Effect of Oxygen Supply on L-Lysine, L-Threonine and L-Isoleucine Fermentations

Kunihiko AKASHI, Hiroshiro SHIBAI and Yoshio HIROSE

Central Research Laboratories of Ajinomoto Co., Ltd., Kawasaki, Japan

Received February 21, 1979

Effect of oxygen tension on L-lysine, L-threonine and L-isoleucine accumulation was investigated. Sufficient supply of oxygen to satisfy the cell's oxygen demand was essential for the maximum production in each fermentation. The dissolved oxygen level must be controlled at greater than 0.01 atm in every fermentation, and the optimum redox potentials of culture media were above -170 mV in L-lysine and L-threonine and above -180 mV in L-isoleucine fermentations. The maximum concentrations of the products were 45.5 mg/ml for L-lysine, 10.3 mg/ml for L-threonine and 15.1 mg/ml for L-isoleucine. The degree of the inhibition due to oxygen limitation was slight in the fermentative production of L-lysine, L-threonine and L-isoleucine, whose biosynthesis is initiated with L-aspartic acid, in contrast to the accumulation of L-proline, L-glutamine and L-arginine, which is biosynthesized by way of L-glutamic acid.

Recently most amino acids are commercially produced by fermentative process, and amino acid fermentation is now an important branch of the fermentation industry in Japan. Genetic induction of an auxotrophic or a regulatory mutant that can overproduce amino acid is of course extremely important, and at the same time, the optimization of culture conditions, especially agitation-aeration effectiveness, is indispensable for the scale-up and commercial production of amino acids in a large scale fermentor. In this paper, the effect of oxygen tension on the product formation was studied in L-lysine, L-threonine and L-isoleucine fermentations employing the mutants of Brevibacterium.

MATERIALS AND METHODS

Microorganisms. The microorganisms that accumulated a large amount of L-threonine or L-isoleucine were the mutants of Brevibacterium flavum 2247 (ATCC No. 14067). The microorganism which produced L-lysine was a mutant derived from Brevibacterium lactofermentum 2256 (ATCC No. 13869). Both of the parent strains are typical producers of glutamate.

Culture method. Fermentations were carried out in a 300-ml jar fermentor at 32°C for about 30~72 hr. The pH of the main culture was automatically maintained with gaseous ammonia at 7.0 in every fermentation. The media for main cultures in the fermentations are shown in Table 1.

Analysis. Measurements of the dissolved oxygen (P_L, atm), respiration rate of cells (r_{ab}, mol of O_2/ml·min), maximum oxygen demand of cells (K_r M, mol of O_2/ml·min) and redox potential of the culture medium (E, mV) were carried out as described previously.

Measurements of glucose, lactate, amino acids and bacterial growth were carried out as reported previously.

RESULTS

Effect of dissolved oxygen on product formation in L-lysine, L-threonine and L-isoleucine fermentations

Typical examples of fermentation kinetics are shown in Figs. 1-a, 1-b and 1-c. In every culture, oxygen was sufficiently supplied to maintain the P_L level between 0.01 and 0.10 atm throughout the fermentation, and the cell's oxygen demand was satisfied. The concentration of the products was 45.5 mg/ml for L-lysine, 10.3 mg/ml for L-threonine and 15.1 mg/ml for L-isoleucine. In order to study the relationship between P_L level and product formation in each fermentation, the agitation,
Table I. Production Medium of Lysine, Threonine and Isoleucine Fermentations

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Fermentation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lysine</td>
</tr>
<tr>
<td>Glucose (g/liter)</td>
<td>130</td>
</tr>
<tr>
<td>KH₂PO₄ (g/liter)</td>
<td>1</td>
</tr>
<tr>
<td>MgSO₄·7H₂O (g/liter)</td>
<td>0.4</td>
</tr>
<tr>
<td>FeSO₄·7H₂O (g/liter)</td>
<td>0.01</td>
</tr>
<tr>
<td>MnSO₄·4H₂O (g/liter)</td>
<td>0.01</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (g/liter)</td>
<td>25</td>
</tr>
<tr>
<td>L-Isoleucine (g/liter)</td>
<td>0.3</td>
</tr>
<tr>
<td>DL-Alanine (g/liter)</td>
<td>0.35</td>
</tr>
<tr>
<td>Biotin (µg/liter)</td>
<td>50</td>
</tr>
<tr>
<td>Thiamine (µg/liter)</td>
<td>200</td>
</tr>
<tr>
<td>Hydrochloride</td>
<td></td>
</tr>
<tr>
<td>Nicotinamide (mg/liter)</td>
<td>0.5</td>
</tr>
<tr>
<td>Soybean hydrolysate</td>
<td>15</td>
</tr>
</tbody>
</table>

*64 g/liter of nitrogen is contained.

