Production of Arginine by Fermentation

Takashi Utagawa2

Fermentation & Biotechnology Laboratories, Global Foods & Amino Acids Company, Ajinomoto Co., Inc., Kawasaki, Japan

ABSTRACT Studies on the production of L-arginine by fermentation using mutants of Corynebacterium (Brevibacterium), Bacillus, and Serratia have been conducted since the 1960s. More recently, the breeding of L-arginine production strains by gene recombination techniques using Escherichia coli has been investigated. To produce L-arginine efficiently by fermentation, it is necessary to breed strains with a strong biosynthetic pathway to L-arginine. Because L-arginine is biosynthesized from the precursor L-glutamic acid through ornithine and citrulline, the use of strains with a high capability for producing L-glutamic acid is desirable. Corynebacterium (Brevibacterium), which is well known in the production of L-glutamic acid, was selected as a starting strain for the breeding of an L-arginine producer and has been used on a commercial scale. Regarding the fermentation conditions, as for other amino acids, L-arginine fermentation is controlled by regulating pH near the neutral point. Due to its high oxygen requirement, L-arginine production is seriously impaired without sufficient oxygen. Advanced purification methods are necessary to obtain highly pure L-arginine from the fermentation broth. After fermentation is complete, bacterial cells and proteins are removed by means of a membrane or centrifugation, and impurities are removed by means of an ion-exchange resin or activated carbon. Highly pure L-arginine crystals can be obtained through concentration at the end of the process.

KEY WORDS: fermentation • mutant • Corynebacterium • Brevibacterium • breeding • purification

L-Arginine was first isolated from a lupine seedling extract in 1886. It was identified as a component of casein in 1895; later it was found to be widely distributed in foods and feed. L-Arginine is broken down by arginase to form L-ornithine and urea in mammals, completing the ammonia excretion system. L-Arginine is recognized as performing an important function in mammals.

The production methods for amino acids are summarized as follows: 1) protein hydrolysis, 2) chemical synthesis, and 3) microbiological synthesis. Most L-arginine has been produced by the direct-fermentation method from natural carbon sources.

There are two types of metabolic control mechanisms in microorganisms (Fig. 1) (1). In type 1, which includes Escherichia coli and Bacillus subtilis, N-acetylglutamate is synthesized from L-glutamate by N-acetylglutamate synthase, and this enzyme is strongly inhibited by L-arginine through a feedback control mechanism. In type 2, which includes Corynebacterium (Brevibacterium), N-acetylglutamate-acetylornithine acetyltransferase, which is not inhibited by L-arginine, catalyzes a transacetylation reaction between glutamate and N-acetylornithine to form N-acetylglutamate and ornithine. However, N-acetylglutamokinase is inhibited by L-arginine in type 2.

Studies on the industrial production of L-arginine by fermentation initially used mutants of Corynebacterium (Brevibacterium). Unlike ornithine or citrulline, L-arginine cannot be produced using auxotrophic mutants because it is an end product of the L-arginine biosynthetic pathway. Although mutants of E. coli and Saccharomyces cerevisiae were reported to secrete L-arginine, it was only during the past 30 y, when regulatory mutants such as analog-resistant mutants were put into full-scale use, that the production of large amounts of L-arginine by fermentation became possible. More recently, transduction or gene engineering was applied to breed highly productive L-arginine producers.

Glucose from tapioca or corn is one of the best carbon sources for L-arginine production. Because 1 molecule of L-arginine contains 4 nitrogen atoms, ammonia is an essential raw material for supplying nitrogen (Fig. 2). Advanced technologies are applied in the isolation and purification process to obtain highly purified L-arginine crystals.

Breeding L-arginine producers

Because L-glutamic acid is an important metabolic precursor for L-arginine biosynthesis, L-glutamic acid producers were used as starting strains for L-arginine producers.

Some amino acid analog–resistant mutants produce their corresponding amino acids. Canavanine-resistant mutants of...
N-acetylglutamate—amounts of L-ornithine and L-citrulline when suboptimum glutamic acid producers, accumulate large amounts of L-arginine. The inversions found many L-arginine producers among mutants which the mechanism regulating L-arginine synthesis is altered. Therefore, mutants of glutamic acid producers in the regulatory mechanisms operating in the arginine biosynthetic pathway. However, the levels of accumulation are comparatively low, probably due to the intrinsic properties of their parental strains.

Conversely, L-arginine auxotrophs of B. subtilis excrete or accumulate L-arginine. How-ever, the levels of accumulation are comparatively low, probably due to the intrinsic properties of their parental strains. 

