Studies on DL-Alanine Formation by Thermophilic Bacteria

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Received April 27, 1967

During the course of the screening of thermophilic microorganisms, several strains were found to accumulate amino-acids. Of those strains that were isolated from feces source with Bennett medium, one strain was found to produce a large amount of an amino acid. This amino acid was isolated in crystalline form and identified as DL-alanine by IR absorption spectrum, specific rotatory power and elementary analysis. The taxonomic studies were carried out and this strain was identified as *Bacillus coagulans*. The strain *B. coagulans* B₉-17 produced DL-alanine as much as 1000 mg/l after 24 hours at 50°C in shake culture.

The yield of DL-alanine was increased up to 16.5 g/l with some improvements.

INTRODUCTION

Thermophilic bacteria are generally defined as those having optimal temperature for growth between 50°C to 65°C.

Thermophilic bacteria are very interesting not only from industrial standpoint but also from physiological one. Studies pertaining to thermophilic bacteria have been made by many researchers, especially about heat-stable enzymes,^{1~5)} cell components^{6,7)} and nutritional requirements.^{8~10)}

However, there appeared almost no studies on the accumulation of metabolic products

and practical use of them except lactic acid fermentation under anaerobic conditions.

This study was carried out to investigate metabolic products of thermophilic bacteria under aerobic conditions, which were scarcely studied in the past.

MATERIALS AND METHODS

1. Microorganisms. *B. coagulans* B₉-17, isolated from feces by the authors, was used. *B. coagulans* B₉-17 indicated a better growth between 50°C and 60°C, and thus this strain should be called thermophilic, rather than heat resistant. This strain grew well on the yeast extract medium, but did not grow on the medium containing either Polypepton, corn steep liquor or casein hydrolysate, instead of yeast extract.

2. Medium. The accumulation medium used in shake culture contained glucose 50 g, ammonium sulfate 4 g, monobasic potassium phosphate 4 g, magnesium sulfate 0.5 g and yeast extract 4 g, per liter. One-hundred-milliliter flasks, containing 30 ml each of the medium were sterilized at 115°C for 3 minutes.

Before cultivation, 1 g of sterile CaCO₃ was added to the medium in the flask.

3. Cultivation. For the investigation of products, 1 platinum loop of cells grown on the glucose ammonium sulfate agar slant (Table I) for 17~20 hours of 50°C was inoculated into 30 ml of the

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3) L. L. Campbell and G. B. Manning, *ibid.*, **236**, (11), 2962 (1961).

4) G. B. Manning, L. L. Campbell and R. J. Foster, *ibid.*, **236**, (11), 2958 (1961).

5) L. L. Campbell and P. D. Cleveland, *ibid.*, **236**, (11), 2766 (1961).

6) E. R. L. Gaughran, *J. Bacteriol.*, **53**, 506 (1947).

7) T. Kobayashi and T. N. Rinker, *Arch. Biochem. Biophys.*, **84**, 342 (1947).

8) H. Baker and H. Sobotka, *J. Gen. Microbiol.*, **9**, 484 (1953).

9) L. L. Campbell and E. E. Sniff, *J. Bacteriol.*, **65**, 267 (1953).

10) H. Baker, O. Frank, B. Black, I. Pasher, H. Hutner and H. Sobotka, *Can. J. Microbiol.*, **6**, 556 (1960).

medium in flask. (Table I)

The culture was incubated at 50°C for 24 hours.

4. Paper chromatography. Paper chromatography was employed for the detection of metabolites and was run for 18 hours on Toyo filter paper No. 50 by the ascending method. Solvent systems used are listed in Table II. The spots were visualized by spraying developing reagents.

TABLE I. COMPOSITION OF MEDIUM EMPLOYED FOR THE INVESTIGATION OF PRODUCTS

Glucose	20.0 g
(NH ₄) ₂ SO ₄	4.0
KH ₂ PO ₄	4.0
MgSO ₄ ·7 aq.	0.5
Yeast ext.	2.0
Agar agar powder	20.0
Dist. Water	1000 ml
pH*	8.5

* Adjusted to the desired level with 2N NaOH solution.

