STUDIES ON THE ISOLEUCINE FERMENTATION IX. ACCUMULATION OF ISOLEUCINE IN THE MEDIUM CONTAINING THREONINE

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IX. ACCUMULATION OF ISOLEUCINE IN THE MEDIUM CONTAINING THREONINE

By

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As reported in the preceeding paper (1, 2), the accumulation of a large amount of isoleucine was observed in the medium containing α-aminobutyric acid (designated as AB), though AB is not considered as a normal intermediate in the biosynthetic pathway of isoleucine. However, wide distribution of the enzyme which transaminate from AB to various α-keto acid was observed in bacteria (3—5). Accordingly, AB might be a precursor for the isoleucine biosynthesis being converted to α-ketobutyric acid which is a normal intermedeate, if the AB$\leftrightarrow$ α-keto acid transaminase and appropriate α-keto acid are present. And further, It was pointed out, that mutant strains of *E. coli* requiring isoleucine indicated abundant growth on addition of AB or α-ketobutyric acid instead of isoleucine (6—8). As to the isoleucine accumluation, it was not so high in threonine or homoserine containing medium as compared to AB containing medium (1, 2), though the normal pathway for isoleucine biosynthesis is considered to proceed via homoserine and threonine (9—14). When we examined the accumulation of isoleucine by bacterial type culture, it was observed that almost all strains tested could not accumulate isoleucine in the medium containing threonine, but *Serratia marcescens* was the only strain which accumulate isoleucine in this medium (2), while Horváth reported the accumulation of isoleucine by *Streptomyces* in the medium containing threonine (15).

In this paper, we describe the accumulaton of isoleucine by *Serratia marcescens* strain LM27.

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Methods

Serratia marcescens strain LM 27 and other strains which are kept in the Laboratory of Microbiology, Faculty of Agriculture, Tohoku University were used throughout this experiments.

The composition of the basal medium is as follows: glucose 10%, urea 0.5%, K_2HPO_4 0.07%, $MgSO_4 \cdot 7H_2O$ 0.03%, $FeCl_3 \cdot 6H_2O$ 0.005%, $CaCO_3$ 2%, pH 7.8. When 1% of *DL*-AB or *DL*-threonine was added, 0.2% of peptone and meat extract were also added to the basal medium.

Usually, 4 *ml* of this medium was placed in a tube with a side arm (17 mm in inside diameter and 110 mm in length), inoculated 0.06 *ml* of seed culture and shaked on a receprocal shaker at 30°C. Seed culture was prepared by shaking for 24 hrs in the same tube containing 6 *ml* of the medium which was composed of 0.5 per cent of peptone, 0.5 per cent of meat extract and 0.25 per cent of NaCl.

After appropriate culture time, an aliquot of the cultured medium was subjected to paper chromatography in a butanol, acetic acid, water (4 : 1 : 1) system. The amount of isoleucine and valine on the paper chromatogram was estimated by eluting the ninhydrin-colored spot according to the method described by Giri (16).

Results and Discussion

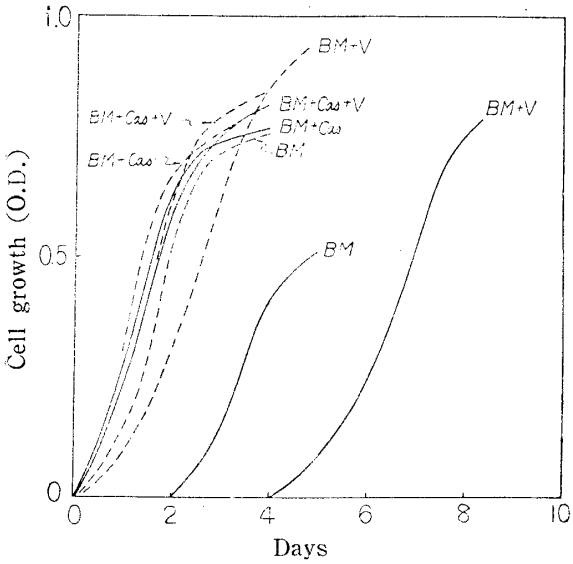

Fig. 1. Cell growth of *Serratia marcescens* strain LM27 in AB containing medium.
Medium: Basal medium(BM), basal medium plus 0.2% of casamino acid(BM+Cas), basal medium plus 9 kinds of B- vitamians (BM+V), basal medium plus casamino acid and B-vitamins (BM+Cas+V), with ——, and without -----AB.

