Studies on the the Isoleucine Fermentation

Part VI. Accumulation of L-Isoleucine by Bacteria

By Masaya HAYASHIBE* and Toshihiko WATANABE*

Laboratory of Microbiology, Faculty of Agriculture, Tohoku University, Sendai

Received August 21, 1961

Accumulation of L-isoleucine and L-valine was studied on 14 genera, 47 species and 110 strains of aerobic bacteria using bacterial type cultures. A large amount of L-isoleucine and a small amount of L-valine accumulated when 1% of DL- α -aminobutyric acid was added to the culture medium. As a rule, facultative aerobes such as *Aerobacter*, *Erwinia*, *Serratia* and *Bacillus* showed good accumulation. In the absence of α -aminobutyric acid, powerful L-isoleucine accumulators produced a large amount of L-valine, although the accumulation of L-isoleucine was scarcely observed under that condition. In the presence of α -aminobutyric acid, the accumulation of L-valine was generally suppressed, but in several strains, on the contrary, the accumulation increased as well as that of L-isoleucine. When DL-threonine was used instead of α -aminobutyric acid, the amount of L-isoleucine accumulated was not as high as that with α -aminobutyric acid in almost all strains except *Serratia marcescens*. It was concluded that a distinct relationship between bacterial genera or species and accumulation of L-isoleucine did not exist, that is, powerful accumulators were limited to special strains, and that the addition of α -aminobutyric acid was necessary for the accumulation of a large amount of L-isoleucine.

According to the recent studies dealing with amino acid accumulating bacteria, strong amino acid accumulators are considered to belong to special genera or species¹⁻¹²⁾,

and the amino acids accumulated in large quantities are glutamic acid, alanine and valine etc.¹⁻¹³⁾ A large number of strains belonging to *Enterobacteriaceae* accumulate L-valine^{1-3, 11-13)}, while L-isoleucine which is supposedly synthesized through an analogous biosynthetic pathway to that of valine¹⁴⁻¹⁷⁾ accumulate only in small quantities^{2, 13)}.

Hayashi et al. have found that a large amount of L-isoleucine was accumulated in the culture medium of *Bacillus subtilis* strain 14 when grown in an α -aminobutyric acid

* Present address: Research Department, Oriental Yeast Industry Co., Ltd, Itabashi, Tokyo.

1) H. Iizuka and K. Komagata, *J. Agr. Chem. Soc. Japan*, **34**, 27 (1960); *Amino Acids*, **1**, 111 (1959).

2) Y. Sasaki and T. Shiio, *Amino Acids*, **2**, 30 (1960).

3) T. Asai, K. Aida and K. Ohishi, *J. Ferm. Assoc. Japan*, **15**, 371 (1957).

4) S. Kinoshita, S. Uda and M. Shimono, *J. Gen. Appl. Microbiol.*, **3**, 193 (1957).

5) S. Kinoshita, K. Tanaka, S. Uda, S. Akita, T. Saito and T. Iwazaki, *J. Ferm. Assoc. Japan*, **16**, 1 (1958).

6) S. Kinoshita, K. Tanaka, S. Uda and S. Akita, *Intern. Symp. Enz. Chem.*, (1957, X. Tokyo and Kyoto).

7) Y.C. Su and K. Yamada, *This Journal*, **24**, 69 (1960); *Amino Acids*, **1**, 38 (1959).

8) C. Ogawa, M. Ōide and Y. Midorikawa, *Amino Acids*, **1**, 45 (1959).

9) S. Ota and M. Tanaka, *J. Ferm. Tech. (Japan)*, **37**, 261 (1959); *Amino Acids*, **1**, 50 (1959).

10) S. Okumura, et al., presented at the 192th meeting of Kanto subdivision of the Agricultural Chemical Society of Japan, 1959.

11) S. Uda and S. Kinoshita, *J. Gen. Appl. Microbiol.*, **5**, 159 (1959).

12) Z. Sugisaki, *ibid.*, **5**, 138 (1959); *Soc. J. Agr. Chem. Japan*, **34**, 153 (1960).

13) Y. Takamura, M. Itoh, Y. Fujii and T. Uemura, *Tohoku J. Agr. Research*, in press.