TABLE II. SOLVENT SYSTEMS AND DEVELOPING REAGENTS FOR THE DETECTION OF METABOLIC PRODUCT

Solvent	Reagent	Metabolic Product
(1) BuOH-Acetic Acid -Water (4 : 1 : 2)	B.P.B*	Organic Acid
	Ninhydrin	Amino Acid
(2) Phenol Saturated with Water	Ninhydrin	Amino Acid

* 0.1% Ammoniac Ethyl Alcohol Solution.

RESULT AND DISCUSSION

1. Isolation of Microorganisms

For isolation of thermophilic bacteria from soils and feces, some different media were tested.

The growth of aerobic bacteria was better on modified Czapek-Dox medium (containing 0.6% of yeast extract)¹¹⁾ and Bennett medium. Therefore, these media were used as isolation medium.

Thermophilic bacteria were isolated from field soils and feces of animals in Ueno zoo. These samples were extracted by thoroughly mixing 1 g of soil or feces with 10 ml of sterile tap water in 30 ml test tube. One ml of the extract was plated on the agar medium in a Petri dish and incubated for 24 to 48 hours at 50°C. Colonies on the plate were fished, purified at 50°C, and stocked as agar slant culture.

Total 340 strains (Table III) were obtained from soil and feces sources. Of the tested bacteria, strain B₉-17 produced a large amount of a single amino acid, where only one ninhydrin-positive spot was detected on the paper chromatogram.

2. Determination of Metabolic Product

After 24 hours, the cultured broth was submitted to the paper chromatography. Using glucose as substrate, a single ninhydrin-positive spot was given by the broth of strain B₉-17.

TABLE III. NUMBER OF STRAINS ISOLATED

Isolation Sources	Media											
	Glucose			Sucrose			Xylose					
	Bennett			Czapeck-Dox			Bennett			Modified Czapeck-Dox*		
	pH											
	4	7	9	4	7	9	4	7	9	4	7	9
Feces	10	14	16	5	24	17	9	23	16	7	19	22
Soils	2	9	—	20	39	—	3	—	27	17	27	—
Isolated at 50°C for 24 to 48 hrs.										Total 340		

* Containing 0.6% of yeast extract.

11) E. Küster and E. C. Locci, *Arch. Microbiol.*, **45**, 188 (1963).

TABLE IV. PAPER CHROMATOGRAM OF PRODUCT

Solvent Systems	R_F value*	
	Product	Authentic
<i>n</i> -BuOH: Acetic acid: Water (4:1:2 v/v)	0.40	0.39
Phenol Saturated with Water	0.56	0.56

* After 17 hours at room temperature.

The metabolic product of strain B₉-17 was assumed to be alanine from R_F values of paper chromatogram using two different solvent systems in the ascending technique (Table IV). Amino acid was determined by Yemm-Cocking method.¹²⁾

3. Isolation and Identification DL-Alanine as Metabolic Product

DL-Alanine accumulated in the cultured broth was isolated, purified and identified as described below.

The cells were separated from the cultured broth by centrifugation. Then the supernatant solution was treated with H⁺ type of Amberlite IR-120 and OH⁻ type of Amberlite IRA-410 to deplete and with CH₃COO⁻ type of Dowex 1×8 to adsorb a very small amount of acidic amino acids.

The solution passed through Dowex 1×8 (CH₃COO⁻ type) which was not contaminated with other amino acid as detected in paper chromatogram, was evacuated to dryness. The crude crystals were recrystallized from aqueous ethyl alcohol. The refined crystals were used for IR absorption spectrum, specific rotatory power and elementary analysis. IR absorption spectrum of this compound corresponded completely to that of an authentic specimen. (Fig. 1). Analysis of this amino acid. Found: C, 40.52; H, 7.56; N, 15.79. Calcd. for C₃H₇NO₂: C, 40.44; H, 7.92; N, 15.72%. Specific rotatory power indicated that this compound was racemic.

Therefore, this amino acid was identified as DL-alanine.

FIG. 1. Infrared Absorption Spectra of the Product.

A: Product R: Authentic DL-Alanine.

FIG. 2. Microscopic Photograph of Strain B₉-17 Grown on Nutrient Agar Slant for 48 hours at 50°C.

4. Taxonomic Studies of the Strain Isolated

The results of taxonomic studies are shown in Fig. 2 and Table V. From these results, it was concluded that this strain belongs to *B. coagulans* with reference to Bergey's Manual of Determinative Bacteriology, 7th ed.

