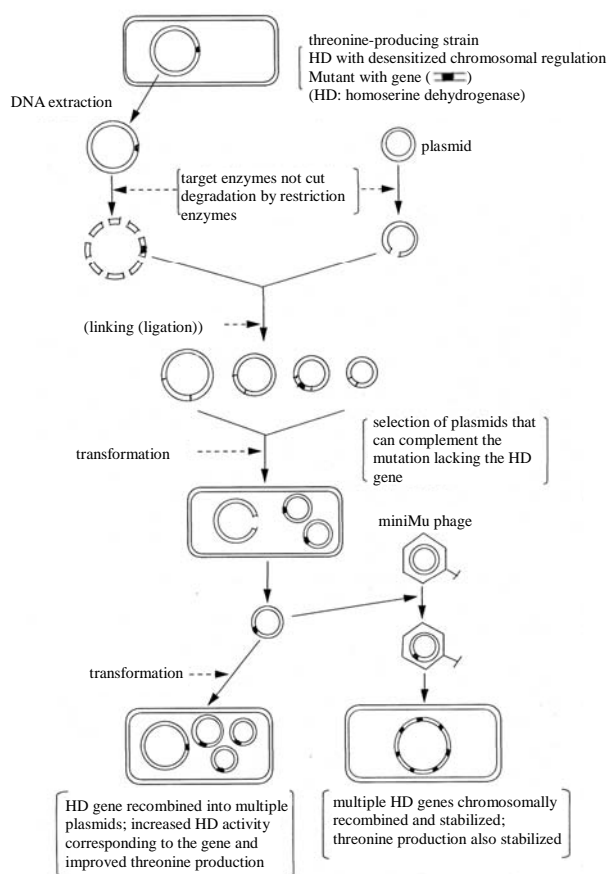


Fig. 4.15 Schematic Diagram of Improvements to Threonine-Producing Strains by Recombinant DNA Technology

4.10 Production of Novel Amino Acids through Application of Genetic Engineering Technology

4.10.1 Hydroxyproline

This amino acid is the main component in animal collagen. Used as an ingredient in pharmaceutical products, hundreds of tons of it have been produced around the world to date by extracting it from collagen

hydrolysate. Since this amino acid is unrelated to microbial biological activity, it cannot be produced by direct fermentation using microbes. However, technology developed by Kyowa Hakko has made this possible by using recombination technology to combine the functions of different microbes. In addition to being D- or L-compounds, the chemical structure of hydroxyproline also has eight isomers, depending on if the hydroxyl group is in position 3 or 4, or if the hydroxyl and carboxyl groups are cis or trans. The biologically active isomer is trans-4-hydroxyproline.

The method developed by Kyowa Hakko involved screening *Dactylosporangium* sp. producing proline 4-hydroxylase, the enzyme that introduces the hydroxyl group at carbon 4 in the proline, cloning this gene and inducing it in a proline-producing strain bred from a different *E. coli*, thereby adding a hydroxyl group to the proline. With a conversion yield of 100 % from the proline in this strain and a fermentation performance of 40 g/L, this method conforms to the industrialization guidelines for genetically-modified organisms and is being implemented in production.⁽⁵⁴⁾

4.10.2 D-p-Hydroxyphenylglycine

The decarbamoyl reaction in the final stage of the D-HPG synthesis process developed by Kaneka, as mentioned in Section 4.8.5, has been established in a production process that involves taking the D-carbamylase gene that catalyzes the reaction from *Agrobacterium* sp., fermenting it with *E. coli* and using these cells as the enzyme source.

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5 | Development of Cultivation Technology and Equipment

Large-scale cultivation involves the scaling up and reproduction of test results for screened or bred microbes obtained at test tube or flask level to large-scale jar fermenter or tank cultivation, as well as the development of cultivation technology that cannot be achieved at test tube or flask level.

Fig. 5.1 shows an outline of the large-scale cultivation equipment commonly currently used in amino acid fermentation. This technology has been established fundamentally based on a submerged cultivation system developed for producing antibiotics.

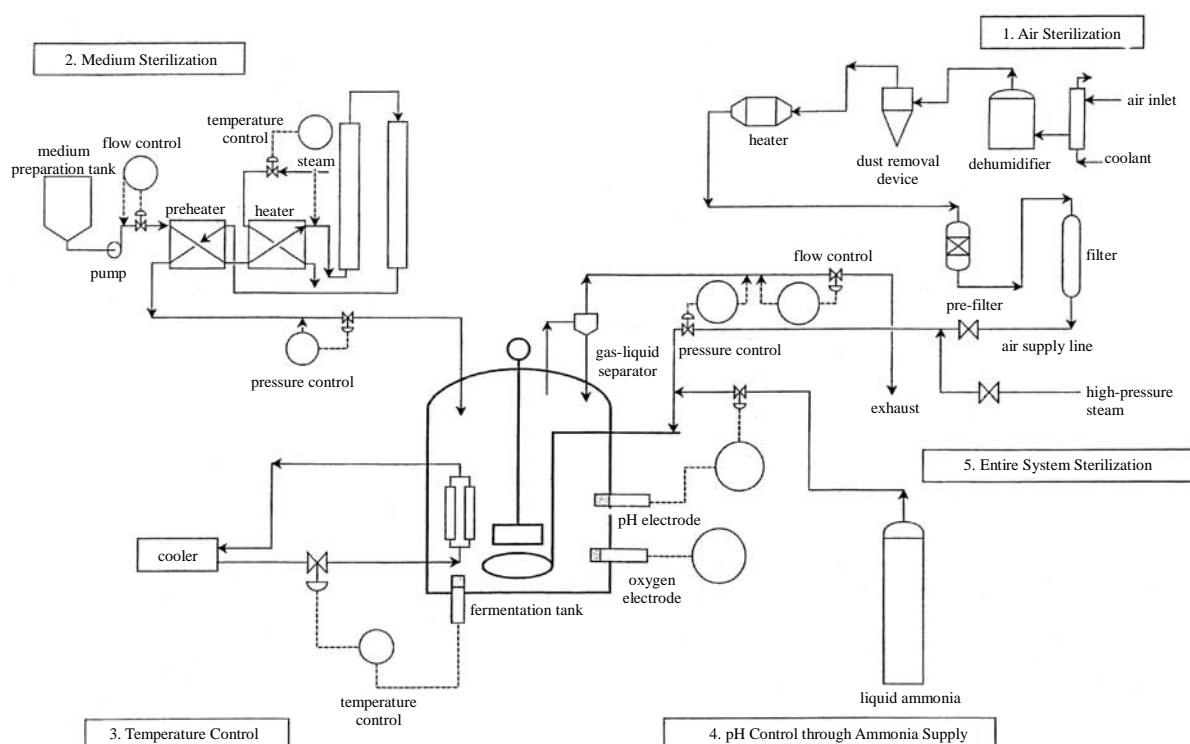


Fig. 5.1 Schematic Diagram of Large-Scale Cultivation Equipment (from ⁽¹⁾)

There are five main elements to the system: (1) air sterilization; (2) medium sterilization; (3) temperature control; (4) pH control; (5) entire system sterilization. Together, these form the system that controls the aeration and agitation culture taking place in the centrally-positioned fermentation tank. This is all controlled by computer. The following technologies have been developed for this system.

