Studies on the Low Temperature Fermentation

Part II. Amino Acid Formation by Facultative Psychrophilic Bacterium*

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With the *Brevibacterium* sp. P 145 isolated from soil, the effect of incubation temperature on the extracellular amino acid accumulation has been examined from cultural and enzymological points of view. The strain was found to accumulate L-glutamic acid up to 5.88 mg/ml and L-alanine 0.38 mg/ml at 5°C, whereas it formed 0.21 mg/ml of L-glutamic acid and 2.54 mg/ml of L-alanine at 28°C.

The accumulation of L-alanine in the medium at 28°C seemed to be related to the thiamine requirement of the strain. In the case of thiamine deficiency, L-alanine was the main product in the culture at 28°C. When the incubation temperature was abruptly shifted from 28° to 5°C or from 5° to 28°C, the amino acid accumulation was also changed to that of the final temperature. L-Alanine dehydrogenase existed even in the cells grown at 5°C but was not active at this low temperature. These results were in accord with the informations obtained from cultural experiments.

In the preceding paper,¹⁾ the authors have reported that a psychrophilic bacterium, *Brevibacterium* sp. P 145 isolated from soil, formed glutamic acid at low temperature and was auxotrophic only at high temperature.

It has often been assumed that the rate of individual metabolic process decreases at the same rate as the growth rate, as the temperature decreases from the optimum. However, increased production of metabolites by microorganisms at suboptimum temperature are known in some cases as pigment^{2,3)}

and dextran production.⁴⁾ Owen and Johnson⁵⁾ reported with *Penicillium chrysogenum* that the best temperature for mycelium-production was at 30°C, while at 20°C the penicillin productivity was the best. Similar phenomenon was utilized to obtain high yields of citric acid by *Aspergillus niger*.⁶⁾ Kosano and Yamada⁷⁾ reported that the ratio of two fermentation products, glutamic acid and lactic acid, was affected by the temperatures for seed-culture and main-culture.

In the present paper, the conversion of fermentation products by incubation temperature for *Brevibacterium* sp. P 145 is decreased,

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4) L. K. Dunican and H. W. Seeley, *J. Bacteriol.*, **86**, 1079 (1963).

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6) Y. Noguchi and O. Arao, *J. Ferment. Technol. Japan*, **38**, 491 (1960).

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and the nature of this conversion is discussed on the basis of the results of cultural and enzymological experiments.

MATERIALS AND METHODS

Microorganism. A facultative psychrophilic bacterium, *Brevibacterium* sp. P145 was used throughout the experiment. The organism has a broad growth temperature range, i.e., from 0° to 37°C.

Medium. The basal medium: glucose, 30 g; urea, 4 g; NH₄Cl, 1 g; K₂HPO₄, 2 g; KH₂PO₄, 1 g; MgSO₄·7H₂O, 0.5 g; phenol red, 2 mg; in 1000 ml of tap water. The pH was adjusted to 7.4. The urea solution was fed not only to supply nitrogen but also to maintain the medium slightly alkaline.

Cultivation. One loopful organism from the slope culture was inoculated into a test tube containing 5 ml of the basal medium. The cultured broth of 5 ml was inoculated into 100 ml of the medium in a 500 ml shaking flask. The incubation was carried out at various temperature on a reciprocal shaker (at 130 reciprocation per min). At intervals, a part of the cultured broth was withdrawn and centrifuged, and the supernatant solution was subjected to chemical analysis.

Analysis. For the quantitative analysis of glutamic acid and alanine, the paper chromatographic method as described previously¹⁾ was adopted. Glucose was determined both by Somogyi-Nelson's method⁸⁾ and by Bertrand's method.⁹⁾

The growth of the organism was routinely estimated by measuring the optical density at 610 m μ of 10-fold diluted broth. The dry weight of cells was computed from the optical density, using a standard curve prepared with the culture grown exponentially at 5°C in a peptone-supplemented basal medium.

Protein was determined by the method of Lowry *et al.*¹⁰⁾

Preparation of cell-free extract. The dialyzed cell-free extract was subjected to the assays of L-glutamic acid dehydrogenase and L-alanine dehydrogenase.

After 4 and 2 days at 5° and 28°C, respectively, the cells were harvested by centrifugation. The cells were washed twice with 0.85% NaCl solution, then suspended in M/15 potassium phosphate buffer, pH 7.8. The cell-free extract was prepared by exposing the suspension to Kaijo Denki Ultrasonic Oscillator (19 Hz, for 30 min) and the cell debris was removed by centrifugation at 12,000 $\times g$ for 30 min. The extract thus obtained was dialyzed overnight against M/15 potassium phosphate buffer, pH 7.8, containing 0.05% 2-mercaptoethanol, at 5°C.