14) E.A. Adelberg, "A Symposium on Amino Acid Metabolism", The Johns Hopkins Press, Baltimore, 1955, p. 415.

15) H.E. Umbarger and B. Brown, *J. Bacteriol.*, **70**, 242 (1955).

16) H.E. Umbarger and B. Brown, *J. Biol. Chem.*, **233**, 1156 (1958).

17) H.E. Umbarger, B. Brown and E.J. Eyring, *ibid.*, **235**, 1425 (1960).

(designated as AB) containing medium¹⁸). Chibata et al. reported an additional observation that strains belonging to genus *Pseudomonas* accumulate isoleucine when AB is added to the medium¹⁹).

We have examined, using bacterial type cultures, whether isoleucine is accumulated by any bacteria whenever AB is added to the medium, or only by limited genera, species or strains of bacteria. Summarized results have been presented already²⁰). In this paper, thereupon, we have described detailed studies on the conditions for isoleucine accumulation.

METHODS

Bacillus subtilis strain 14 and 110 strains of bacterial type cultures which are kept in the Laboratory of Microbiology, Faculty of Agriculture, Tohoku University (called with a symbol of LM), the Institute of Applied Microbiology, Tokyo University (IAM), and the Institute for Fermentation, Osaka (IFO) were used. Besides bacteria, about 50 strains of yeast and 15 strains of mold kept in the Laboratory of Microbiology, Faculty of Agriculture, Tohoku University were also tested.

GB.AB medium with a composition as indicated in Table I was employed throughout this experiments. Peptone and meat extract were added to the medium to support abundant growth in the AB containing

medium. It had been pointed out that AB was an anti-metabolite of such amino acids as valine, threonine, leucine etc.^{21,22}) and that *B. subtilis* strain 14 could not grow without peptone and meat extract in the presence of a high concentration of AB²⁰). Other media described in Table I were also used for various purposes. Six ml of GB.AB medium were placed in a tube with a side arm (17 mm in inside diameter and 110 mm in length), inoculated with a strain of organism from the stock culture, and incubated at 30°C on a reciprocal shaker which was set for 160 oscillations per min with an amplitude of 12 cm. Kd of this system was 6.8×10^{-6} g mol O₂ per atm., ml, min. After 5 days' culture, an aliquot of culture medium was subjected to paper chromatography in a butanol-acetic acid-water (4:1:1) system.

When a large amount of isoleucine or valine was present on examination of chromatogram, it was estimated after elution of the ninhydrin-colored spot according to the method described by Giri²³). The strains which accumulated a large amount of isoleucine or valine were further studied. Bacterial growth was followed estimating the optical density at 660 m μ . At 48 and 96 hrs after the growth reached its stationary phase, isoleucine and valine accumulated were estimated by paper chromatographic method of Giri as well as pulp disc method of microbiological assay using *L. arabinosus*²⁴).

RESULTS AND DISCUSSION

I. Accumulation of Isoleucine and Valine in the Medium Containing α -Aminobutyric Acid.

Accumulation of Isoleucine and Valine by Yeast and Mold.

Accumulation of isoleucine and valine was examined on about 50 strains of *Saccharomyces*, *Candida*, *Rhodotorula*, *Hansenula*, *Pichia* and 15 strains of *Aspergillus*, *Rhizopus*, *Penicillium*. Most strains of yeast and mold tested could grow in the GB.AB medium, but could scarcely accumulate isoleucine and valine. About 10 yeast strains accumulated 200~500 μ g/ml of isoleucine and a small amount of valine.

TABLE I. COMPOSITION OF THE MEDIA

	GB medium*	V ₁ medium**
Glucose	10.0%	5.0%
Urea	0.5	
NH ₄ Cl		2.0
Peptone	0.2	0.2
Meat extract	0.2	
K ₂ HPO ₄	0.07	0.1
MgSO ₄ ·7H ₂ O	0.03	0.05
FeCl ₃ ·6H ₂ O	0.005	
CaCO ₃		3.0

* GB medium with 1% of DL-AB or DL-threonine are called GB.AB and GB. Thr medium, respectively.

** V₂ medium is the one omitted of peptone from the V₁ medium.

18) K. Hayashi, Y. Watanabe, Y. Fujii and K. Shimura, *Amino Acids*, **1**, 89 (1959).