Fig. 1-a. Time Course Curves of L-Lysine Fermentation in a Jar Fermentor.
Conditions of oxygen supply: Agitation speed, 1200 rpm; air flow rate, 1/2 vvm.

Fig. 1-b. Time Course Curves of L-Threonine Fermentation in a Jar Fermentor.
Conditions of oxygen supply: Agitation speed, 1200 rpm; air flow rate, 1/2 vvm.

Relationship between product formation and redox potential of culture medium

As another index to oxygen supply, the $E$ value was very useful in knowing the effect of extremely low oxygen tension on the fermentation result as previously reported. The $E$ value was proportional to the logarithm of $P_L$ where the rate of cell respiration was constant at the pH and temperature-controlled culture. The value of $E_{crit}$, which corresponded to $P_{L_{crit}}$, was determined on the basis of...
Effect of Oxygen Supply on L-Lysine, L-Threonine and L-Isoleucine Fermentations

FIG. 1-c. Time Course Curves of L-Isoleucine Fermentation in a Jar Fermentor. Conditions of oxygen supply: Agitation speed, 1200 rpm; air flow rate, 1/2vvm.

FIG. 2. Relationship between Dissolved Oxygen and Amino Acid Accumulation. ○—O, lysine; △—△, isoleucine; □—□, threonine.

the relationship between the two. \( E_{\text{crit}} \) was \(-170\) mV in L-lysine and L-threonine and \(-180\) mV in L-isoleucine fermentations. As shown in Figs. 3-a, 3-b and 3-c presenting the relationship between the \( E \) value and the product formation, the largest amount of the products accumulated above \( E_{\text{crit}} \) in each fermentation and the extent of the inhibition of the productivity increased as the \( E \) value decreased.

Relationship between degree of oxygen satisfaction (\( r_{\text{oxygen}} / K_M \)) and product formation

The degree of satisfaction of cells' oxygen demand had physiological meanings in contrast to the \( E \) value having physical meanings. As
FIG. 3-c. Relationship between L-Isoleucine Production and Redox Potential.

presented in Figs. 4-a, 4-b and 4-c, the value of \( \frac{r_{ab}}{KrM} \) was closely related to the fermentation results. In each fermentation, the value of \( \frac{r_{ab}}{KrM} \) optimum for the production was 1.0. An extremely oxygen-deficient condition at an \( \frac{r_{ab}}{KrM} \) value of less than 0.3 led to the excretion of lactic acid at the expense of the amino acids accumulated.

FIG. 4-a. Relationship between Microbial Products and Degree of Oxygen Satisfaction.

\( O--O \), lysine; \( \bullet--\bullet \), lactate.

\( KrM, 11.6 \times 10^{-7} \) mol of O\(_2\) ml\(^{-1}\) min\(^{-1}\).

FIG. 4-b. Relationship between Microbial Products and Degree of Oxygen Satisfaction.

\( O--O \), threonine; \( \bullet--\bullet \), lactate.

\( KrM, 22.0 \times 10^{-7} \) mol of O\(_2\) ml\(^{-1}\) min\(^{-1}\).

FIG. 4-c. Relationship between Microbial Products and Degree of Oxygen Satisfaction.

\( O--O \), isoleucine; \( \bullet--\bullet \), lactate.

\( KrM, 1.5 \times 10^{-7} \) mol of O\(_2\) ml\(^{-1}\) min\(^{-1}\).

DISCUSSION

We have been studying the effect of agitation and aeration on the product formation in amino acid fermentations. We already re-
ported on the effect of oxygen tension on L-valine,7) L-leucine,4) L-phenylalanine,8) L-proline5) and L-glutamine8) accumulation. We tried to classify amino acid fermentations from the viewpoint of oxygen effect and to consider the relationship between amino acid productivities and oxygen supply in relation to the characteristics of amino acid biosynthesis, particularly the amount of NAD(P)H2 generated in the synthesis of each amino acid. As far as studied, amino acid fermentations could be classified, from the viewpoint of oxygen effect, into two groups. One included valine, leucine and phenylalanine fermentations. Maximum production in these fermentations occurred when the cell respiration was inhibited by oxygen limitation, while the product formation under sufficient oxygen supply were inhibited. The other included L-proline, L-glutamine, L-lysine, L-threonine and L-isoleucine fermentations where maximum accumulation was obtained under sufficient oxygen supply. These fermentations, however, were subdivided into two groups according to the degree of the inhibition caused by oxygen shortage.