The Course of Cell Growth and Isoleucine Accumulation by *Serratia marcescens* Strain LM27

The course of culture of this strain was traced under shaking, where the composition of eight kinds of media was as follows: basal medium, basal medium plus 0.2 per cent casamino acid, basal medium plus nine kinds of B-vitamins, basal medium plus casamino acid and B-vitamins, with and without addition of 1 per cent of AB. As shown in Fig. 1, the nutritional requirement of this strain was rather simple, though some sorts of B-vitamins improved its maximum growth.

In AB containing basal medium,

this strain requires a lag period in order to initiate its growth as observed in *B. subtilis* strain 14, though adaptation or mutation period to AB is shorter than *B. subtilis* (17). This lag period for adaptation or mutation disappeared on the addition of casamino acid, hence AB might act as an antimetabolite to some kinds of amino acids also in *Serratia marcescens*.

The course of isoleucine accumulation in threonine containing medium by this strain is indicated in Fig. 2, where the culture of *B. subtilis* and *Ps. aureofaciens* which indicated a large amount of isoleucine accumulation in AB containing medium are also presented for the comparison.

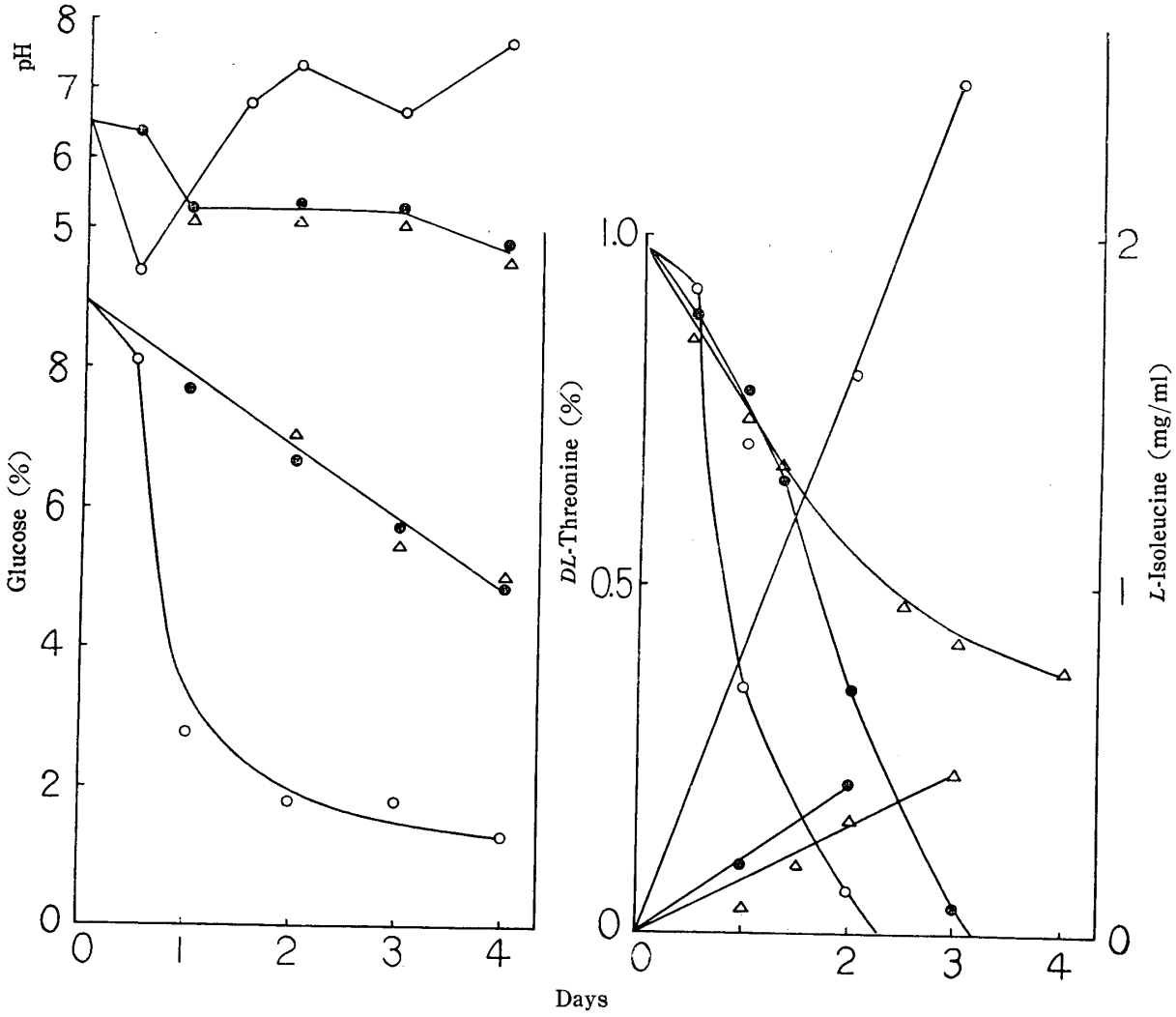

Fig. 2 Course of isoleucine accumulation in threonine containing medium.

Medium: Basal medium supplemented with peptone and meat extract (each 0.2%) and *DL*-threonine (1%).

Strain: *Serratia marcescens* strain LM27 —○—○—, *B. subtilis* strain 14 —△—△—, and *Ps. aureofaciens* IAM 1001 —●—●—.

Glucose was estimated by Somogyi's method with slight modification and threonine by paper chromatographic method as described in the text.

In the case of *Serratia marcescens*, glucose and threonine were rapidly consumed within 72 hrs and the amount of isoleucine increased reversely. A considerable amount of isoleucine was accumulated by this strain, while not by *Bacillus* and *Pseudomonas*.