FIGURE 1 Metabolic control of L-arginine biosynthesis, types 1 and 2: 1) N-acetylglutamate synthase, 2) N-acetylglutaminase, 3) N-acetylglutamate-γ-semialdehyde dehydrogenase, 4) N-acetylimid-thione-δ-aminotransferase, 5) N-acetylornithinase, (5′) N-acetylglutamate-acetyloynithine acetyltransferase, 6) ornithine carbamoyl trans- ferase, 7) arginosuccinate synthetase, and 8) argininosuccinase. Abbreviations: Cit, L-citrulline; Glu, L-glutamate; Orn, L-ornithine.

FIGURE 2 Biological synthesis of L-arginine.

E. coli and S. cerevisiae and arginine hydroxamate–resistant mutants of B. subtilis excrete or accumulate L-arginine. However, the levels of accumulation are comparatively low, probably due to the intrinsic properties of their parental strains. Conversely, L-arginine auxotrophs of Corynebacterium (Brevibacterium), glutamic acid producers, accumulate large amounts of L-ornithine and L-citrulline when suboptimum concentrations of L-arginine are added to the medium. These facts show that such glutamic acid producers have the potential ability to produce large amounts of arginine as well. However, this ability is genetically masked by the feedback regulatory mechanisms operating in the arginine biosynthetic pathway. Therefore, mutants of glutamic acid producers in which the mechanism regulating L-arginine synthesis is altered are expected to produce large amounts of L-arginine.

Kubota et al. (2) tried to apply canavanine and other L-arginine analogs, such as homoarginine and nitro-L-arginine, to Brevibacterium flavum, one of the glutamic acid–producing bacteria. Canavanine (6 g/L) inhibited the growth of B. flavum almost completely. Homoarginine and nitro-L-arginine also inhibited the growth of the bacteria. These chemical-resistant mutants did produce a small amount of L-arginine. The investigations found many L-arginine producers among mutants resistant to amino acid analogs such as thienylserine, D-serine, ethionine, and thiazolealanine; mutants resistant to sulfa drugs such as sulfamerazine and sulfisoxazole; and mutants resistant to a purine base analog, chloropurine.

The most prominent L-arginine producer was derived through several mutation steps from a guanine auxotroph mutant. This mutant was treated with N-methyl-N'-nitro-N-nitrosoguanidine, a mutagen, to obtain a 2-thiazolealanine–resistant mutant. This mutant was treated with diethyl sulfate and an L-arginine producer was isolated (Fig. 3).

Utilizing recent advances in recombinant DNA technology, Momose et al. (3) obtained E. coli strains with acquired or enhanced L-arginine production capability. E. coli AJ 11534, lacking the arginine operons repressor gene and resistant to arginine hydroxamate, produced 0.75 g L-arginine/L. A library of chromosomal DNA from AJ 11534 was constructed in pBR322 and was introduced into a strain lacking N-acetylglu-tamate synthase (argA) gene activity to isolate the argA gene. The colonies carrying the cloned argA gene were selected by complementation of the L-arginine auxotroph. The argA plasmids were introduced into AJ 11534, and L-arginine accumulation in this strain increased to 1.9 g/L. These results suggest that amplification of argA gene activity is an effective means of increasing L-arginine production.

FIGURE 3 Mutant lineage of L-arginine producer. Abbreviations: NG, N-methyl-N'-nitro-N-nitrosoguanidine; TA, 2-thiazolealanine.

Raw materials

Starches from tapioca and corn are the main raw materials for amino acid fermentation. Sugar and sugar syrup are also used. Starches are first hydrolyzed with enzymes such as liquid amylase and glucose amylase to form liquid glucose. After filtration, the glucose solution is >95% dextrose.

Another important raw material is ammonium sulfate. The L-arginine molecule is 32% nitrogen, the highest nitrogen content of any amino acid. This means that an ample nitrogen source must be supplied to produce a large amount of L-arginine.

Effect of oxygen on fermentation

Hirose et al. (4) and Akashi et al. (5) examined the effect of partial pressure of oxygen on the fermentation of various amino acids. The production of L-arginine is strongly inhibited by the lack of oxygen, because oxygen is one of the raw materials for L-arginine. Oxygen dissolves in culture broth through several mutation steps from a guanine auxotroph mutant. This mutant was treated with N-methyl-N'-nitro-N-nitrosoguanidine, a mutagen, to obtain a 2-thiazolealanine–resistant mutant. This mutant was treated with diethyl sulfate and an L-arginine producer was isolated (Fig. 3).

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with a saturation value as low as 7.5 ppm at atmospheric pressure, so air must constantly be introduced into the fermentation vessel.

The various conditions of oxygen supply to fermenters are governed by changes in airflow and agitation speed. The partial pressure of oxygen in the liquid is determined constantly by an automatic oxygen analyzer.

The cell respiration rate ($r_{ab}$) was calculated by measuring the partial pressure of oxygen in the effluent gas. The $r_{ab}$ reaches the maximum oxygen demand ($K_{R,M}$) of the cells when dissolved oxygen is above the critical value for cell respiration.