19) I. Chibata, M. Kisumi and Y. Ashikaga, *ibid.*, **2**, 90 (1960).

20) M. Hayashibe, M. Itoh, T. Watanabe and T. Uemura, *ibid.*, **2**, 100 (1960).

21) K. Dittmer, *Ann. N.Y. Acad. Sci.*, **52**, 1274 (1950).

22) S. Friedman, *J. Bacteriol.*, **76**, 278 (1956).

23) K.V. Giri, *Nature*, **170**, 1025 (1952).

24) H. Kojima et al., *J. Agr. Chem. Soc. Japan*, **32**, 33, 38, 100, 106, 189 (1959).

Accumulation of Isoleucine and Valine by Bacteria.

All bacterial strains were examined for isoleucine and valine accumulation in GBAB medium in a group of 16~24 strains using *B. subtilis* strain 14 as a control culture. Because the culture conditions under which every experiment was performed were not completely controlled, isoleucine accumulation by the control culture deviated according to the maximum growth of the culture obtained, as shown in Table II. Owing to these deviations in the control culture, it was difficult to compare the isoleucine accumulations by strains of different groups. Accordingly the re-ex-

TABLE II. ACCUMULATION OF ISOLEUCINE IN *B. subtilis* STRAIN 14-2 (AMOUNT OF ISOLEUCINE ACCUMULATION AND MAXIMUM GROWTH IN EACH SERIES OF EXPERIMENT)

Exp. No.	Max. Growth (O. D.)	pH	Ileu Accumulated ($\mu\text{g/ml}$)	Val Accumulated ($\mu\text{g/ml}$)
1	0.76	5.4	3300	310
2	0.74	5.4	2310	300
3	0.71	5.6	2440	280
4	0.64	5.4	2800	200
5	0.64	5.2	2940	180
6	0.63	5.4	3000	480
7	0.63	5.4	2680	200
8	0.59	5.6	1940	160
9	0.55	5.4	1700	320
10	0.53	5.4	1340	280
11	0.47	5.6	1420	260

TABLE III. ACCUMULATION OF ISOLEUCINE AND VALINE BY BACTERIA

Genus, Species, Strains	Max. Growth (O. D.)	pH	Ileu Accumulated ($\mu\text{g/ml}$)	Val Accumulated ($\mu\text{g/ml}$)
<i>Pseudomonas</i>				
<i>Ps. aureofaciens</i> IAM 1001	0.95	4.4	5460	400
<i>Ps. ovalis</i> IAM 1002	0.20	3.4	230	140
<i>Ps. cruciviae</i> IAM 1048	0	—	—	—
<i>Ps. shuylkilliensis</i> IAM 1154	0.73	4.8	1530	±
<i>Ps. trifolii</i> IAM 1309	0.33	3.8	240	240
<i>Ps. stutzeri</i> (Petroleum) IAM 1476	0.15	4.4	180	160
<i>Ps. fragi</i> IFO 3458	0.91	2.4	600	160
<i>Ps. reptilivora</i> IFO 3461	0.39	2.6	160	±
<i>Ps. riboflavina</i> IFO 3140	0.15	6.8	140	160
<i>Ps. fluorescens</i> IFO 3081	0	—	—	—
<i>Ps. solanacearum</i> IAM plant pathogenic	0	—	—	—
<i>Xanthomonas</i>				
<i>X. citri</i> IFO 3781	±	5.4	±	±
<i>X. pruni</i> IAM plant pathogenic	0.31	5.2	300	±
<i>X. oryzae</i> IAM plant pathogenic	±	5.6	±	±
<i>X. malvacearum</i> IFO 3383	0	—	—	—
<i>Acetobacter</i>				
<i>A. melanogenum</i> IFO 3190	±	1.8	120	—
<i>A. rancens</i> IFO 3191	0	—	—	—
<i>A. suboxydans</i> IFO 3130	0	—	—	—
<i>A. gluconicum</i> IFO 3171	0	—	—	—
<i>Achromobacter</i>				
<i>A. liquidum</i> IFO 3084	0.12	6.4	100	100
<i>Flavobacterium</i>				
<i>F. flavescens</i> IFO 3085	±	6.8	120	120
<i>Micrococcus</i>				
<i>M. luteus</i> IAM 1097	0.24	6.4	180	120
<i>M. flava</i> IAM 1409 (Petroleum)	0.75	6.8	730	±
<i>M. flavus</i> IFO 3242	0.70	5.4	200	±
<i>M. candidus</i> LM 23	0.64	4.8	430	130
<i>M. aureus</i> LM 24	0.75	5.6	1220	410