It was reported by Baker et al.¹⁰⁾ that *B. coagulans* generally required methionine and some of them required other amino acids (glutamic acid, alanine, histidine) instead of methionine. However, it was not determined whether the newly isolated strain requires methionine or not.

¹²⁾ E. W. Yemm and E. C. Cocking, *Analyst*, **80**, 209 (1955).

TABLE V. TAXONOMIC STUDIES

Morphological characteristics	Rod, 0.6 to 0.8 by 3.0 to 4.0 microns, occurring in filaments. Spore 0.8 by 1.5 microns, oval to cylindrical, thin-walled, subterminal. Sporangia not definitely swollen. Gram positive, motile.
Nutrient agar colony	Circular, rough, wrinkled, lobate, yellowish gray, butyrous.
Nutrient agar slant	Growth moderate, filiform, flat, wrinkled, yellowish gray.
Glutamate agar slant	No growth.
Nutrient gelatin stab	No liquefaction.
B.C.P milk	Softly coagulate.
M.R. test	Negative.
Product of acetyl methyl carbinol	Negative.
Hydrolysis of starch	Positive.
Product of HS	Positive.
5% NaCl nutrient broth	No growth.
Acid production from sugars	Acid from glucose, fructose and maltose under both aerobic and anaerobic conditions. No acid from glyceroxylose, arabinose, sucrose, lactose and starch.
Glucose, gluconate, citrate, succinate, <i>p</i> -hydroxy-benzoate and protocatechuate are not utilized as a sole source of carbon.	
Cytochrome oxidase	Negative.
Catalase	Positive.
Grow well between 50°C~55°C.	No growth at 65°C.

All the tests were carried out at 50°C.

5. Some Aspects of DL-Alanine Formation by Strain B₉-17

1) **Influence of different glucose concentrations.** It is quite probable that the yield of DL-alanine was influenced by the concentra-

tion of glucose in the medium, so that this was investigated using the accumulation medium, so that this was investigated using the accumulation medium. The result is shown in Fig. 3.

The increase in glucose concentration failed to enhance the yield of DL-alanine as shown in Fig. 3. An increase in glucose concentration exceeding 5% retarded consumption of glucose and resulted in poor DL-alanine formation. The optimal glucose concentration was about 5%.

2) **Influence of initial pH of the medium.** In order to investigate the influence of pH of the culture, pH of medium was controlled as follows. A range of pH values from 5.5 to 8.5 in increment of approximately 0.5 pH unit was investigated. (Fig. 4) The initial pH of the growth medium was adjusted to the desired level, and then was allowed to respond the microbial growth, adding no buffers to the medium.

The medium of pH 5.5 was incubated for

FIG. 3. Influence of Glucose Concentration.

Volume of Medium; 100 ml/500-ml flask
Incubation Time; 72 hours
Temperature; 50°C
Composition of Medium; (NH₄)₂SO₄ 18 g,
Glucose 50 g, KH₂PO₄ 4 g, MgSO₄·7 aq. 0.5 g,
Yeast ext. 4 g, Dist. Water 1000 ml.
●—● Yield of alanine
○—○ Residual glucose.

FIG. 4. Influence of Initial pH.

Volume of Medium; 100 ml/500-ml flask
 Incubation Time; 72 hours
 Temperature; 50°C
 Composition of Medium; $(\text{NH}_4)_2\text{SO}_4$ 18 g,
 Glucose 50 g, KH_2PO_4 4 g, $\text{MgSO}_4 \cdot 7 \text{ aq.}$ 0.5 g,
 Yeast ext. 4 g, Dist. Water 1000 ml.
 ●—● Yield of alanine
 ○—○ Residual glucose.

comparing the influence of other pH values. pH of the growing culture decreased at early stage of growth to about pH 6.0 to 7.0. The optimal pH value for formation of DL-alanine was about pH 5.5 to 6.5, though it was not definitely accurate owing to a slight decline of the pH value during incubation.

But the consumption of glucose by strain B₉-17 was best at pH 8.5. In contrast, the yield of DL-alanine was little.

3) **Influence of incubation temperature.** Fig. 5 indicates the influence of incubation temper-

FIG. 5. Influence of Incubation Temperature.