Enzyme assay. The enzyme activities were determined by measuring the change in optical density at 340 m μ according to the method reported by Olson and Anfinsen.¹¹⁾ The increase or decrease of the optical density at 340 m μ was followed in a Hitachi-Perkin-Elmer Model 139 Spectrophotometer.

Specific activities of L-glutamic acid and L-alanine dehydrogenases were expressed as ΔE per gram of protein, where ΔE was the increase or decrease of optical density of NADH or NADPH at 5° and 28°C during the initial 1 min incubation.

RESULTS AND DISCUSSION

Identification of L-glutamic acid and L-alanine

L-Glutamic acid and L-alanine accumulated in the cultured broth were identified as des-

TABLE I. IDENTIFICATION OF L-GLUTAMIC ACID AND L-ALANINE

Sample	Method			
	Chromatographic		Biological	
	Glutamic acid mg/ml	Alanine mg/ml	L-Glutamic acid mg/ml	L-Alanine mg/ml
1*	5.50	0.72	5.20	1.00
2**	0.07	2.54	0.09	2.25

* Broth cultured at 5°C for 10 days.

** Broth cultured at 28°C for 4 days.

The L-amino acids were determined by paper chromatographic and biological methods. The biological methods were the manometric method using L-glutamic acid decarboxylase of *E. coli* for L-glutamic acid and the microbiological method using *Leuco. citrovorum*, for L-alanine.

8) M. Somogyi, *J. Biol. Chem.*, **160**, 61, 74 (1945).

9) G. Bertrand, *Bull. Soc. Chem. Paris*, **35**, 1285 (1906).

10) O. H. Lowry, N. J. Rosenbrough, A. L. Forr and R. T. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

11) J. A. Olson and C. B. Anfinsen, *J. Biol. Chem.*, **197**, 67 (1952).

cribed below. For the identification of L-glutamic acid, the manometric method using L-glutamic acid decarboxylase prepared from *Escherichia coli*¹²⁾ was employed. For L-alanine, microbiological analysis using *Leuconostoc citrovorum*¹³⁾ was carried out. Results were summarized in Table I. The values obtained enzymatically or biologically coincided to those measured by paper chromatography.

Cultural condition

Some cultural conditions favoring the accumulation of L-glutamic acid at 5°C were examined.

1) *Growth substance.* Various growth promoting substances shown in Table II were added to the basal medium. It was demonstrated that peptone and meat extract gave a considerable stimulative effect on both growth and L-glutamic acid accumulation, whereas yeast extract and casamino acid did not show any noticeable effect.

TABLE II. EFFECT OF GROWTH SUBSTANCE ON GLUTAMIC ACID FORMATION AT 5°C

Growth substance	(%)	Growth mg/ml	Glutamic acid	
			mg/ml	Comparison
None		2.0	0.72	1.00
Peptone	0.2	3.8	3.90	5.41
Yeast extract	0.2	2.7	1.14	1.58
Meat extract	0.2	4.3	3.78	5.25
Casamino acid	0.2	2.8	1.88	2.61

Cultures were performed with addition of each growth substance (0.2%) to basal medium at 5°C for 8 days.

2) *Glucose concentration.* The effect of glucose concentration in the medium on the amino acid accumulation and the growth was investigated. The results are shown in Table III. It was observed that the optimum concentration of glucose was 3% and that

the higher concentration caused the lower yield of L-glutamic acid as well as growth.

TABLE III. EFFECT OF GLUCOSE CONCENTRATION ON GLUTAMIC ACID FORMATION AT 5°C

Glucose (%)	Growth		Glutamic acid (mg/ml)
	0	10 days	
3.0	0.2	4.3	5.88
5.0	0.2	3.8	4.08
7.0	0.2	1.2	0.86
10.0	0.2	1.4	1.77

Cultures were performed with the medium supplemented with 0.2% peptone at 5°C for 8 days.

Effect of temperature

Five milliliters of the broth cultured in the basal medium for 3 days at 5°C were inoculated into 100 ml of the medium in a 500-ml flask.

The culture was incubated at 5°, 15° or 28°C. As shown in Table IV, the highest extent of growth was obtained at 15°C. Although the maximum growth extent reached most rapidly at 28°C (after 2 days), the cell yields were lower than those at other temperatures. As regard to the amino acid accumulation at 5°C, L-glutamic acid was mainly accumulated and L-alanine was only slightly, whereas at 28°C, the main product was L-alanine but not L-glutamic acid. At 15°C, both amino acids were accumulated in the same extent which was almost the mean of those observed at 5° and 28°C.

