Agr. Biol. Chem., **39** (6), 1319~1322, 1975

Production of L-Valine by 2-Thiazolealanine Resistant Mutants Derived from Glutamic Acid Producing Bacteria†

Takayasu TSUCHIDA, Fumihiro YOSHINAGA, Koji KUBOTA and Haruo MOMOSE

Central Research Laboratories, Ajinomoto Co., Inc., Suzuki-cho, Kawasaki, Japan

Received March 3, 1975

Potent L-valine producers were screened among 2-thiazolealanine resistant mutants derived from three typical L-glutamic acid producing bacteria: *Brevibacterium lactofermentum*, *Corynebacterium acetoacidophilum*, *Arthrobacter citreus*. By strain No. 487, the best producer derived from *Brevibacterium*, 31 mg/ml of L-valine was produced after 72 hr when 10% glucose was supplied as a carbon source, thus giving the yield of 31% from glucose. Accumulation of the other amino acids was negligible. The addition of L-isoleucine and L-leucine in the culture medium did not reduce the L-valine production, indicating that the L-valine biosynthesis is insensitive to these end products in the L-valine producer.

Excretion of L-valine by mutants resistant to valine analogues has been known in *E. coli*,[1] *Salmonella typhimurium*,[2] and *Serratia marcescens*.[3] In the previous paper,[4,5] the authors reported that a mutant of *Brevibacterium lactofermentum* 2256 resistant to 2-thiazolealanine, which has been known as a histidine analogue,[6] produced large amounts of L-leucine from glucose. Based on the assumption that this analogue is generally available for altering genetically the control mechanism of branched-chain amino acid synthesis, the authors further attemptd to screen L-valine producers among 2-thiazolealanine resistant mutants of several L-glutamic acid producing strains from the industrial point of view.

The present paper describes the isolation of such mutants and cultural conditions for L-valine production.

MATERIALS AND METHODS

Microorganisms. *B. lactofermentum* 2256 (ATCC No. 13869), *Corynebacterium acetoacidophilum* 410 (ATCC No. 13870) and *Arthrobacter citreus* 13-2A (ATCC No. 17775) were used throughout this work. These strains are typical L-glutamic acid producers, respectively, in each bacterial genus.

Media. The following media were employed. The complete medium contained 0.5% glucose, 1% peptone, 1% yeast extract, 0.5% NaCl.

The minimal medium was composed of 2% glucose 1% $(NH_4)_2SO_4$, 0.2% urea, 0.1% KH_2PO_4, 0.3% K_2HPO_4, 2 ppm Fe^{2+}, 2 ppm Mn^{2+}, 50 μg/liter of biotin and 300 μg/liter of thiamine hydrochloride. This medium was used for the isolation of analogue resistant mutants.

The standard medium for L-valine production contained 8% glucose, 4% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.04% $MgSO_4 \cdot 7H_2O$, 2 ppm Fe^{2+}, 2 ppm Mn^{2+}, 1% of Mieki (solution of amino acid mixture prepared from soybean hydrolysate; total nitrogen, 24 g/liter), 50 μg/liter of biotin, 300 μg/liter of thiamine hydrochloride, and 5% $CaCO_3$. Calcium carbonate was sterilized separately.

The pH value of each medium was adjusted to 7.0.

Mutation and selection of mutants resistant to 2-thiazolealanine. Overnight grown cells in 5 ml of the complete medium were harvested, washed with 1/15 M phosphate buffer (pH 7.0) and resuspended in an equal volume of the same buffer containing 200 μg/ml of N-methyl-N'-nitro-N-nitrosoguanidine. After 30 min incubation at 31.5°C with shaking, the treated cells were washed twice with, and resuspended in the same buffer. The viable cells decreased from 1.5×10^9 to 1.0×10^7

† Studies on the Fermentative Production of Branched-chain Amino Acids. Part III. See references 4) and 5).

This work was presented at the Annual Meeting of the Agricultural Chemical Society of Japan held on April 1st, 1973 in Tokyo.

per ml by the nitrosoguanidine treatment. The diluted cell suspension was spread on a minimal plate containing 1000 μg/ml of 2-thiazolealanine. Cultivation was carried out for 10 days at 31.5°C. The colonies thus appeared were picked up, and checked again for the analogue resistant character.

Culture for L-valine production. Culture methods were essentially the same as described in the previous paper[4] except that the standard medium mentioned in this text was used.

Analytical methods. Quantitative analysis of L-valine in culture broth was carried out by microbiological assay using *Leuconostoc mesenteroides* ATCC 8042.

Analysis for amino acids was carried out by using a Hitachi Amino Acid Analyzer Model KLA-2.