5.1 Oxygen Supply

Oxygen is not only required for energy acquisition through microbe respiration, but it is also the second-highest consumed raw material in the composition of amino acid next to sugar, which provides the carbon source. If there is not enough oxygen in glutamic acid fermentation, succinic acid and lactic acid will be produced and the amino acid

yield will decrease significantly. Extensive investigation has been carried out on the impact of oxygen concentration on amino acid production by amino-acid-producing strains derived from *Brevibacterium flavum* and *B. lactofermentum*(*C. glutamicum*). High oxygen concentrations produced the greatest yield of glutamic acid and its synthesis products glutamine, alanine, and proline, while low oxygen concentrations produced the greatest yield of valine and phenylalanine.⁽²⁾

Since oxygen has a low solubility, the oxygen supply can form a bottleneck in fermentation productivity if a high oxygen concentration is required. This technical issue was successfully resolved by using large turbine blades for culture agitation and increasing the oxygen supply capacity.⁽³⁾ There are ongoing investigations into improving the oxygen supply by using liquid oxygen to increase the oxygen partial pressure in the air or by using oxygen enrichment devices.

5.2 Fed-Batch Cultivation

This method is an improved batch culture method in which the target product concentration and batch-wise productivity is increased by adding a special reserve of sugar to continue cultivation once the culture has consumed all of the sugar content in the main raw material. While this method was developed for antibiotic fermentation, Ajinomoto Co., Inc. applied this technology to the production of glutamic acid and other amino acids, increasing productivity by extracting liquid in proportion to the amount of liquid added.⁽³⁾

5.3 Regulating the Osmotic Pressure of the Culture Fluid

Ajinomoto Co., Inc. also discovered that the amount of glutamic acid or other amino acid accumulated reaches a plateau after a certain time. While this is due to a decline in microbial production activity, one cause was shown to be an increase in the osmotic pressure of the culture fluid. Countermeasures to increase production speed included gradually adding to the sugar concentration or adding glycine betaine.

5.4 Controlling Foaming

Large amounts of foaming can occur, depending on the type of fermentation. The causes of foaming are complex and have not been identified, but depend on a combination of factors, including medium composition, cell composition, amino acid or other product, temperature, and pH. Therefore, it is not uncommon for

foaming to occur unexpectedly due to differences in the type of amino acid or microbe. Where there is a lot of foaming, the fermentation liquid can spurt out of the fermentation tank, causing the microbes to become contaminated and requiring the volume of fermentation liquid to be reduced. Reducing the volume of liquid means reducing the productivity. To solve this issue, technology has been developed that treats the foam with an anti-foaming agent or mechanically breaks it down by sending it to the cyclone separator along with the exhaust emissions.⁽³⁾

As mentioned previously, these technologies have essentially taken the knowledge gained from major advances in the industrialization of penicillin fermentation and applied it to amino acid fermentation. Although developed mainly for glutamic acid fermentation, they have of course also been widely adopted into fermentation processes for other amino acids as well.

Large-scale cultivation and separation/purification processes require a lot of equipment. This is all controlled by computer.⁽⁴⁾

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6 | Development of Amino Acid Separation and Purification Technology

The so-called “downstream” technology of amino acid fermentation is the process of separating, purifying, and collecting the amino acid from the culture fluid and bioreactor reaction solution. This includes decoloring and crystallizing the amino acid, as well as labor-saving technologies. This is an important process that determines the quality of the product, while the quality of the yield significantly impacts the cost. While there are very few academic reports on this technology, there is a lot of expertise in the field that can be drawn from resources found in company histories and similar sources. The following sections outline separating/purifying glutamic acid and lysine from fermentation liquid and separating/purifying aspartic acid from fumaric acid using the fermentation method. Processes for other amino acids are generally the same as these, with only minor differences.

6.1 Monosodium Glutamate

Fig. 6.1 shows an overview of this separation/purification process. This method has been used since the early days of glutamic acid fermentation to collect the glutamic acid as a salt from the fermentation mother liquor. Later, technology was developed to improve the elimination of impurities by transferring the glutamic acid α -form crystals into β -form crystals. Technology was also developed to collect the α -form crystals from the mother liquor by concentrated crystallization. These technologies have increased the recovery rate of glutamic acid and also have the advantage of suppressing dispersal of the ammonia formed in the concentration of the culture fluid.

6.2 Lysine Hydrochloride

Fig. 6.2 shows an overview of the separation/purification process for lysine hydrochloride. This process also faced major technical development issues in terms of increasing production, conserving energy and minimizing environmental impact. This process centers around ion exchange resin treatment. In the early days, this involved filling a large resin column with resin and draining out the culture fluid, eluent, and regenerating solution in turn by switching valves. As this method was time-consuming and produced low yields, new technology was developed to allow continuous operation by having the culture fluid, eluent, and regenerating solution drain quickly, with smaller columns for each fluid switched in turn. The resin operation was further simplified when *E. coli* was introduced as a producing strain and microfiltration membranes started being used for the filtration of this strain.⁽³⁾