The accumulation of L-proline and L-glutamine, which are biosynthesized by way of glutamic acid, was strongly inhibited by oxygen deficiency. On the other hand, the production of L-lysine, L-threonine and L-isoleucine, which are biosynthesized via L-aspartic acid, decreased only slightly under insufficient oxygen supply. Comparison of the two groups in relation to the productivity under an oxygen-deficient condition is presented in Fig. 5. It is very interesting that the biosynthesis of amino acids belonging to the L-aspartic acid family was resistant to oxygen shortage in marked contrast to the sensitiveness of accumulation of amino acids belonging to the glutamic acid family. L-Glutamic acid producer of Brevibacterium is hardly able to convert α-ketoglutaric acid into succinic acid, and this property is considered to favor the overproduction of glutamic acid.9) Two kinds of routes are considered for the biosynthesis of glutamic acid. One involves a glyoxylate cycle in which glutamic acid is biosynthesized by way of

\[ \text{pyruvate, citrate and } \alpha\text{-ketoglutarate as shown in Fig. 6. NADH}_2 \text{ was used for the sum of } \text{NADH}_2 \text{ and } \text{FADH}_2 \text{ produced through assimilation of glucose. In this process, } 6 \text{ mol of } \text{NADH}_2 \text{ and } 2 \text{ mol of ATP are generated.} \]
by the assimilation of 1 mole of glucose (eq. 1).

Glucose $\rightarrow 2/3$ Glutamic acid $+ 2$ ATP  
$+ 6$ NADH$_2$ $+ 2/3$ CO$_2$  \( \text{(1)} \)

The other involves a phosphoenolpyruvate (PEP) carboxylation system in which glutamic acid is formed by way of PEP, oxaloacetic acid, isocitric acid and $\alpha$-ketoglutaric acid as shown in Fig. 7. In this process, 3 mol of NADH$_2$ and 1 mol of ATP are generated by the assimilation of 1 mol of glucose (eq. 2).

Glucose $\rightarrow$ Glutamic acid $+ 3$ NADH$_2$ $+ $ ATP  \( \text{(2)} \)

In glutamic acid fermentation, it was experimentally shown that the product was accumulated according to eq. (3).

Glucose $\rightarrow$ 0.82 Glutamic acid $+ 1.94$ CO$_2$  \( \text{(3)} \)

Judging from the amount of carbon dioxide evolved during the cultivation, the amino acid was produced from glucose employing both the glyoxylate cycle and PEP carboxylation system. Biosynthesis of glutamic acid generates NAD(P)H$_2$ whether it is performed through the glyoxylate cycle or PEP carboxylation system. This characteristic of glutamate biosynthesis was considered to cause the marked inhibition of the product formation in oxygen-limited culture, because oxygen was required in amino acid fermentation mainly to reoxidize NADH$_2$ generated in the process of amino acid. Biosynthesis of aspartic acid is also made through the glyoxylate cycle or PEP carboxylation system. Biosynthesis of amino acid through the glyoxylate cycle is an NAD(P)H$_2$ generating process in marked contrast to that through the PEP carboxylation system which is an NAD(P)H$_2$ consuming one. Table II is a summary of NAD(P)H$_2$ formation and ATP consumption in relation to the biosynthesis of amino acids belonging to the aspartate and glutamate families. In lysine, threonine and isoleucine fermentations, the degree of decrease in product formation was shown to be less significant than that in amino acid fermentations of the glutamic acid family. Biosynthesis of these amino acids of the aspartic acid family might be performed by way of PEP and oxaloacetate including carbon dioxide fixation in oxygen-limited culture.

### Table II. Calculation of NAD(P)H$_2$ and ATP Generation in Biosynthesis of Amino Acids by Way of Glyoxylate Cycle and PEP Carboxylation Pathway

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Glyoxylate cycle</th>
<th>CO$_2$ fixation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAD(P)H$_2$</td>
<td>ATP</td>
</tr>
<tr>
<td>Glu</td>
<td>$+ 6$</td>
<td>$+ 2$</td>
</tr>
<tr>
<td>Gin</td>
<td>$+ 6$</td>
<td>$+ 1/3$</td>
</tr>
<tr>
<td>Pro</td>
<td>$+ 5/3$</td>
<td>$+ 1/3$</td>
</tr>
<tr>
<td>Arg</td>
<td>$+ 4/3$</td>
<td>$- 2/3$</td>
</tr>
<tr>
<td>Lys</td>
<td>$+ 2/3$</td>
<td>$+ 2/3$</td>
</tr>
<tr>
<td>Ile</td>
<td>$+ 2$</td>
<td>$+ 2/3$</td>
</tr>
<tr>
<td>Thr</td>
<td>$+ 4$</td>
<td>$0$</td>
</tr>
</tbody>
</table>

REFERENCES