Volume of Medium; 30 ml/100-ml flask
 Composition of Medium; $(\text{NH}_4)_2\text{SO}_4$ 18 g,
 KH_2PO_4 4 g, $\text{MgSO}_4 \cdot 7 \text{ aq.}$ 0.5 g, Yeast extract
 4 g, Glucose 50 g, Dist. Water 1000 ml
 Yield of alanine { at 56°C ●—●
 at 50°C ●---●
 Residual glucose { at 56°C ○—○
 at 50°C ○---○

ature on DL-alanine formation and consumption of glucose by *B. coagulans* B₉-17. The organism B₉-17 was incubated at different temperatures (37°C, 50°C, 56°C, 62°C), but there was no growth at 37°C and 62°C.

The yield of DL-alanine was higher at 56°C than at 50°C, and further, accumulation of DL-alanine at 56°C was initiated at earlier stage than at 50°C. Therefore, the strain was not only heat-stable, but thermophilic.

4) **Influence of different nitrogen sources on DL-alanine formation.** In order to compare various nitrogen sources, ammonium sulfate of basal medium was replaced by the equimolar concentration of various nitrogen sources and ten kinds of nitrogen sources were examined for their effect on DL-alanine formation. The results obtained after three days are shown in Table VI. In this table, DL-alanine yields in two nitrogen concentrations corresponding to 1.0% and 2.0% of $(\text{NH}_4)_2\text{SO}_4$ are indicated. As shown in Table IV, nitrates and urea were inferior to ammonium salt as to the yield of DL-alanine, and strain B₉-17 did not grow on the organic nitrogen medium.

TABLE VI. INFLUENCE OF NITROGEN SOURCES

N-Sources	Added	*Residual glucose	Alanine
$(\text{NH}_4)_2\text{SO}_4$	1.00%	0.00%	5.41 g/l
	2.00	0.00	7.52
NH_4Cl	0.90	0.99	12.99
	1.80	1.46	9.97
NaNO_3	1.40	1.83	trace
	2.80	2.39	trace
NH_4NO_3	0.65	1.74	4.53
	1.30	0.47	11.84
Urea	0.50	2.33	trace
	1.00	2.33	trace
NH_4 -acetate	1.10	5.00	0.00
	2.20	5.00	0.00
NH_4 -phosphate	1.90	5.00	0.00
	3.80	5.00	0.00
NH_4 -lactate	1.50	2.57	2.77
	3.00	3.53	2.10
Peptone	1.30	5.00	0.00
	2.60	5.00	0.00

* Residual glucose after 70 hour's incubation at 50°C.

Initial concentration was 5%.

Volume of the medium was 100 ml/500-ml flask.

Of the nitrogen sources tested, ammonium sulfate, ammonium chloride and ammonium nitrate were good for the yield of DL-alanine. The highest yield of DL-alanine was obtained with ammonium chloride, which however retarded the consumption of glucose remarkably, in contrast with a little effect of ammonium sulfate in this respect.

5) Influence of the concentration of nitrogen source. In order to find the suitable concentration of nitrogen source in the medium, ammonium sulfate and ammonium chloride were used as nitrogen source for DL-alanine accumulation.

The result is presented in Fig. 6. As obviously seen from the figure influence of the

FIG. 6. Influence of Nitrogen Concentration.

Temperature; 56°C
 Incubation Time; 45 hours (using (NH₄)₂SO₄ as nitrogen source)
 50 hours (using NH₄Cl as nitrogen source)
 Volume of Medium; 30 ml/100-ml flask
 Composition of Medium except nitrogen source;
 Glucose 50 g, KH₂PO₄ 4 g, MgSO₄·7 aq. 0.5 g,
 Yeast ext. 4 g, Dist. Water 1000 ml.
 ●—● Yield of Alanine ○—○ Residual Glucose.

concentration of nitrogen source was very remarkable and the optimal concentration for the yield of DL-alanine was about 1.3% w/v for ammonium chloride and about 2.0% (w/v) for ammonium sulfate. The highest yield of DL-alanine was obtained with 1.3% of ammonium chloride and it was 16.5 g per liter. About 40% (w/v) of the consumed glucose was converted into DL-alanine.