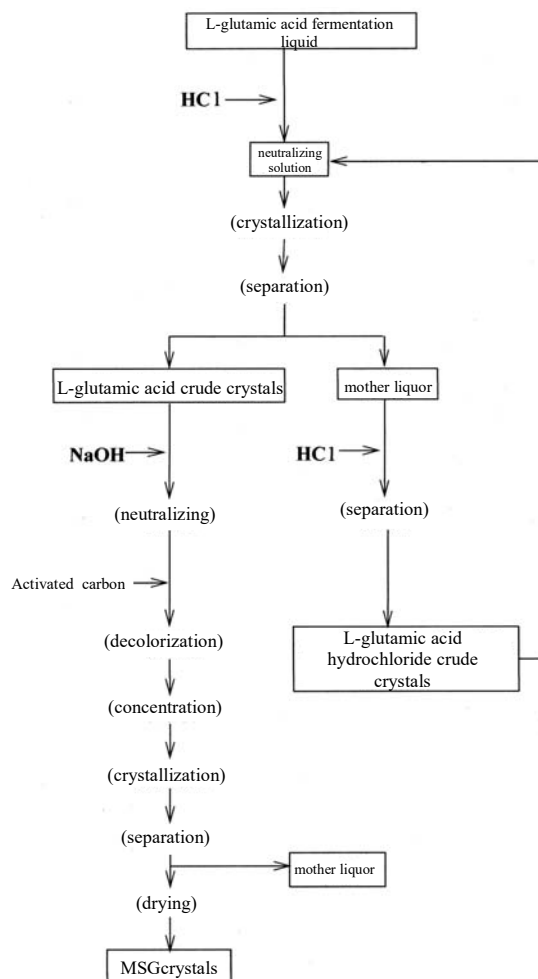


Fig. 6.1 L-Monosodium Glutamate Purification and Separation Process⁽¹⁾

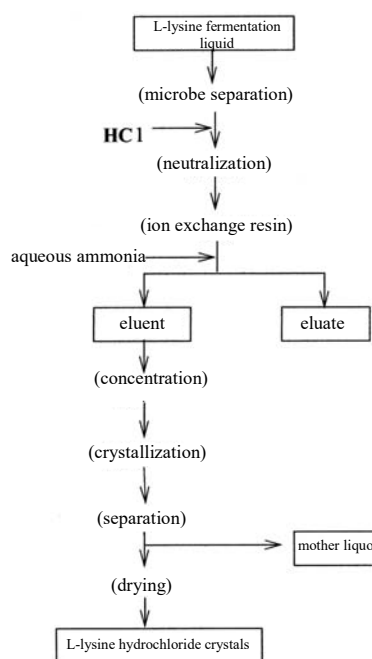


Fig. 6.2 L-Lysine Hydrochloride Purification and Separation Process⁽²⁾

6.3 Aspartic Acid

Since aspartic acid is produced from fumaric acid in a bioreactor using immobilized *E. coli* with aspartase activity, there are no microbial cells, fermentation medium, or byproducts secreted from the microbes such as those found in the glutamic acid or lysine culture fluid. Accordingly, the reaction solution is far simpler in composition than the culture fluid and it is easier to process. Fig. 6.3 shows an overview of the aspartic acid separation/purification process.

<References>

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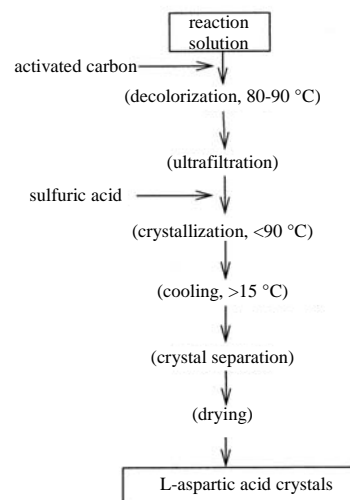


Fig. 6.3 L-Aspartic Acid Purification and Separation Process ⁽⁴⁾

7 | Amino Acid Standards

Amino acid standards have been listed in all kinds of documents (including the Japanese Pharmacopoeia, the Japanese Pharmaceutical Codex, the Japan Chemical Additives Specifications, and the Japanese Standards of Food Additives). Companies have also determined their own standards. Here, we shall outline the amino acid test standards adopted by Ajinomoto Co., Inc. The main items and details are as follows.⁽¹⁾

Specific optical rotation $[\alpha]_D^{20}$

A means of determining the optical purity of amino acids, measured using a polarimeter. Optical rotation measurements are carried out at a temperature of 20 °C, a layer length of 100mm and the light used is the sodium D line.

Dissolved state

A limit test of the turbidity and color of the sample solution. Test methods include visually checking the turbidity and color against a white background or using a spectrophotometer to measure the transmittance at a measurement wavelength of 430nm using a 10mm cell with a solvent for contrast.

Chloride (Cl)

A limit test of the chloride (Cl) mixed in with the sample or a quantitative test of the chloride present in the sample composition. Silver nitrate is used for the limit test, while the Volhard method is used for the quantitative test.

Ammonium (NH₄)

A limit test of the ammonium (NH₄⁺) mixed in with the sample. After the sample solution has been distilled, the indophenol method is used with sodium ferrous phenol pentacyanonitrosyl and a mixture of sodium hypochlorite and sodium hydroxide.

Sulfate (SO₄)

A limit test of the sulfate (SO₄) mixed in with the sample, using barium chloride.

Iron (Fe)

A limit test of the iron (Fe) mixed in with the sample, using 1,10-phenanthroline.

Heavy metals (Pb)

A limit test of the heavy metals mixed in with the sample. These heavy metals are metallic contaminants that are colored by acidic sodium sulfide solution; the limit is expressed as the amount of lead.

Arsenic (As₂O₃)

A limit test of the arsenic mixed in with the sample. The limit is expressed as the amount of arsenic trioxide (As₂O₃).

Other amino acids

A method of separating and detecting other amino acids mixed in with the sample. The test method used is thin-layer chromatography, in which a layer of silica gel is thinly spread onto a glass plate is used as the stationary phase. The sample is spotted onto this and dried and the lower part dipped into an eluting solvent solution. The sample then separates and moves in the space between it and the solvent. The piece is sprayed with a ninhydrin reagent and the ratio between the distance travelled by the colored amino acids and the distance travelled by the solvent is used to identify the amino acids contained in the sample.

Drying loss

Measuring the amount of moisture content, part or all of the crystallization water and volatile substances lost during drying.

Moisture content

A method of measuring the moisture content in the sample using titration (Karl Fischer method).

Ignition residue (sulfate)

Measuring the amount of residual substance that did not volatilize when ignited to constant weight at 550-650 °C as a sulfate.

Content

Non-aqueous titration: Titrating an amino acid dissolved in a non-aqueous solvent (usually acetic acid) as a base in perchloric acid and measuring the change in potential difference.

Neutralization titration: Titrating an acidic amino acid (aspartic acid, glutamic acid) dissolved in water as an acid in a sodium hydroxide solution and quantifying it with the indicator phenolphthalein or measuring the change in potential difference.

