APPLIED MICROBIOLOGY, Apr. 1973, p. 636-640
Vol. 25, No. 4

Printed in U.S.A.

Production of D-Alanine by *Corynebacterium fascians*

SHIGEKI YAMADA, HARUKO MAESHIMA, MITSURU WADA, AND ICHIRO CHIBATA

Department of Biochemistry, Research Laboratory of Applied Biochemistry, Tanabe Seiyaku Co., Ltd., Kashima-cho, Higashiyodogawa-ku, Osaka, Japan

Received for publication 3 January 1973

A strain identified as *Corynebacterium fascians* was found to accumulate extracellular D-alanine from glycerol. Cultural conditions for the accumulation of D-alanine were investigated and, as a result, a yield of 7 g of D-alanine per liter was obtained after a 96-h incubation in a medium containing 5% glycerol, 4% $(NH_4)_2HPO_4$, and 0.3% corn steep liquor. Optical purity of D-alanine was dependent upon the concentration of corn steep liquor. At the optimal condition, almost optically pure D-alanine was formed and readily isolated (5 g/liter) from the fermentation broth. The product was not contaminated with any detectable amount of other amino acids, except for glycine which was present at a concentration of less than 1 percent.

In recent years, the microbial production of amino acids has attracted considerable attention in the fermentation industry (4). Many studies have been reported on the microbial production of alanine from carbohydrates (2-4, 6, 7, 9-11, 13-15, 17). In most studies, alanine produced was optically inactive or the optical purity was unknown. A few reports have shown the production of L-alanine (14, 15); however, neither the accumulation of D-alanine nor the formation of alanine from glycerol have been reported.

During the investigation of microorganisms utilizing petrochemicals, we found that a strain of *Corynebacterium fascians* accumulated large amounts of D-alanine in a medium containing glycerol as the sole carbon source. This paper reports screening of microorganisms, optimum cultural conditions for production, isolation of the product, and identification of D-alanine, which may provide a workable procedure for the industrial production of D-alanine.

MATERIALS AND METHODS

Microorganisms. From the collection of microorganisms maintained in our laboratory, a strain of *C. fascians* was selected as a D-alanine-producing bacterium. This strain was deposited in the Fermentation Research Institute, Inage, Chiba, Japan, and was designated *C. fascians* FERM-P no. 1511. For stock cultures this strain was grown at 27 C for 2 days on an agar slant containing 0.5% glucose, 0.5% meat extract, 1.25% yeast extract, 1% polypeptone, 0.5% NaCl, and 1.5% agar adjusted to pH 7.0 and stored at 5 C. Freshly prepared slant cultures were used for seed cultures.

Media. Unless otherwise noted, media shown in Table 1 were employed.

Cultivation method. Screening tests were carried out in test tubes with shaking at 30 C for 120 h. For the production of alanine, 100 ml of the media were distributed in 500-ml shaking flasks, autoclaved at 120 C for 10 min, and inoculated with 5% of seed cultures incubated for 2 days. All cultures were incubated at 30 C on a reciprocating shaker operating at 140 strokes/min with an 8-cm stroke. Unless otherwise noted, the fermentations were carried out for 96 h.

Estimation of cell growth. The growth was determined turbidimetrically, at 660 nm in a Hitachi electric photometer EPO-B, and was expressed as dry-cell weight (mg/ml) calculated from a standard curve.

Determination of amino acids, glycerol, and pyruvate. Identification and determination of amino acids in the experiments for screening of amino acid-producing organisms were performed by paper chromatography (1-butanol:acetic acid:water, 4:1:1, v/v/v) and, for more detail, by a Hitachi amino-acid analyzer (KLA-3B). Total alanine was measured microbiologically by using *Leuconostoc citrovorum* ATCC 8081 (16). D-Alanine was determined manometrically by using D-amino-acid oxidase prepared from hog kidney by the method of Brumby and Massey (1). Pyruvate was determined by the Friedemann-Haugen method (5). Glycerol was measured by the procedure of Iwai et al. (8).

Determination of optical purity of D-alanine. Optical purity of D-alanine in the broth was calculated by the equation in Table 7 and that of the crystalline D-alanine was determined with a Perkin-Elmer 141 polarimeter.

TABLE 1. *Composition of media*[a]

Components	Medium		
	Screening (%)	Seed culture (%)	Alanine production (%)
Glycerol	2	1	5
$(NH_4)_2HPO_4$	1	1	4
Corn steep liquor	0.05	0.15	0.3

[a] Components were dissolved in tap water, and media were adjusted to pH 7.0.

Chemicals. All chemicals were obtained from commercial sources.

RESULTS

Screening of microorganisms forming amino acids from glycerol. Two hundred sixty-five strains, which had been tested for their use of petrochemicals and maintained in our laboratory, were inoculated in medium I and cultured for 120 h. As shown in Table 2, a number of strains used glycerol as the sole carbon source. Some strains were observed to produce amino acids, especially alanine. Of the tested organisms, *C. fascians* FERM-P no. 1511 produced a large amount of alanine and therefore was used for the experiments described below.