Althogh pH of the medium was always high in the culture of *Serratia marcescens* compared with *Bacillus* and *Pesudomonas*, the amount of isoleucine accumulation was not seriously improved even when the pH of the medium was maintained above 7.5 in the culture of *Bacilus* and *Pseudomonas*. The consumption of threonine was almost complete in *Serratia* and *Pseudomonas*, whereas it stopped at the level of about 50 per cent consumption in *Bacilus*. It was observed that the enzyme preparation of *B. Subtilis* strain 14 could dehydrate only *L*-threonine, but not *D*-threonine (18). From the above results, it might be expected to utilize not only *L*- but *D*-threonine in *Serratia* and *Pseudomonas*, and indeed it was observed that D-threonine as well as *L*-threonine served as the precursor for isoleucine accumulation by *Serratia marcescens*. These results are presented in Table 1.

Table 1. Accumlation of isoleucine in *L*- or *D*-threonine containing medium.

Medium	Culture time	pH	Ileu accumulated (μg/m*l*)
Basal medium + *L*-threonine (1%)	48	7.8	2130
Basal medium + *D*-threonine (1%)	72	8.0	2340
Basal medium + *DL*-threonine (1%)	72	7.6	2160

Although it is not yet clear why threonine can not serve as the precursor for the accumulation of a large amount of isoleucine in almost all strains tested except *Serratia marcescens*, it is interesting that these results might suggest the role of the precursor in isoleucine fermentation.

Effect of Organic Nitrogen Source on the Accumulation of Isoleucine and Valine

Sugisaki reported that valine accumulation by a strain belonging to *Aerobacter aerogenes* lowered when the organic nitrogen source was present in the medium (19). Tanaka *et al.* pointed out that concentration of biotin had a serious effect on the accumulation of glutamic acid by *Micrococcus glutamicus* (20). Considering these facts, we examined the effect of these materials on the accumulation of isoleucine and valine.