Iodometric titration: Titration of excess iodine in sodium thiosulfate using the reaction of cysteine with iodine.

pH

Measured using a glass-electrode pH meter.

Endotoxins (injected)

A method used for detecting endotoxins from Gram-negative microbes using a lysate reagent prepared from components extracted from horseshoe crab blood cells.

<References>

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8 | Main Raw Materials for Amino Acid Fermentation

Besides the carbon source, which is the main raw material for amino acid fermentation, other raw materials include ammonia and amino acid mixtures as nitrogen sources, phosphoric acid, potassium, magnesium, iron, or manganese as mineral components and biotin, thiamine, or other vitamins. Surfactants and β -lactam antibiotics are also used in glutamic acid fermentation. The carbon source occupies the largest proportion of the raw materials. Since the other raw materials are substantially lower in quantity than the carbon source, they are omitted from the present description. While this report includes the enzyme method of amino acid production as production by fermentation in the broader sense, it does not cover the raw materials for the enzyme method, as these are wide-ranging, depending on the type of amino acid. Accordingly, the main raw material for the direct fermentation method refers to the main carbon source.

Table 8.1 shows the current situation and points of issue related to the main carbon source materials and potential raw materials. The main raw material for the fermentation process is glucose, either starch-based or sugar-based. Starch-based glucose is converted to sugar with an enzyme (amylase). Sugar-based glucose can be either raw sugar from sugarcane or molasses. Molasses is the crystallization mother liquor that is left after the raw sugar crystals have been collected. It can be made relatively cheaply from sugarcane or sugar beet; the former is called cane molasses, while the latter is called beet molasses. Both are composed of sucrose, glucose, and fructose. Of the materials shown in Table 8.1, acetic acid and ethanol are able to be used (assimilated) by the glutamic acid bacteria. Special screening has been carried out on microbes able to assimilate methanol and n-paraffin and producing strains are being bred from these.

A major current issue is a sudden price rise for starch, sugar, and molasses due to a growing demand for these as a source of bioethanol due to rising oil prices worldwide. Accordingly, a future issue will be developing biomass, methanol, carbon dioxide, and other underused source materials to avoid a competition between food and energy.

Table 8.1 Current Situation and Points of Issue Regarding Currently Used and Potential Main Carbon Sources for Amino Acid Fermentation

Carbon Source	Current Situation and Points of Issue
Starch-based Corn, cassava, sago palm, etc.	Assimilated by enzymes (amylase); the most widely used in current amino acid fermentation. Price increase due to competition with bioethanol a significant point of issue.
Sugar-based Cane molasses Beet molasses Raw sugar (sucrose)	Molasses is the crystallization mother liquor from sugar and is made up of sucrose, glucose, and fructose. Used in glutamic acid and lysine fermentation. Price increase a significant point of issue.
Other raw materials	
Acetic acid	A superior raw material for usability and amino acid purification than colored mixtures such as molasses due to its solubility and purity. Used briefly for fermentation of glutamic acid, lysine, and threonine, but its use was discontinued after it rose in price following the oil crisis (1980).
Methanol	Superior for usability and amino acid purification due to its purity. Currently receiving some attention due to rising prices of starch-based and sugar-based sources, but present productivity is low. For further study.
Ethanol	Superior for usability and amino acid purification due to its purity. Studied briefly for practical implementation but this is currently discontinued.
n-paraffin (n-alkane)	Received attention as a potential source material prior to the oil crisis (1980), but was discontinued beyond the research stage due to price increase and consumer aversion.
Cellulose-based materials (biomass)	Receiving attention as a renewable resource from rice straw, chaff, bagasse, scrap wood, etc. Bottlenecked at collection and sugar conversion.
Carbon dioxide	Thought to be the ultimate ideal raw material, but has low production yield in its present state. For further study.

9 Overseas Expansion of Amino Acid Fermentation Technology

Kyowa Hakko exported its glutamic acid fermentation technology developed in 1957 to US company Merck in 1958 and to Taiwan company Ve Wong around the same time. The company also exported its lysine fermentation technology to Merck in 1958.

Meanwhile, Ajinomoto Co., Inc. used a strategy of establishing factories to expand its glutamic acid fermentation technology overseas. This expansion made active progress, with plants successively established in Thailand in 1961, Malaysia, the Philippines, and Italy in 1962, Peru in 1968, Indonesia in 1969, Brazil in 1974, and then into the United States, China, Vietnam, and other countries. Ajinomoto Co., Inc. also established subsidiary company Eurolysine in France in 1974 and began fermentation production of lysine for use in animal fodder. The company also established Heartland Lysine Inc. in the United States (Iowa) in 1984, followed by further expansion into Brazil, Thailand, Italy, and China. In France and the United States, production began on threonine and tryptophan for animal fodder in addition to lysine. This successful overseas expansion of amino acid fermentation technology led to an increase in amino acid production volume worldwide, as mentioned in Section 2.4.

Meanwhile, although Kyowa Hakko advanced its lysine technology into Hungary and its lysine, threonine, and tryptophan technology into the United States and Mexico, according to the *Nikkei-Bio Yearbook*,⁽¹⁾ it closed down its operations in Mexico in March 2004, sold all of its stock in Hungary, including its intellectual property related to lysine, threonine, and tryptophan, to Degussa and faced the challenge of restructuring its BioKyowa facilities in the United States. This shows a stark contrast between the two companies.

Ajinomoto Co., Inc. started increasing its production of amino acids for pharmaceutical products at its plant in the United States (North Carolina), followed by China and Brazil. By contrast, although Kyowa Hakko faced a slump in the United States, as mentioned above, it was increasing its production in China (Shanghai Kyowa).

Table 9.1 shows an outline of the distribution, product lines and production capacity (including estimates) of the main amino acid plants around the world.

Most of the processes used at these factories are based on Japanese technology; in many cases, the overseas manufacturer has access to a cheap supply of raw materials and is using Japanese technology that has come off patent. China is a major player in MSG production and consumption, consuming around one third of the MSG produced around the world. Production volumes, around 30,000 tons in 1980, increased by around 20 times to around 600,000 tons in 1997. While further increases in demand can be expected as living standards rise and eating habits improve, there are around 100 manufacturers in the industry in addition to the major ones listed in Table 9.1, meaning intense competition in which only the strong will survive. There is a growing demand to respond to environmental issues and the major players are beginning to consolidate.⁽²⁾ In contrast to the flourishing overseas expansion, there is very little amino acid being produced within Japan at present (2007). This is a natural consequence of the dependence of amino acid fermentation on raw materials, as well as the large market and low cost of labor overseas.