(Continued)

Genus, Species, Strains	Max. Growth (O.D.)	pH	Ileu Accumulated ($\mu\text{g/ml}$)	Val Accumulated ($\mu\text{g/ml}$)
<i>Sarcina</i>				
<i>S. lutea</i> LM 25	0.47	5.4	470	570
IAM 1099	0.53	5.0	220	$\pm$
IFO 3232	0.72	7.2	800	$\pm$
<i>S. aurantiaca</i> IAM 1059	0.17	4.4	100	100
IFO 3064	0.18	4.6	$\pm$	$\pm$
<i>Staphylococcus</i>				
<i>S. aureus</i> LM 22	0.46	5.4	$\pm$	$\pm$
<i>Escherichia</i>				
<i>E. coli</i> K 12	0.29	4.2	160	—
LM 40	0.58	4.0	410	480
LM 42	0.22	4.4	220	—
LM C 7	$\pm$	5.0	$\pm$	—
LM C 10	0.16	4.6	—	—
LM C 16	0.13	4.6	$\pm$	—
<i>Aerobacter</i>				
<i>A. aerogenes</i> IAM 1019	0.77	6.8	800	1000
IAM 1063	0.25	4.0	$\pm$	$\pm$
IAM 1102	0.92	5.8	1540	660
IAM 1160	0.47	3.6	$\pm$	$\pm$
IFO 3319	0.45	1.8	260	$\pm$
LM 47	0.48	4.0	220	130
<i>Erwinia</i>				
<i>E. aroideae</i> IAM 1068	0.15	4.8	$\pm$	$\pm$
<i>E. carotovora</i> IAM 1024	0.13	4.6	$\pm$	$\pm$
LM 23	$\pm$	5.6	$\pm$	—
LM E 24	0.68	6.4	1380	480
LM E 25	0.23	5.6	370	—
LM E 30	0.70	6.8	1520	540
<i>Serratia</i>				
<i>S. marcescens</i> LM 27	0.90	7.0	2060	360
<i>Bacteroides</i>				
<i>B. succinicum</i> LM 38	0.65	7.0	$\pm$	$\pm$
<i>Bacillus</i>				
<i>B. subtilis</i> strain 14-2	0.64	5.2	2940	180
strain 14-5	0.71	5.2	3460	240
<i>B. subtilis</i> LM 8-2	0.30	5.6	990	180
LM 8 B 3	0.54	5.6	1620	250
LM 46	0.67	5.6	540	$\pm$
LM 49	0.69	5.4	80	$\pm$
LM 62	0.68	6.6	$\pm$	$\pm$
<i>B. subtilis</i> strain Tracy	0.63	5.8	940	200
<i>B. circulans</i> strain 21	0.80	7.6	1500	200
<i>B. licheniformis</i> LM 57	0.70	5.4	240	480
<i>B. pumilus</i> LM 58	0.89	4.6	$\pm$	130
<i>B. megatherium</i>	0.76	4.0	430	—
<i>B. cereus</i> LM 56	0.52	4.8	160	—
<i>B. firmus</i> LM 64	$\pm$	5.4	$\pm$	—
<i>B. brevis</i> LM 69	0.11	5.4	$\pm$	—
<i>B. mesenteroides</i> LM 14	0.22	4.8	$\pm$	—
<i>B. anthracis</i> LM 44	0.23	5.0	$\pm$	—

 $\pm$: Amino acid accumulation under 100 $\mu\text{g/ml}$.

amination of several strains which indicated good accumulation compared with the control culture was performed (Fig. 1). The results are presented in Table III.

Several strains could scarcely grow in GB.AB medium and these strains could not accumulate amino acids. It seemed likely that the growth of these strains was inhibited by the presence of AB, but pH of the culture medium was lowered in some of these strains, especially in *E. coli*. Since the biosynthesis of isoleucine and valine had been studied in detail in *E. coli*, isoleucine and valine accumulation in *E. coli* was re-examined with the addition of CaCO_3 to prevent the lowering of pH. As shown in Table IV, the lowering of pH was prevented to some extent, but the accumulation of isoleucine and valine was not seriously improved.