Isoleucine and valine accumulation in the basal medium with and without *DL*-AB or *DL*-threonine was examined. As organic nitrogen source, a mixture of equal parts of peptone and meat extract was used, and the concentration of these materials was each 0, 0.1, 0.2, 0.4 per cent. As shown in Fig. 3, valine accumulation in the basal medium was seriously affected by the presence of

these materials, that is, when 0.2 per cent of these materials were present, valine accumulation could scarcely occurred. Isoleucine accumulation, on the contrary, increased in the presence of these materials in threonine containing medium. Similar results were obtained about isoleucine accumulation in AB containing medium, though the effect of organic nitrogen source on isoleucine accumulation could not sufficiently be ascertained, because cell growth was not immediately initiated. Isoleucine was accumulated also in this case.

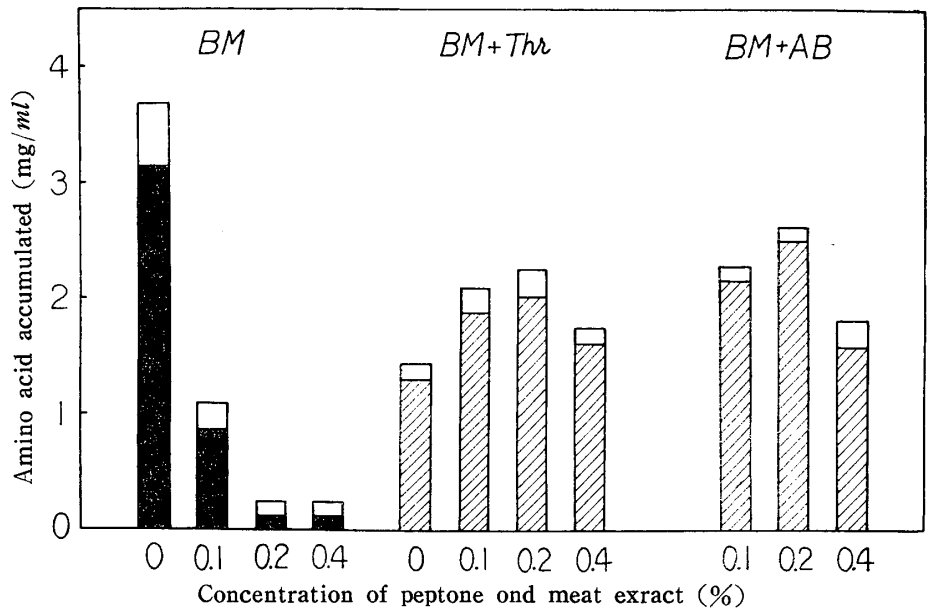

Fig. 3. Effect of organic nitragen on the accumulation of isoleucine and valine.
Medium: Basal medium (BM), BM plus 1% *DL*-threonine (BM+Thr) and BM plus 1% *DL*-AB (BM+AB).
Peptone and meat extract was used as organic nitrogen source, concentration of these material was 0, 0.1, 0.2, 0.4 % (each).
■ ▨ Amino acid accumulation at 27 hrs, and □ at 96 hrs, ■ valine, ▨ Isoleucine.

It was also ascertained that a small amount of orgnic nitrogen derived from the seed culture did not seriously affect on the accumulation of valine in basal medium, where the final concentration of these materials became 0.005 per cent.

Inorganic Nitrogen Source and Accumulation of Isoleucine and Valine

Ammonium salts of inorganic and organic acid were added to the basal medium instead of urea and the accumulation of isoleucine and valine was observed in these media, where 0.2 per cent of peptone and meat extract was added in the case of threonine or AB containing medium. The amount of inorganic nitrogen was 0.23 per cent N. Ammonium chloride, sulfate, phosphate, carbonate, formate, tartarate, oxalate and citrate were used as inorganic nitrogen source.

The amount of amino acid accumulation was markedly low, when ammonium carbonate, formate and oxalate were used. In the medium containing inorganic

ammonium salts or urea, advantages could not be found in any case, because variation in the amount of accumulation was observed between and within every experimental series, that is, ammonium sulfate was the best one in one experiment as shown in Fig. 4, while urea was the best in other cases. When organic ammonium salt such as ammonium tartarate or citrate was used, the amount of amino acid accumulation was always constant in a high level in each experimental series. Valine accumulation in the basal medium was highly increased in the medium containing tartarate or citrate as inorganic nitrogen. An example of these results is presented in Fig. 4.