Table 9.1 Distribution, Product Lines and Production Capacity of the Main Amino Acid Plants Worldwide

Country	Company (conglomerate)	Region	Products	Production Capacity (tons/year) (including estimates)
Brazil	Ajinomoto	Sao Paulo	Glu	90,000
	Interamericana	“ Laranjal Paulista Valparaíso Pederneras	various	2,000
	Ajinomoto Biotina		Glu	80,000
			Lys	60,000
			Thr	13,000
			Lys	60,000
China	Linghua MSG Group	Jining, Shandong	Glu	140,000
	Lianhua MSG Group	Xiangcheng, Henan	“	120,000
	Hongmei MSG Group	Shenyang, Liaoning	“	50,000
	Guangzhou Orsan	Guangzhou,	“	40,000
	Gourmet Powder	Guangdong	“	40,000
	Jiangsu	Zhangjiagang, Jiangsu		
	Chrysanthemum			
	Gourmet Powder			
	Group			
	GBT		Lys	240,000
	GBT Technology	Changchun	“	120,000
	Group			
France	BBCA Biochemical	Anhui	“	20,000
	Juneng Group	Shandong	“	20,000
	Sichuan Chunhua	Chengdu, Sichuan	“	30,000
	Ajinomoto			
	Ajinomoto	Shanghai	Various	800
	Kyowa Hakkō	“	“	800
Germany	Ajinomoto	Amiens	Lys	70,000
	Eurolysine			
		“	Thr	35,000
		“	Trp	3,000
Hungary	Ajinomoto Foods	Nales	Glu	65,000
	Europe			
Italy	Degussa	Frankfurt	Met	222,000
Indonesia	Kyowa Hakkō	Kaba	Lys	(withdrawn)
	Ajinomoto Bioitalia	Bottorighe	Lys	30,000
	Cheil Sugar	Pasuruan	Glu	50,000
		“	Lys	100,000
		“	Thr	10,000
	Ajinomoto	Slavaya	Glu	50,000
	SASA	-	Glu	65,000
	Miwon Indonesia	-	Glu	80,000
Japan	Ajinomoto	Kawasaki	various	-
	“	Saga	Phe, Arg, Asp, Cys	-
	Kyowa Hakkō	Yamaguchi	various	-
South Korea	Cheil Sugar	-	Glu	60,000
	Desan	-	Phe	4,000
	BASF	-	Lys	70,000
Malaysia	Malaysia Ajinomoto	Kuala Lumpur	Glu	15,000
Mexico	Kyowa Hakkō	-	Lys	(withdrawn)
			Thr	“
Philippines	Ajinomoto	Manila	Glu	20,000
Peru	Ajinomoto	Lima	Glu	10,000
Slovakia	Degussa	Slovenska Lupca	Lys	10,000
		“	Thr	3,000
		“	Trp	400
Thailand	Thailand Ajinomoto	Bangkok Kamphaengphet Patontani	Glu Glu Lys	90,000 40,000 50,000
Taiwan	Vedan	-	Glu	100,000
United States	USA Ajinomoto	Iowa	Glu	40,000
		North Carolina	various	-
	Ajinomoto Heartland	Iowa	Lys	50,000
		“	Thr	40,000
	BioKyowa	Missouri	Lys	(withdrawn)
		“	Thr	“
Vietnam	ADM	Illinois	Lys	160,000
		“	Thr	10,000
Vietnam	Vietnam Ajinomoto	-	Glu	60,000
	Vedan	-	“	200,000
	Miwon	-	“	20,000

<http://www.ajinomoto.co.jp/kyushu/info.html>
<http://www.ajinomoto.co.jp/kawasaki/profile.html>
<http://www.kyowa.co.jp/csr/sustain/report/houfu.html>
http://www.ajinomoto.com/ar/i_r/pdf/fact/Feed-useAA-Oct2007.pdf?search=
 Based on the above websites as well as articles in the *Health Food Journal*, *Nikkan Kogyo Shimbun*, *The Chemical Daily* and other sources.

Although Tanabe Pharmaceuticals actively published research on amino acid fermentation up until the 1980s, it is no longer doing this. It has had a change in management policy due to its enterprise integration with Mitsubishi and it is thought to be likely to focus its research and development goals towards pharmaceutical products.

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- (1) *Nikkei-Bio Yearbook 2005*: p. 538.
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10 Amino Acids and Environmental Issues

10.1 Fermentation Process

The fermentation process itself is a natural process, like brewing sake or soy sauce, and has no problematic impact on the environment. Systems are being developed that are even more actively in harmony with the environment. For example, in the process of producing glutamic acid by fermentation from sugarcane, Ajinomoto factories in Indonesia and Brazil produce a waste liquid byproduct containing microbes, ammonia, phosphoric acid, calcium, and magnesium. They have established a system of returning this byproduct to the land in the form of a near-complete fertilizer. This resource-circulation type of production process is called a bio-cycle. Fertilizer made from this byproduct has been shown to effectively increase the yield and quality of the agricultural produce and is not only used on sugarcane, but also on corn, coffee, fruit trees, and flowers ⁽¹⁾.

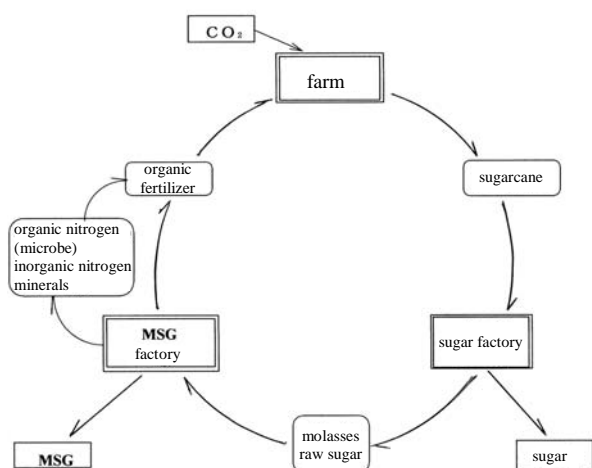


Fig. 10.1 Raw Materials and Byproduct Cycle System at an Amino Acid Fermentation Factory (revised from ⁽¹⁾)

10.2 Livestock Ammonia Emission Reduction Effect from Amino Acid Supplements in Animal Fodder

Emissions of nitrogen (ammonia) from animal excrement are thought to be a leading cause of environmental pollution, affecting the air quality or the water quality of rivers and lakes. Nitrogen emissions are caused by an imbalance in the amino acids in the animal fodder. The amino acid composition in the corn and other grains used for animal feed lacks particular amino acids when compared to the amino acid composition of eggs or milk, meaning a poor amino acid balance. Accordingly, even if there are a lot of other amino acids present, they will be broken down into ammonia and excreted rather than being used. This is the cause of nitrogen emissions. Consequently, supplementing the animal fodder with the lysine and threonine lacking in the corn and other grains not only improves nutrition, but also increases the utilization of the amino acids and significantly reduces the amount of nitrogen emissions. ⁽²⁾ Thus, effective use of amino acids helps to conserve the environment.