The degree of satisfaction of cell oxygen demand ($r_{ab}/K_{R,M}$) is used as an index of oxygen supply under conditions of extremely low oxygen tension. The cells produce a large amount of the end products with a sufficient supply of oxygen at $r_{ab}/K_{R,M} < 1.0$. L-Arginine formation decreases markedly at $r_{ab}/K_{R,M} > 1.0$. Lactic acid accumulates in a culture where oxygen is extremely deficient. In aerobic amino acid fermentation, oxygen is one of the raw materials that must be supplied in large amounts. Therefore, quantitative evaluation of oxygen transfer during the fermentation is indispensable for optimal operation. Aerobic microorganisms require oxygen mainly to reoxidize NAD(P)H$_2$ or FADH$_2$ in order to form NAD(P) or FAD and to effectively generate ATP for its metabolism.

Effect of carbon dioxide on fermentation

Akashi et al. (6) examined the effect of carbon dioxide on amino acid fermentation. L-Arginine accumulation peaked when cell respiration was satisfied at $r_{ab}/K_{R,M} = 1.0$. Therefore, the influence of carbon dioxide on L-arginine biosynthesis was investigated under conditions of sufficient oxygen supply. L-Arginine formation was maximal at gas-phase carbon dioxide tension $\sim 0.12$ atm (12.1 kPa) whereas gas tension $> 0.15$ atm (15.2 kPa) was inhibitory. The inhibition caused by carbon dioxide led to the accumulation of L-alanine at the expense of L-arginine.

L-Arginine biosynthesis requires carbon dioxide fixation. It is interesting that the production of L-arginine is influenced by carbon dioxide. If carbon dioxide is regarded as one of the substrates for L-arginine biosynthesis, high carbon dioxide tension might favor the efficient production of L-arginine instead of having an inhibitory effect. The mechanism of the inhibitory effect of carbon dioxide on arginine formation is not clear. Adequate aeration is necessary to dilute the inhibitory effect of carbon dioxide.

Inhibitory effect of high dissolved oxygen tension in the growth phase

Although oxygen shortage markedly inhibits cell growth, high dissolved oxygen tension also affects cell growth. The cells grow more rapidly at a dissolved oxygen level (pyridoxal)}
of 0.01 to 0.05 atm (1.0 to 5.1 kPa) than at 0.32 to 0.42 atm (32.4 to 42.5 kPa). In addition, high oxygen tension during the growth phase has a continued influence on product formation, so that the cells grown under high oxygen tension have a poor ability to produce arginine (Table 1).

**Isolation and purification of L-arginine**

The isolation and purification process of L-arginine is diagrammed in Figure 4. After L-arginine fermentation is completed, the microorganisms are separated by centrifugal separation or membrane filtration. The supernatant or filtrate is charged to the resin column to separate organic acids or other amino acids. Both cation and anion types of resin are used to minimize the impurities in the L-arginine crystals. Active carbon powder is used for decoloration. After filtration through active carbon powder, the filtrate is concentrated to form the L-arginine crystals, which are separated by centrifugation. The crystals are dissolved in pure water and then ultrafiltered. The pure crystals are obtained by concentration and cooling. Because L-arginine dissolves well in hot water (Fig. 5), the concentrated solution must be cooled to ~10°C to obtain L-arginine crystals. The crystals are dried with a dryer to minimize the moisture concentration at <0.5%. The obtained crystals (Fig. 6) are >98.5% pure (Table 2).

**LITERATURE CITED**


**TABLE 2**

*Specification of L-arginine*

<table>
<thead>
<tr>
<th>Item</th>
<th>Limit</th>
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<tbody>
<tr>
<td>Specific rotation ([α]_D^20)</td>
<td>+26.9 to +27.9\° (C = 8.6 mol/L HCl)</td>
</tr>
<tr>
<td>State of solution (transmittance)</td>
<td>Clear and colorless ≥98.0%</td>
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<tr>
<td>Chloride (Cl)</td>
<td>≤0.020%</td>
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<tr>
<td>Ammonium (NH₄)</td>
<td>≤0.02%</td>
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<tr>
<td>Sulfate (SO₄)</td>
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</tr>
<tr>
<td>Iron (Fe)</td>
<td>≤10 µg/kg</td>
</tr>
<tr>
<td>Lead (Pb)</td>
<td>≤10 µg/kg</td>
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<tr>
<td>Arsenic (As₂O₃)</td>
<td>≤1 µg/kg</td>
</tr>
<tr>
<td>Loss on drying</td>
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<tr>
<td>Residue on ignition (sulfated)</td>
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<tr>
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<td>Endotoxin</td>
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