TABLE IV. EFFECT OF TEMPERATURE ON AMINO ACID FORMATION

Incubation temperature °C	Maximum cell yield		Amino acid		
	mg/ml	after (day)	Glutamic acid mg/ml	Alanine	after (day)
5	5.2	4	5.88	0.38	8
15	5.7	3	1.88	1.00	6
28	4.0	2	0.21	2.54	4

Cultures were performed with the medium supplemented with 0.2% peptone.

12) V. A. Najjar and J. Fisher, *J. Biol. Chem.*, **206**, 215 (1954).

13) H. E. Sauberlich, *ibid.*, **177**, 545 (1949).

FIG. 1. Time-Course of Amino Acid Formation at 5° and 28°C.

Medium contained 30 g of glucose, 4 g of urea, 1 g of K_2HPO_4 , 2 g of KH_2PO_4 , 0.5 g of $MgSO_4 \cdot 7H_2O$, 2 g of peptone and 2 mg of phenol red in 1000 ml of tap water (pH 7.4).

The time course of amino acid accumulation at 5° and 28°C is presented in Fig. 1.

Effect of the addition of methionine, thiamine and biotin

As described in the preceding paper,¹¹ *Brevibacterium* sp. P 145 requires methionine essentially, and thiamine and biotin accessorially.

Relationship between productivity of amino acid and nutritional requirement of the organism was investigated. In the experiment, the inoculum was prepared from the washed cells grown fully in the basal medium at 5°C. When DL-methionine (200 µg/ml) was added in the basal medium the growth was rather lower. Although the yields of the amino acids were lower compared with the control, main products were consistently L-glutamic acid at 5°C and L-alanine at 28°C. Biotin did not affect the amino acid formation. On the other hand, the L-alanine

accumulation at 28°C seemed to be influenced in some extent by the concentration of thiamine added. If the excess thiamine, (5.0 µg/ml) was added to the basal medium supplemented with methionine, the rate of L-alanine accumulation decreased. In the case of thiamine deficiency, L-alanine was the main product of the culture at 28°C (Table V).

In the alanine accumulation, thiamine might play an important role. Tanaka and Kinoshita¹⁴ reported with *Bacillus lentus* that the alanine production increased with the suboptimum concentration of thiamine even if the biotin level was high. The fact that alanine was accumulated 28°C by the strain P 145 seemed to agree with the accessory requirement of thiamine at 28°C as the case of *B. lentus*.

14) K. Tanaka and S. Kinoshita, *Nippon Nogei-kagaku Kaishi*, **34**, 600 (1960).

TABLE V. EFFECT OF METHIONINE, BIOTIN AND THIAMINE ON AMINO ACID FORMATION

Basal medium	Addition	(μg/ml)	Culture condition			
			at 28°C for 4 days		at 5°C for 10 days	
			Glutamic acid	Alanine	Glutamic acid	Alanine
			mg/ml		mg/ml	
GU	(Basal medium)		(no growth)		0.70	tr
GU	DL-Methionine	20	tr	0.68	0.62	tr
GU	DL-Methionine	200	tr	0.67	0.90	tr
GU	DL-Methionine	2000	tr	0.72	0.80	tr
GUM	Biotin	0.005	tr	0.60	0.88	tr
GUM	Biotin	0.05	tr	0.72	0.60	tr
GUM	Biotin	0.5	tr	0.80	0.70	tr
GUM	Thiamine HCl	0.5	tr	0.60	0.98	tr
GUM	Thiamine HCl	1.0	tr	0.60	0.90	tr
GUM	Thiamine HCl	5.0	tr	tr	1.00	tr
GUM	Peptone	2000	0.60	2.34	4.61	0.70

The basal medium mentioned in materials and methods symbolized by GU and the basal medium supplemented with DL-methionine was symbolized by GUM. tr: trace.

Effect of abrupt temperature shift on amino acid formation

This series of experiment was made to establish whether, after temperature shifted, the amino acid accumulation by the organism was changed or not. *Brevibacterium* sp. P 145 was cultured aerobically in the peptone-supplemented medium at 5° and 28°C. After these cultures reached the certain stages, the temperature was rapidly changed to 28° and 5°C respectively. Figure 2 represents the effect of the temperature shift from 5° to 28°C (a) and from 28° to 5°C (b) on the accumulations of L-glutamic acid and L-alanine. Although L-alanine was scantily produced at 5°C, after the shift from 5° to 28°C the production of L-alanine occurred abruptly. As to L-glutamic acid, the accumulation continued even after the shift to 28°C, although the rate of the formation was considerably depressed comparing with the culture at 5°C. In the case of shift down (from 28° to 5°C) the formation of L-alanine stopped completely and L-glutamic acid was produced progressively. From these results, it is assumed that the conversion of main products by changing the incubation temperature reflects the sub-

FIG. 2-a. Effect of Abrupt Temperature Shift from 5° to 28°C on the Formation of Amino Acid.