6) Influence of oxygen supply. At 55°C, oxygen will be dissolved in water about a half comparing the case of at 30°C. In order to investigate the influence of oxygen supply on the formation of DL-alanine, the shake culture experiments were performed under the different conditions by varying the volume of the medium in flask shaken at 140 recip./min with 50 mm stroke.

Judging from the results shown in Fig. 7,

FIG. 7. Influence of Oxygen Supply.

Temperature; 56°C
 Incubation Time; 50 hours
 Composition of Medium; NH₄Cl 13.5 g, Glucose 50 g,
 KH₂PO₄ 4 g, MgSO₄·7 aq. 0.5 g, Yeast ext. 4 g,
 Dist. Water 1000 ml.
 ●—● Yield of Alanine ○—○ Residual Glucose.

the influence of oxygen supply was not remarkable. However, it was considered that oxygen supply was a controlling factor; excess of this seemed to be rather inhibitory for DL-alanine formation.

7) Influence of KH₂PO₄ and MgSO₄·7 aq. concentrations on DL-alanine formation. It is quite probable that the fermentation products are generally influenced by the concentration of KH₂PO₄ and MgSO₄·7 aq., so that the influence of these inorganic salts is to be studied. The results obtained in this respect are shown in Fig. 8 and Fig. 9. In case of KH₂PO₄, the best result was obtained at the concentration of 0.4% in both the media of (NH₄)₂SO₄ and of NH₄Cl, whereas the concentration of KH₂PO₄ exceeding 0.4% made the growth unstable. On the other hand, increase of MgSO₄·7 aq. concentration in the medium

FIG. 8. Influence of Concentration of KH_2PO_4 .Incubation temperature; 56°C Nitrogen sources: 1.35% of NH_4Cl or
2.0% of $(\text{NH}_4)_2\text{SO}_4$

Volume of medium; 25 ml/100-ml flask

Composition of medium except nitrogen and KH_2PO_4 ;Glucose 50 g, $\text{MgSO}_4 \cdot 7 \text{ aq.}$ 0.5 g, Yeast ext. 4 g,
Dist. Water 1000 ml.

Yield of alanine

●—● (using NH_4Cl as nitrogen source)○—○ (using $(\text{NH}_4)_2\text{SO}_4$ as nitrogen source). FIG. 9. Influence of Concentration of $\text{MgSO}_4 \cdot 7 \text{ aq.}$

Incubation time; 50 hours

Temperature; 56°C

Volume of medium; 25 ml/100-ml flask

Composition of medium except $\text{MgSO}_4 \cdot 7 \text{ aq.}$,
 NH_4Cl 13.5 g, Glucose 50 g, KH_2PO_4 4 g, Yeast
ext. 4 g, Dist. Water 1000 ml.

●—● Yield of alanine

○—○ Residual glucose.

was rather inhibitory.

8) **Culture under optimal conditions.** The most favourable culture conditions (Table VII)

TABLE VII. OPTIMAL MEDIUM AND CONDITION

Glucose	50.0 g
NH_4Cl	13.5
KH_2PO_4	4.0
$\text{MgSO}_4 \cdot 7 \text{ aq.}$	0.5
Yeast ext.	4.0
Dist. Water	1000 ml
Volume of the medium	25 ml/100-ml flask
Incubation temperature	56°C

FIG. 10. Culture under Optimal Condition
Condition as Table VII.●—● Yield of alanine
○—○ Residual glucose
○--○ Growth of cells.

for DL-alanine accumulation were investigated by the fermentation experiments. The results are shown in Fig. 10. Glucose was almost exhaustively consumed in 65 hours and the rate of conversion to DL-alanine attained more than 40% w/v. The maximum yield of DL-alanine was attained in 56.5 hours culture and residual glucose was about 1%.

SUMMARY

1. One strain of thermophilic bacillus, which produced an amino acid, was isolated

from a feces source with Bennett medium.

2. This strain was identified to *Bacillus coagulans*.

3. The amino acid produced by this strain was identified as DL-alanine.

4. The cultural conditions were investigated, and ammonium chloride was favourable for the amino acid production.

Acknowledgement. We wish to express our sincere gratitude to Dr. K. Komagata of the Central Research Laboratory of Ajinomoto Co., Inc., for his help in the taxonomic studies. We also wish to thank Mr. N. Fukuda and Mr. A. Awata of Ueno Zoo for the supply of feces sources used in this work.