RESULTS AND DISCUSSION

Sensitivity to 2-thiazolealanine of B. lactofermentum 2256

The growth of *B. lactofermentum* 2256 was completely inhibited by the addition of 2-thiazolealanine at the concentrations of more than 1000 μg/ml when cultured in the minimal medium at 31.5°C for 24 hr (Fig. 1). Similar results were obtained with *C. acetoacidophilum* 410 and *A. citreus* 13-2A. As shown in Table I the growth inhibition by 1000 μg/ml of the analogue was recovered by the addition of L-valine, L-leucine, L-isoleucine, L-methionine, L-aspartic acid, L-histidine, L-phenylalanine, L-tyrosine or L-tryptophan. Especially, L-valine, L-leucine or L-tryptophan was most effective.

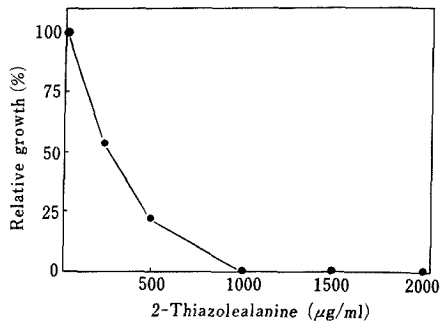

FIG. 1. Growth Inhibition of *Brevibacterium lactofermentum* 2256 by 2-Thiazolealanine.

Derivation of 2-thiazolealanine resistant mutants

Nitrosoguanidine treated cells of *B. lactofermentum* 2256 were spread on a minimal plate containing 1000 μg/ml of 2-thiazolealanine.

The resistant mutants appeared with frequency of 10^{-6}

The best producers, strain No. 487, accumulated 23.1 mg/ml of L-valine in the standard medium. Figure 2 shows the sensitivity of strain No. 487 to 2-thiazolealanine as well as that of the parent strain No. 2256. No. 487 was completely resistant to 2000 μg/ml of the analogue, but still sensitive to more than 10000 μg/ml.

Similar procedure was applied to the other two bacterial strains described above, *i.e., C. acetoacidophilum* and *A. citreus*. L-Valine producers among 2-thiazolealanine resistant mutants tested in each case were found with

TABLE I. RECOVERY OF THE GROWTH OF *Brevibacterium lactofermentum* 2256 FROM 2-THIAZOLEALANINE INHIBITION BY AMINO ACIDS

Amino acid added (1000 μg/ml)	Recovery of growth (%)	Amino acid added (1000 μg/ml)	Recovery of growth (%)
Glycine	0	L-Asparagine	0
L-Alanine	0	L-Glutamic acid	0
L-Valine	100	L-Glutamine	0
L-Leucine	100	L-Lysine	0
L-Isoleucine	50	L-Arginine	0
L-Serine	0	L-Histidine	80
L-Threonine	0	L-Phenylalanine	98
L-Cysteine	0	L-Tyrosine	75
L-Methionine	90	L-Tryptophan	100
L-Aspartic acid	90	L-Proline	0

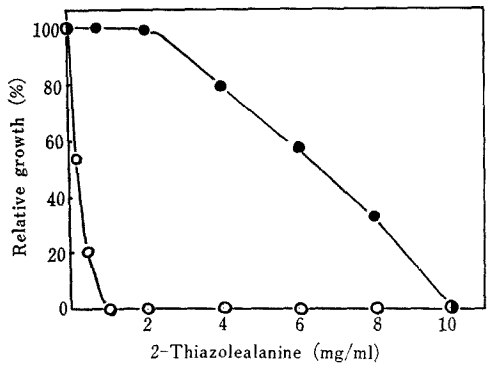

FIG. 2. Growth Inhibition of *Brevibacterium lactofermentum* 2256 and No. 487 by 2-Thiazolealanine.
○—○, No. 2256; ●—●, No. 487.

the approximate same frequency as that in the case of *B. lactofermentum* 2256, and the amounts of L-valine accumulated by the best producer in each genus were found to be roughly comparable to that of strain No. 487, the best producer in *Brevibacterium* (Table II).

TABLE II. PRODUCTION OF L-VALINE BY 2-THIAZOLEALANINE RESISTANT STRAINS

Strains	L-Valine produced (mg/ml)
Brevibacterium lactofermentum No. 487	23.1
Brevibacterium lactofermentum No. 215	22.3
Corynebacterium acetoacidphilum No. 130	20.5
Corynebacterium acetoacidphilum No. 75	18.1
Arthrobacter citreus No. 51	18.8
Arthrobacter citreus No. 73	9.5

These results indicate that the control mechanism operated in L-valine synthesis is very similar or essentially the same between three species. Thus it was verified that 2-thiazolealanine was also effective to obtain potent L-valine producers from typical L-glutamic acid producing strains.