TABLE IV. ACCUMULATION OF ISOLEUCINE AND VALINE BY *Coli-aerogenes* BACTERIA

Strain	pH	Ileu Accumulated ($\mu\text{g/ml}$)	Val Accumulated ($\mu\text{g/ml}$)
<i>Escherichia coli</i>			
K 12	4.8	130	100
LM C 1	5.2	—	—
IAM 64	5.0	380	180
Ki 563	5.8	250	200
Crooks	4.6	540	$\pm$
Najjar	4.6	1320	$\pm$
LM 40	5.0	360	$\pm$
LM 41	5.0	460	320
LM 52	4.0	920	$\pm$
<i>Aerobacter aerogenes</i>			
LM 47	4.0	430	$\pm$
IAM 1102	8.8	1500	830
IAM 1063	4.0	780	180
IAM 1019	8.4	480	1180
IAM 1160	4.0	830	180

Medium used: GB.AB medium with 3% of CaCO_3 .

In the strains belonging to genus *Pseudomonas*, the amount of isoleucine and valine accumulated was generally small except a few strains which accumulate a large amount of

isoleucine. Strains belonging to genus *Xanthomonas*, *Acetobacter*, *Achromobacter*, *Flavovacterium* could not grow under the given conditions. Cocci such as *Micrococcus* and *Sarcina* generally indicated abundant growth and amino acid accumulation. As to *Bacillaceae*, only several strains such as *B. subtilis*, *B. circulans* and *B. megatherium*, showed a high accumulation of amino acids. Fermentative bacteria such as *Escherichia*, *Aerobacter*, *Erwinia*, *Serratia* generally accumulated a large amount of isoleucine and valine.

It is generally conceived that an amino acid accumulator appears frequently in *Brevibacterium*⁷⁻¹⁰), *Micrococcus*³⁻⁶), *Bacillus*³), *Aerobacter*^{11,12}). The present results agreed with these results as is shown above. Iizuka et al. pointed out from the taxonomical view-point that an amino acid accumulator appeared frequently in facultative anaerobes such as *Escherichia*, *Bacillus*, *Micrococcus*, and scarcely appeared in a strictly aerobe such as *Pseudomonas*¹). The present results also agreed with their results, indicating that active carbohydrate metabolism was necessary for the accumulation of amino acid under the aerobic conditions in the presence of high concentration of glucose.

From the above results, it is concluded that isoleucine accumulating strains under given conditions were distributed broadly in many genera, species and strains, but it is also concluded that isoleucine accumulation was not a specific property of definite genus or species.

II. Metabolism of Related Amino Acids by Isoleucine Accumulator.

Several strains which accumulated a large amount of isoleucine and/or valine in AB containing medium were selected on re-examination and the isoleucine and/or valine accumulation in a variety of the media was examined as indicated in Table I.

It was pointed out that the biosynthesis of

isoleucine and valine proceeded in a similar mechanism from the experimental results using mutant strains of *E. coli* and *Neurospora*^{14,15}, and that in some strains its terminal several steps proceeded by the same enzymes^{16,17}. On the other hand, it was also pointed out that valine accumulators appeared more frequently than isoleucine accumulators. It was thought of interest to examine whether isoleucine accumulator does accumulate valine in the absence of AB. Valine accumulation was shown to decrease when organic nitrogen source or urea was used¹², so a possibility was considered that strains which did not show valine accumulation in GB.AB or GB medium could accumulate valine in

accumulation occurred with all the strains examined. Takamura et al. observed that a number of bacteria isolated from soil, animal diet, wine mash (Saké Moromi) and sheep rumen accumulated valine but not isoleucine and that these valine accumulators accumulated isoleucine when AB was added to the medium¹³. It is inferred from these results that a close relationship between isoleucine and valine accumulation might exist, that is, valine is accumulated by such strains that have some mechanisms in favor of valine accumulation²⁵, whereas isoleucine can not be accumulated by these strains until AB is added to the medium in order to supply the intermediate for the isoleucine biosynthesis.

FIG. 1. Accumulation of Isoleucide and Valine by Selected Strains in Various Media.