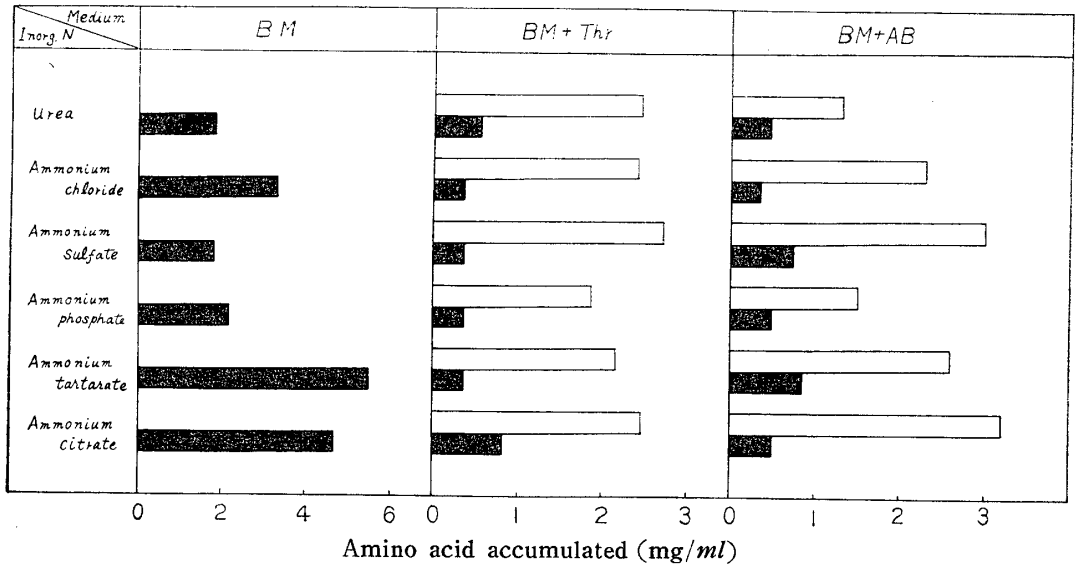

Fig. 4. Inorganic nitrogen source and accumulation of amino acid.
Medium: BM, BM+Thr or AB+peptone and meat extract (each 0.2%). As inorgonic nitrogen source various ammonium salts were added at the concentration of 0.23% N.
□ Isoleucine accumulation and ■ valine accumulation at 72 hrs' cultre.

One of the authors observed previously in the isoleucine fermentation by *B. subtilis* strain 14 that the accumulation of isoleucine increased and fixed in a constant level on the addition of sodium glutamate (21), and a similar effect of tartarate and citrate was observed in the isoleucine and valine accumulation by *Serratia marcescens*.

Effect of Aeration on the Accumulation of Isoleucine aud Valine

It was pointed out that the level of aeration is an important factor in the amino acid fermentation and controls not only the amount of the accumulation of metabolite, but the nature of metabolite accumulated (19, 20, 22). The amount of isoleucine accumulated by *B. subtilis* was also markedly affected by the level of aeration under which the fermentation proceeded (21), and this conforms to the isoleucine and valine accumulation by *Serratia marcescens* as shown in

Fig. 5. The level of aeration was set by changing the volume of the medium in the culture tube to 2, 4 and 6 *ml.*