Effect of nutrients on L-valine production

Investigations were made to improve the cultural condition for larger amount of L-valine production by strain No. 487. The addition of nucleic acid bases (adenine, guanine, uracil or cytosine; 500 μg/ml) rather decreased the L-valine productivity. The effects of 17 amino acids were shown in Table III. It was found

TABLE III. EFFECT OF AMINO ACID ON L-VALINE PRODUCTION BY *Brevibacterium lactofermentum* No. 487

Amino acid	L-Valine produced (mg/ml) Amino acid added 0.1%	0.5%
None	23.1	23.1
Glycine	23.0	20.5
L-Alanine	22.7	23.2
L-Leucine	23.2	23.8
L-Isoleucine	23.0	23.1
L-Serine	24.3	25.3
L-Threonine	22.7	23.2
L-Cysteine	24.5	25.7
L-Methionine	23.0	23.1
L-Aspartic acid	22.4	24.4
L-Glutamic acid	24.8	25.7
L-Lysine·HCl	23.5	24.1
L-Arginine	21.5	19.3
L-Histidine	19.5	19.2
L-Phenylalanine	21.5	20.9
L-Tyrosine	21.3	21.4
L-Tryptophan	21.0	19.2
L-Proline	23.1	26.4

that L-serine, L-cysteine or L-glutamic acid (0.1 or 0.5%) have apparently stimulatory effects, and L-arginine, L-histidine, L-phenylalanine, L-tyrosine and L-tryptophan had slightly inhibitory effects. In a previous reports[4] the stimulation by L-cysteine and the inhibition by L-phenylalanine or L-tryptophan have been observed also in L-leucine production. The branched-chain amino acids, L-leucine or L-isoleucine, had no effect on the L-valine production. This result suggests that L-valine synthesis in the L-valine producer is insensitive to these end products. The regulatory mechanisms operated in L-valine synthesis in the parent and mutant strains will be reported elsewhere.

Time course of L-valine production

An example of the time course of the

fermentation by strain No. 487 was presented in Fig. 3. The fermentation medium is the same as the standard medium except that

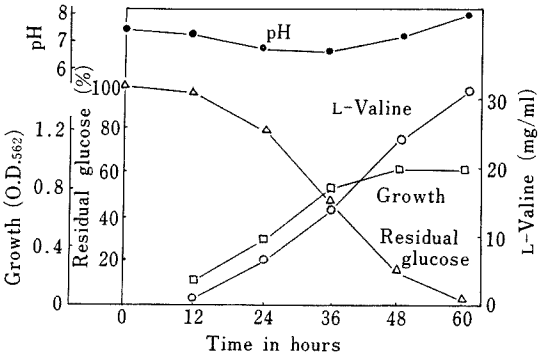

FIG. 3. Time Course of L-Valine Production.

glucose was added up to 10% instead of 8%. The growth began to increase exponentially after about 12 hr and reached the stationary phase at 48 hr, when the accumulation of L-valine commenced and then increased linearly until 72 hr. The amount of L-valine produced finally was 31.0 mg/ml. Glucose was consumed almost completely.

The amounts of other amino acids accumulated into the culture were negligible compared with that of the main product, L-valine: 2.1 mg of glutamic acid, 1.5 mg of alanine, 4 mg of lysine, 1 mg of leucine, 1 mg of isoleucine per ml, and much less of the other amino acids. Thus, the basis of commercial process for L-valine production by mutants of L-glutamic acid producing bacteria has been established. It should be noted that leucine and isoleucine, the branched-chain amino acids other than valine, were not produced practically by No. 487. This means that in strain No. 487, the biosynthesis of L-leucine and L-isoleucine are still controlled by their own regulatory mechanisms. This strain contrasts well with strain No. 218,[4,5] a leucine producer, in which the biosynthesis of L-valine is thought to be still controlled in connection with the genetic alteration of regulatory mechanism specifically operated in L-leucine biosynthesis.

Acknowledgement. The authors are indebted to Drs. T. Tsunoda and S. Okumura of this Company, Drs. M. Takahashi, H. Okada and Y. Hirose of this laboratories for their encouragement. The authors are also indebted to Miss M. Ito for her technical assistance.

REFERENCES

1) O. Karlstrom, *Biotechnol. Bioeng.*, **7**, 245 (1965).
2) J. M. Calvo, M. Freundlich and H. E. Umbarger, *J. Bacteriol.*, **97**, 1272 (1969).
3) M. Kisumi, S. Komatsubara and I. Chibata, *Amino Acid and Nucleic Acid*, No. **19**, 1 (1969).
4) T. Tsuchida, F. Yoshinaga, K. Kubota, M. Momose and S. Okumura, *Agr. Biol. Chem.*, **38**, 1907 (1974).
5) T. Tsuchida, F. Yoshinaga, K. Kubota, M. Momose and S. Okumura *ibid.*, **39**, 1149 (1975).
6) H. S. Noyed, *J. Biol. Chem.*, **244**, 2816 (1969).