Isoleucine (▨, □) and valine (■, □) accumulation at 48 hrs. and 96 hrs. after the growth reached to its maximum.

* Val accumulation in V₂ medium.

urea and/or organic nitrogen free media. As shown in Fig. 1, these selected strains which showed isoleucine and/or valine accumulation in AB containing medium, did not generally accumulate isoleucine in AB free medium except accumulation of a small amount of isoleucine in *B. subtilis* strain 14, *Serratia marcescens*, *Aerobacter aerogenes*, but valine ac-

These results might be due to the complicated pathway for the biosynthesis of isoleucine as compared to valine biosynthesis. As to the precursors for the isoleucine accumulation, Hayashi et al. observed in *B. subtilis* strain 14 that homoserine or threonine could not lead to accumulation of as large an amount of

25) Unpublished.

isoleucine as AB¹⁸⁾, in spite of the nature of threonine and homoserine as the normal intermediates in the biosynthetic pathway²⁶⁻³⁰⁾. As shown in Fig. 1, similar results were obtained in almost all isoleucine accumulators tested, that is, AB was always superior as the precursor for the isoleucine accumulation. This superiority of AB might be considered to supply α -ketobutyric acid independently of the feedback inhibition which is controlling the biosynthesis of isoleucine, because α -ketobutyric acid formation from threonine is strongly inhibited by isoleucine³⁰⁻³²⁾. On the other hand, however, the superior quality of AB might be due to another reason considering an experiment in which threonine as well as AB was rapidly consumed by *B. subtilis* to the same degree either in threonine or AB containing medium³²⁾. *Serratia marcescens* was the only strain which accumulated nearly the same amount of isoleucine with threonine addition as well as with AB addition.

As shown in Fig. 1, most strains accumulated as much valine in GB medium than in GB.AB medium. AB is considered as anti-metabolite of valine etc.^{21,22)} and this was confirmed with *B. subtilis*²⁰⁾. Accordingly this confirmed with *B. subtilis*²⁰⁾. Accordingly this may account for the superior quality of AB as the precursor of isoleucine accumulation, because pyruvate is directed to

isoleucine biosynthesis by the presence of AB which keeps pyruvate from being utilized for valine biosynthesis. In some strains belong to *Erwinia*, *Aerobacter* and *Pseudomonas*, on the other hand, valine accumulation was higher in GB.AB medium than in GB medium. This was presumably because the enzyme activities for isoleucine biosynthesis were enhanced under the influence of AB²⁰⁾. The accumulation of isoleucine and valine, therefore, might be increased by the action of this enhanced enzyme system, if the supply of pyruvic acid is abundant. But if the supply of pyruvic acid is limited; the valine accumulation might decreased as a result of deficiency of pyruvic acid which was pulled toward isoleucine biosynthesis whose intermediates are present abundantly.

It was concluded that emphasis has to be placed on the presence of AB as well as the specificity of the strains.

Acknowledgements. The authors express their sincere thanks to Prof. Uemura for his constant guidance throughout this work, and also to Prof. Aso and Prof. Shimura for their kind advices and encouragements. Generous supply of the bacterial type cultures was indebted to Prof. H. Iizuka, the Institute of Applied Microbiology, Tokyo University, and Dr. T. Hasegawa, the Institute for Fermentation, Osaka. The authors' thanks are also due to the continuous encouragement and the permission for the publication of this report given by Mr. T. Shoda, the Director of Oriental Yeast Industry Co., and Mr. K. Okazawa, the Chief of Research Department.

26) M. Strassman, A.J. Thomas, L.A. Locke and S. Weinhouse, *J. Am. Chem. Soc.*, **76**, 4241 (1954).

27) E.A. Adelberg, *ibid.*, **76**, 4241 (1954).

28) P.H. Abelson, *J. Biol. Chem.*, **206**, 335 (1954).

29) H.E. Umbarger, *Federation Proc.*, **15**, 374 (1956).

30) H.E. Umbarger and B. Brown, *J. Biol. Chem.*, **233**, 415 (1958).

31) M. Hayashibe and T. Uemura, *Nature*, **191**, 1417 (1961).

32) M. Hayashibe and T. Uemura, *Amino Acids*, **3**, 108 (1961).