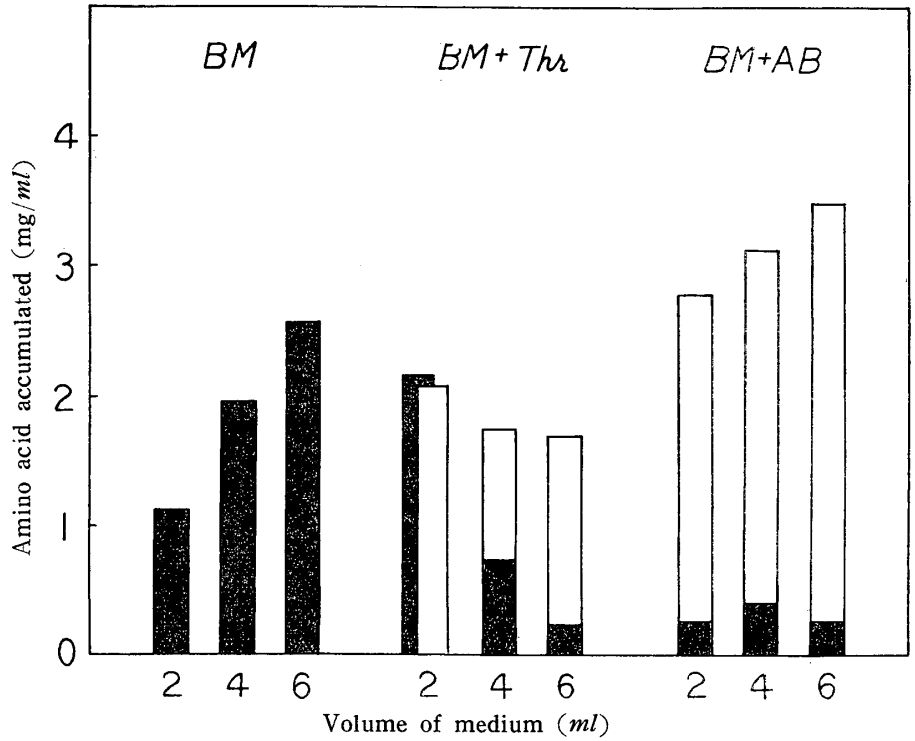

Fig. 5. Effect of aeration on the accumulation of amino acid.
Medium: BM, BM+Thr or AB+peptone and meat extract (each 0.2%). The level of aeration was set by changing the volume of medium.
□, ■ Same as in Fig. 4.

The level of aeration used had no notable effect on the amount of isoleucine accumulated in AB or threonine containing medium, whereas valine accumulation was markedly affected. Valine accumulation in the basal medium tended to decrease as the oxygen level increased, on the contrary, the reverse relation was obtained in threonine containing medium.

As indicated in the above experiments, the amount of valine accumulation was markedly affected by the presence of organic nitrogen, the kind of ammonium salts and the level of aeration, whereas the amount of isoleucine accumulation was not so. It might be generally conceived that valine accumulation was more seriously affected by the environmental factor than isoleucine accumulation. The authors speculated the unstable nature inside the amido acid accumulation, and this unstable nature seemed to be exposed in valine accumulation by *Serratia marcescens.* Although isoleucine was not accumulated under ordinary conditions by almost all strains tested, it was accumulated with constancy on the addition of AB or threonine by these strains which can accumulate valine(2). Substances such as AB or threonine might serve as the stabilizer to fix the unstable nature of metabolite accumulation, together with

the precursor for the isoleucine biosynthesis. Further observation on the stabilizing effect of some compounds such as glutamate, citrate and tartarate might suggest a complicate nature of amino acid accumulation.

Summary

Accumulation of isoleucine and valine by *Serratia marcescens* strain LM27 was examined using the medium with and without the addition of AB or threonine, where this strain was the only one, which accumulated a large amount of isoleucine in the threonine containing medium.

1. *Serratia marcescens* strain LM27 was able to grow in the medium containing glucose, urea, mineral salts (basal medium), but some sorts of B-vitamins improved its maximum growth. If AB was added to the basal medium, some days' induction period was required to initiate cell growth, while this induction period disappeared on the addition of casamino acid. AB might also act as an antimetabolite in this strain.

2. When *Serratia marcescens* strain LM27 was inoculated in threonine containing medium, glucose and threonine was rapidly consumed and isoleucine accumulated reversely as the culture proceeded, where the consumption of threonine was almost complete. Isoleucine was accumulated not only in *L*-threonine contatning medium but also in *D*-threonine containing medium.

3. When a small amount of organic nitrogen was present in the medium, valine accumulation decreased markedly, while these materials were required for isoleucine accumulation in AB or threonine containing medium. The amount of isoleucine and valine accumulation was fixed in a high level when ammonium tartarate or citrate was used as the inorganic nitrogen source. The level of aeration had a marked effect on the accumulation of valine, while isoleucine was not so affected.

4. It was speculated that the unstableness is present inside valine accumulation, whereas isoleucine accumulation indicated considerable constancy owing to the presence of AB or threonine.

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