<References>

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11 Safety of Amino Acids

11.1 Debate over the Safety of MSG

Amino acids are nutrients routinely ingested as food or fodder in the form of protein or in isolated form. They are believed to be extremely safe compared with non-routinely ingested products such as medicines. However, concern and anxiety over the safety of chemical compounds used in pharmaceutical products and food additives spread throughout the world from the late 1960s onwards, extending to amino acids as well. Amidst these circumstances, the following two reports emerged on MSG.

(1) A report on the so-called Chinese restaurant syndrome claiming that excessive ingestion of MSG in Chinese cuisine caused hot flashes, headaches, heart palpitation and other symptoms ⁽¹⁾

(2) A report claiming that newborn juvenile mice forcibly administered with large amounts of MSG (0.5-4.0 g/kg bodyweight) incurred neuronal cell death in the hypothalamus and inner layers of the retina ⁽²⁾ These reports were widely picked up on by newspapers and other media, with *Asahi Shimbun* in particular reporting in a one-sided and sensational manner, “Et Tu, Monosodium Glutamate?” (likening it to sodium cyclamate, which was banned from being sold due to carcinogenic issues). Based on results by J. W. Olney, author of report (2) above, the United States Senate Select Committee on Nutrition and Human Needs recommended that MSG not be used in baby food.

In June 1970, the WHO/FAO Joint Expert Committee on Food Additives (JECFA) recommended an acceptable daily intake (ADI) of 120 mg/kg bodyweight for MSG. With the consumer movement led by R. Nadar intensifying in the United States, re-evaluation began on food additives and pharmaceutical products that had been designated as GRAS (Generally Recognized as Safe).

Meanwhile, MSG manufacturers’ committees around the world formed into the International Glutamate Technical Committee to demand a verifiable judgement, promoting the collection of scientific and impartial data and holding open symposia. Consequent reports stated that there were no signs of neurotoxicity resulting from oral ingestion of MSG and that a double-blind trial using a placebo had proven that there was no correlation between MSG ingestion and the adverse symptoms reported above (the so-called Chinese restaurant syndrome). ^{(3), (4)} In May 1980, the FDA announced its final conclusion that MSG was “safe as a food additive at its current usage level,” even for baby food.

Even with this scientific “safety declaration” for MSG,

there is still a persistent theory that MSG is harmful, with products and stores using catchphrases assuring that “no chemical seasonings are used in our products.” A survey carried out by the author on university students revealed that over 90 % of students have the impression that MSG is “not good for health.” It is the author’s belief that proper understanding is necessary and that publicity activities would play a major role in this. There is a problem in the current situation with a widespread, deep-rooted sense of antagonism based on misunderstanding and completely removed from people’s likes or dislikes.

11.2 Amino Acid Safety Data

Table 11.1 shows the median lethal dose LD₅₀ (the dosage required to kill 50 % of the members of a tested population = acute toxicity) of various amino acids. The values for the salts and acetic acids used in everyday food are also given here for the sake of comparison; the values for most of the amino acids are higher than these, in other words, they are far safer. Subacute toxicity testing (testing involving 1-3 months of continuous administration to rats and then checking for weight change and histopathology of visceral organs), two-year chronic toxicity testing on mice, rats, and dogs, and carcinogenicity testing have proven that no changes occurred at all compared to the control groups. ⁽⁶⁾

Table 11.1 LD₅₀ Values of Amino Acids and Other Food Ingredients ⁽⁵⁾

Substance	LD ₅₀ (g/kg bodyweight, orally administered to rats)
Salt	3.0
Cys	3.1
Acetic acid	3.3
Vitamin B ₁₂	4.0
Sodium carbonate	4.1
Niacin	7.0
Lys	10.6
(Cys) ₂	11.2
Citric acid	11.7
Vitamin C	11.9
Glycerol	12.6
Ser	14.0
Arg, Phe, Gly	16.0
Ala, Asn, Ile, Gln, Glu, Thr, Tyr, Trp, Val, His, Pro, Met, Leu	>16.0
Sucrose	29.7

All of the amino acids examined were L-compounds.

11.3 Issues with Chemical Compounds Derived from Tryptophan

There is a case of an incident presumed to be caused by secondary substances produced from amino acids. In the early 1980s, there were instances of eosinophilia-myalgia syndrome (EMS) believed to be caused by a particular batch of tryptophan, resulting in over 30 fatalities. The substances thought to be the cause were identified within a relatively short space of time to be ethylene-bis (tryptophan) and (phenylamino) alanine. These compounds were detected in products that used a particular batch of tryptophan that had low purity and are believed to have formed as the tryptophan transformed in the presence of impurities. The supplier, Showa Denko, had to pay a high amount of indemnity because of this incident. While it does depend on the type of amino acid, this incident particularly highlights the importance of product purity and content quality control.

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12 | Systematization of Amino Acid Fermentation Technology

It has been a century since the beginning of industrial production of amino acid, and 50 years since the beginning of industrial production by fermentation. Both of these technologies are original to Japan. While amino acids are now mainly produced by fermentation, this is one of three production methods, along with protein hydrolysis and chemical synthesis. Each method was developed with a lot of support from science and technology. Table 12.1 summarizes the progression of this technology, focusing on the fermentation method, the main topic of this report. The first amino acid commercialized was glutamic acid in 1908, produced by protein hydrolysis. Production using this method continued until around 1960; in other words, although increasingly fewer amino acids were produced using the protein hydrolysis method due to the establishment of the revolutionary fermentation method around that time, the method is still used today for some amino acids, including tyrosine, cysteine, and asparagine. The amino acid separation and purification technology established for this method was also used in the fermentation method.