Medium was the same as described in Fig. 1.

- (a): Culture was performed at 5° for 8 days.
- (b): Incubation temperature was shifted after 4 days culture at 5°C.
- (c): Incubation temperature was shifted after 2 days culture at 5°C.
- (d): Incubation temperature was shifted after 1 day culture at 5°C.

FIG. 2-b. Effect of Abrupt Temperature Shift from 28° to 5°C on the Formation of Amino Acid.

Medium was the same as described in Fig. 1.

- (a): Culture was performed at 28°C for 4 days.
- (b): Incubation temperature was shifted after 3 days culture at 28°C.
- (c): Incubation temperature was shifted after 2 days culture at 28°C.
- (d): Incubation temperature was shifted after 1 day culture at 28°C.

stantial difference of metabolic processes at each temperature. Such alteration by incubation temperature is remarkable especially in L-alanine formation. It is presumed that the organism has a cold-sensitive step in the L-alanine biosynthesis.

Effect of temperature on L-glutamic acid and L-alanine dehydrogenase activities

In order to explain enzymatically the conversion of fermentation by incubation temperature, the presence and activity of L-glutamic acid and L-alanine dehydrogenases in the cells which might be the last steps of biosynthesis of L-glutamic acid and L-alanine, were examined. As shown in Fig. 3, the

FIG. 3. L-Glutamic Acid Dehydrogenase Activity at 5° and 28°C.

Reaction mixture contained 50 μ moles of L-glutamic acid, 1 μ mole of NADP, 200 μ moles of phosphate buffer (pH 8.3) and 3 mg (as protein) of enzyme in total volume of 3.5 ml. The enzyme was prepared from cells grown at 5°C.

FIG. 4. L-Alanine Dehydrogenase Activity at 5° and 28°C.

Reaction mixture contained 50 μ moles of L-alanine, 1 μ mole of NADP, 200 μ moles of phosphate buffer (pH 8.3) and 3 mg (as protein) of enzyme in total volume of 3.5 ml. The enzyme was prepared from cells grown at 5°C.

dehydrogenase activity for L-glutamic acid was observed at both 5° and 28°C. On the other hand, L-alanine dehydrogenase activity

TABLE VI. STOICHIOMETRY OF L-GLUTAMIC AND L-ALANINE DEHYDROGENASES

	L-Glutamic acid dehydrogenation μ mole	L-Alanine dehydrogenation μ mole
NADPH oxidized	0.40	0.32
	L-Glutamic acid	L-Alanine
Amino acid formed	0.36	0.30

Oxidation of NADPH was measured by a decrease in OD at 340 m μ . Glutamic acid and alanine formation were determined by paper chromatographic method. Reaction mixture contained 50 μ moles of α -ketoglutarate or Na-pyruvate, 120 μ moles of (NH₄)₂SO₄, 0.5 μ mole of NADPH, 200 μ moles of phosphate buffer (pH 7.8) and 3 mg (as protein) of enzyme in total volume of 3.5 ml. The reaction was carried out at 28°C for 10 min.

TABLE VII. SPECIFIC ACTIVITIES OF L-GLUTAMIC ACID AND L-ALANINE DEHYDROGENASES AT 5° AND 28°C

Reaction temp. °C	Substrate	Enzyme from cells grown			
		at 5°C		at 28°C	
		coenzyme		coenzyme	
		NAD	NADP	NAD	NADP
5	L-Glutamate	0.00	8.33	0.00	8.33
	L-Alanine	0.00	0.00	0.00	0.00
28	L-Glutamate	0.00	41.6	0.00	36.7
	L-Alanine	0.00	30.0	0.00	22.1

Reaction mixtures were the same as described in Figs. 3 and 4.

was not apparent at 5°C (Fig. 4).

The stoichiometry of the reverse reactions is shown in Table VI which demonstrates

that the amount of reduced NADP is approximately equivalent to the amount of L-glutamic acid and L-alanine formed.

Using the cell-free extracts prepared from cells grown at 5° and 28°C, the specific activities of both dehydrogenases were determined at 5° and 28°C. As for co-enzyme specificity, the enzymes were found to be specific for NADP in the dehydrogenations of both amino acids, but NAD could not replace NADP (Table VII).

As shown in Table VII, regardless of the temperature of the cultivation, the cell-free extract showed the dehydrogenase activities at 28°C for both amino acids but at 5°C, L-alanine dehydrogenase activity was not observed. The extract from cells grown at 5°C showed L-alanine dehydrogenase activity in the reaction at 28°C but not at 5°C. It follows from these results that the organism is able to synthesize the enzyme at 5°C but is not active at the low temperature. The results obtained at enzyme level are in accord with the information from cultural experiments (Fig. 2-a, b).

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