The method of producing glutamic acid by chemical synthesis was developed to overcome a few issues with the proteolysis method. This technology originated from synthetic chemistry and although it was industrialized for a short time, it was not well received by consumers and was largely discontinued. Currently some amino acids are still produced using the chemical synthesis method, including glycine, DL-methionine, and DL-alanine.

The fermentation trend began with the major discovery of glutamic-acid-producing microbes and glutamic acid fermentation, which still occupies a central position in amino acid fermentation. This technology was undergirded by interest from many researchers due to the fact that there was already a successful business for the attractive target product, namely sodium glutamate (“Ajinomoto”). The technology was also supported by existing science and technology that could be used as

an approach to glutamic acid production, namely microbe screening and cultivation technology in the fields of microbiology, biochemistry, and molecular biology. The technological development is thought to have been significantly influenced by prior antibiotics production methods using fermentation.

It appears that it was not considered possible at the time to produce glutamic acid directly from glucose and the successful isolation of a new microbe called a glutamic acid microbe was deemed a successful “defiance of common sense.” The key to the success lay in the screening method. Interestingly, this technology was produced by Kyowa Hakko, a company that specialized in fermentation technology rather than glutamic acid. Technology developed for the protein hydrolysis method was used for separating and purifying the glutamic acid from the culture solution.

The method of inducing auxotrophic mutants and analog-resistant strains, particularly the latter, from glutamic acid microbes resulted in many amino-acid-producing microbes and amino acid fermentation suddenly skyrocketed. At the same time, another production method was established involving the use of a bioreactor, screening the microbes used in fermentation and developing the enzyme method. Genetic engineering technology that emerged in the 1980s has already made major progress on existing producing strains as a “booster” to the existing technologies and further significant technological developments can be expected in future. Around 75 % of amino acid products and 85 % of amino acid product volume is produced using the fermentation and enzyme methods.

Amino acid fermentation will continue to increase quantitatively as the population increases. Further significant developments are also likely with the recent establishment of peptide synthesis technology, produced from amino acids.

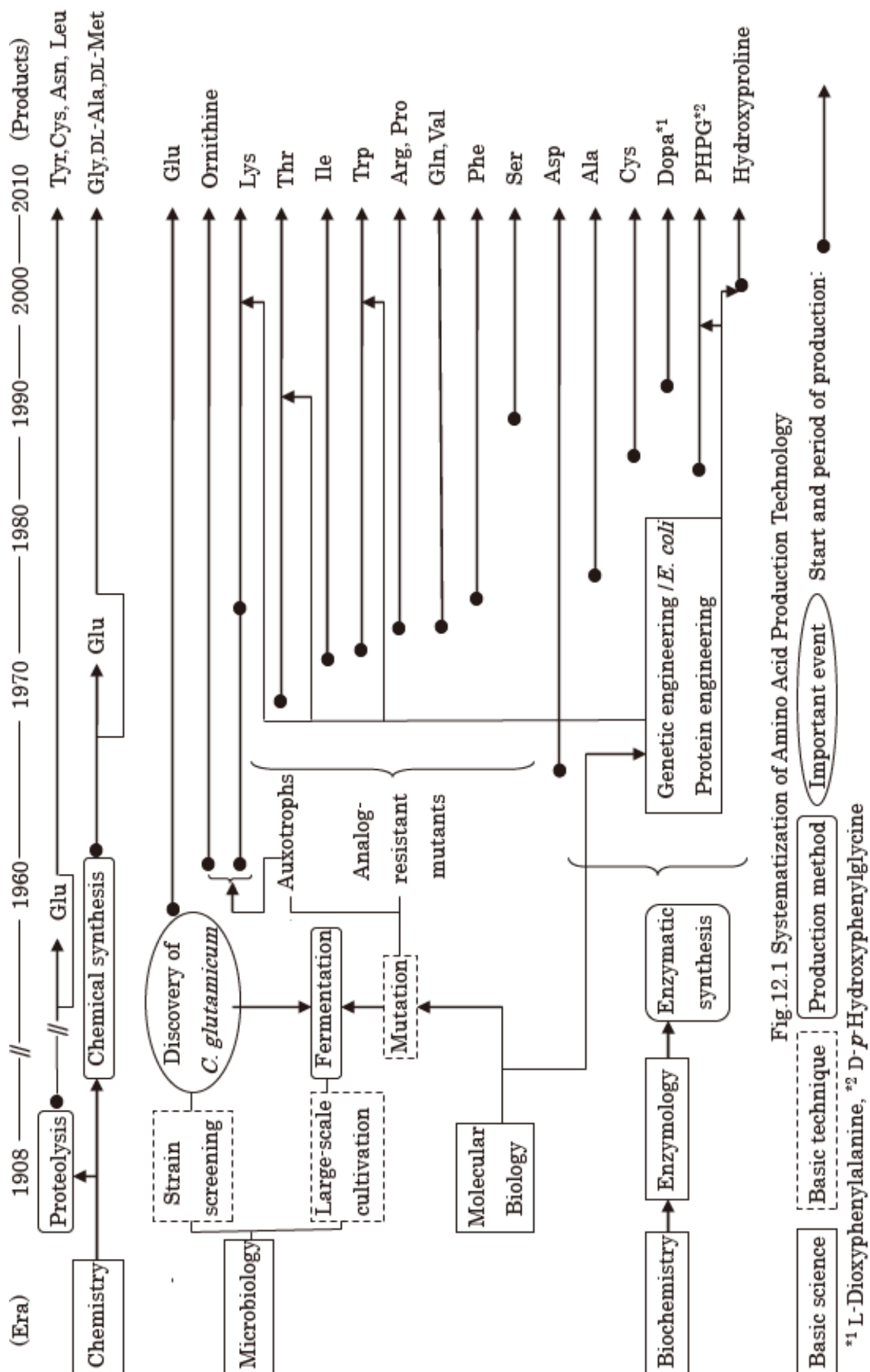


Fig.12.1 Systematization of Amino Acid Production Technology

13 | Future Developments

Amino acid fermentation is original Japanese technology that has now spread around the world, contributing to medical care and food supplies. Japan is at the center of these technologies and leads the world in this industry. Amino acids are essentially natural compounds and there is no alternative protein compound for them, especially nutritionally. The demand for them will certainly increase, given the expected future worldwide population growth and food shortage.

Although the belief is that large growth is expected in the market and that superior technology will produce large profits, the fact is that this is not always the case. The main reasons for this are increases in the price of raw materials and decreases in product prices. This is particularly true of glutamic acid and lysine, which are produced in large quantities and the expense of raw materials takes up a large proportion of the cost. The basic patents for the technology have already expired, meaning that Japan no longer has the advantageous position it held to begin with and is now in increasingly fierce competition with overseas manufacturers, who have access to cheap raw materials. The cost increase can no longer simply be incorporated into the product price, necessitating further cost reductions and optimized production efficiency through increased yields, efficient use of main auxiliary materials, reductions in energy, reductions in byproducts, and development of environmentally friendly processes. Technological developments have a very high relative importance in cost reduction. Let us consider what kind of possibilities there are for improvement and development.

13.1 Strain Breeding / Improving Existing Strains

A field that also considers possibilities for technological improvements. In other words, it is based on new technology that utilizes DNA information and DNA technology and involves the use of DNA and mRNA to comprehensively analyze the movements of proteins and intermediates as well as the metabolic flow within microbial cells, with the ultimate aim of trying to optimize the entire metabolism of the producing strain. Some of these attempts have already produced some powerful results. For example, there is a general trend for production performance to reach a peak in the late stage of the culture and the *rmf* gene characteristically increases its level of expression during this stage. Knocking out this gene made it possible to suppress the peaking and boost amino acid production performance. Research has shown that knocking out the *arc* gene, which regulates the expression of enzyme genes related to and including the TCA cycle, boosts enzymatic activity related to the TCA cycle and boosts the production performance of

glutamic acid, arginine, and lysine, which are biosynthesized through the TCA cycle.

A gene has even been identified as related being to the secretion of substance from the cell, which often presented issues in using microbes to produce substances. Amplifying this gene has been shown to improve the secretion efficiency, or in other words, to improve the amino acid production performance.⁽¹⁾ A bold attempt is also being undertaken to create microbes as minimum genome factories with the minimum genomes (sets of genetic instructions) necessary for producing useful substances, with the aim of efficient production.⁽²⁾ These research findings have been put into practical use and can be expected to increase production even further.

13.2 Strain Breeding / Developing and Using New Raw Materials

While bioethanol has suddenly become a talking point in recent years, as long as the price of crude oil continues to rise, starch and sugar, the main raw materials used in amino acid fermentation, will always compete with bioethanol and food, and price increases will be an issue. Therefore, there is a need to consider using raw materials other than starch and sugar. For example, it is worth investigating breeding producing strains from microbes that can assimilate methanol, or from photosynthetic bacteria that can utilize CO₂. Consideration is also being given to using rice straw, chaff, sugarcane stalks, wood scraps, and other types of biomass. Although there is the problem of sugar conversion and collection from these materials, further development of raw materials for amino acid fermentation can be expected.

13.3 Cultivation Technology

Since continuous cultures have unresolved issues with microbe contamination and the like, no such production processes have been implemented other than activated sludge processes. This is the most anticipated technology in terms of energy and resource conservation.

13.4 Separation / Purification Technology

This technology is quite complete and there is considered to be little prospect for any radical improvements. Acidic cultivation is being considered as a means of reducing the byproduct at the purification stage. For example, cultivation for glutamic acid

fermentation is now carried out under neutral conditions by neutralizing with ammonia; sulfuric acid is added for crystallization, then it is finally neutralized with NaOH to produce MSG. This produces ammonium sulfate as a byproduct. The idea is that if cultivation could be carried out in acidic conditions, there would be no ammonium sulfate byproduct.⁽¹⁾

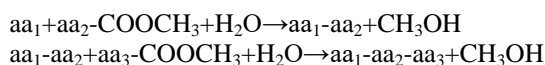
13.5 Environmental Issues

This report mentioned that environmentally friendly systems have been created to recycle raw materials and byproducts at fermentation plants. These systems are now expected to be more complete than ever. Ajinomoto Co., Inc. has already launched its “Awareness Project for High Utilization Technology of Cassava” in collaboration with agricultural experiment stations in Indonesia, aimed at intensively utilizing the waste liquid from its fermentation plants as fertilizer.⁽¹⁾ Future issues for consideration include converting sugar-extracted sugarcane (biomass) into sugar or fuel and reducing CO₂ emissions from factories. Faced with these technological challenges, hopefully Japan will maintain and even advance its predominant position in amino acid technology.

13.6 Development of Peptide Technology

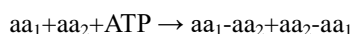
Peptides are substances that are closely linked to amino acids. Peptides link amino acids and form when the carboxyl group of one α -amino acid joins to the amide group of another α -amino acid through dehydrated condensation (see Fig. 2.2). While these have a number of physiological functions, such as hormones, and have potential use as pharmaceutical products or similar, to date only small-scale production has been carried out through chemical synthesis. There are complex issues involved, such as introducing/eliminating protecting groups to/from functional groups, and no practical production methods are being carried out this way. By contrast, biochemical production methods have recently been developed and are gaining attention. One

such method has been developed by Ajinomoto Co., Inc. and has been used to successfully screen microbial enzymes that catalyze the following peptide synthesis reaction from isolated amino acid (aa₁) and amino acid methyl ester (aa₂-COOCH₃).⁽³⁾



This method thus successively lengthens the peptide chain.

A method developed by Kyowa Hakko performs an enzymatic reaction⁽⁴⁾ using ATP, with isolated amino acids as the energy source. This produces both forward and reverse peptides using the following bonding method and is thought to lower the yield of intended particular peptides.



The fact that these technological developments have made peptide production possible indicates a greater likelihood of discovering promising peptides from among unlimited combinations of amino acids and it is very likely that peptide fermentation will emerge as a new technological field in the near future.

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Japan Bioindustry Association	Eikou Shimizu

List of Registration Candidates

No.	Name	Format	Location	Manufacturer	Year	Comments
1	Japan's oldest sample of glutamic acid (prepared by Kikunae Ikeda)	exhibit	Ajinomoto Corporate Museum, Minato, Tokyo	Kikunae Ikeda	1908	The first glutamic acid extracted from <i>kombu</i> , which became the major product "Ajinomoto"
2	Domyōji-game pots	original item	Ajinomoto Corporate Museum, Minato, Tokyo	unknown	1912-1925	Memorable ceramic pots developed as acid-resistant reaction vessels to withstand hydrochloric acid
3	immobilized enzyme cutting machine	original item	Tanabe Seiyaku Yamaguchi Co., Ltd., Sanyo-Onoda	Tanabe Pharmaceuticals	1974-1976	A memorable product produced by Tanabe Pharmaceuticals, a pioneer in enzyme immobilization

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