Utilization of noncarbohydrate substances and alanine production. *C. fascians* was cultured in medium I containing noncarbohydrate substances in place of glycerol as carbon source (Table 3). After 120 h, the growth was poor and, except from glycerol, practically no alanine was accumulated (Table 3). Experiments on the accumulation from the other carbon sources are in progress, and a few carbohydrates such as glucose and fructose also have been found to be effective for D-alanine production. However, in this paper, only the accumulation of alanine from glycerol will be described.

Effect of nitrogen sources. $NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl, H_2NCONH_2, and $(NH_4)_2HPO_4$ were studied as nitrogen sources. The cell growth and alanine accumulation in medium III, containing the nitrogen sources in place of $(NH_4)_2HPO_4$, were very poor (Table 4). Alanine was accumulated only in the medium containing $(NH_4)_2HPO_4$. The higher concentration of $(NH_4)_2HPO_4$ markedly increased the production of alanine.

Effect of $(NH_4)_2HPO_4$ level. To determine the optimal level of $(NH_4)_2HPO_4$ in medium III, its concentration was varied (Table 5). Although the growth was not reduced, even at the lowest concentration of $(NH_4)_2HPO_4$, alanine production was markedly decreased at lower levels. A concentration of 4% and higher is required for increased yields.

Effect of glycerol level. The effect of glycerol concentration on alanine formation in medium III was examined (Table 6). At 2 to 3%, the cell yield reached a maximum, but the amount of alanine accumulated was very small. With the increase of the glycerol level, the alanine yield sharply increased, and at 5% almost maximum production was achieved. Further increase of glycerol concentration had no stimulatory effect on alanine production.

TABLE 2. *Screening of microorganisms forming amino acids from glycerol*

Genus	No. of strains			
	Tested	Grown	Accumulated amino acid	Accumulated alanine
Achromobacter	17	14	1	1
Bacillus	17	14	0	—
Brevibacterium	4	4	0	—
Corynebacterium	3	3	2	2
Micrococcus	17	7	0	—
Pseudomonas	56	54	0	—
Serratia	9	9	1	1
Candida	6	6	0	—
Rhodotorula	3	3	2	1
Others[a]	113	81	4	2

[a] These were isolated from soil and were not identified.

TABLE 3. *Assimilation of noncarbohydrate substances and alanine production by Corynebacterium fascians*

Noncarbohydrate substances added	Growth (mg/ml)	Total alanine accumulated (mg/ml)
Acetic acid	0	Trace
Adipic acid	1.5	Trace
Anthranilic acid	0.6	Trace
Benzoic acid	0.3	Trace
Cinnamic acid	0.9	Trace
Sulfanilic acid	0.9	Trace
Ethanol	0	Trace
Methanol	0	Trace
Propanol	0	Trace
Ethylene glycol	0.9	Trace
Propylene glycol	1.5	Trace
Glycerol	6.2	2
Ethanolamine	0.9	Trace
Cyclohexylamine	0.3	Trace
Ethyl acetate	0.9	Trace

TABLE 4. *Effect of nitrogen sources on alanine production by Corynebacterium fascians*

Nitrogen source added	Percentage of nitrogen source	Growth (mg/ml)	Total alanine accumulated (mg/ml)
$NaNO_3$	2	4.0	Trace
	4	3.7	Trace
NH_4NO_3	2	3.1	Trace
	4	3.1	Trace
$(NH_4)_2SO_4$	2	3.1	Trace
	4	1.8	Trace
NH_4Cl	2	2.8	Trace
	4	2.8	Trace
NH_2CONH_2	2	0.9	Trace
	4	0.9	Trace
$(NH_4)_2HPO_4$	2	17.0	2.4
	4	15.5	6.5

TABLE 5. *Optimal $(NH_4)_2HPO_4$ level for alanine production*

$(NH_4)_2HPO_4$ added (%)	Growth (mg/ml)	Total alanine accumulated (mg/ml)
1	17.6	0.7
2	17.0	2.4
3	16.7	4.7
4	15.5	6.8
5	16.4	7.0
6	17.6	7.0
7	17.3	6.7

TABLE 6. *Optimum glycerol level for alanine production*

Glycerol added (%)	Growth (mg/ml)	Total alanine accumulated (mg/ml)
1	9.0	0.5
2	14.2	1.2
3	15.8	2.6
4	15.5	5.0
5	15.5	6.8
6	15.5	6.7
7	14.5	6.8
8	14.5	7.0
9	12.7	7.0
10	13.0	6.0

Effect of corn steep liquor. The amount of total alanine and its optical purity were dependent upon the corn steep liquor concentration (Table 7). At the lower concentrations the amount of total alanine, but not of the D-isomer, increased. There was a decrease in optical purity as the concentration of corn steep liquor was decreased. At over 0.3%, optically pure D-alanine could be exclusively produced.

Chemical changes under the optimal condition. A typical change of alanine production in medium III is shown in Fig. 1. After 96 h, maximum growth was obtained, and the production of D-alanine also reached the maximum, 6.8 mg/ml. Detectable accumulation of L-alanine did not occur. Amount of D-alanine produced gradually decreased after 120 h. Glycerol was rapidly utilized and completely consumed at 96 h. The accumulation of pyruvate in the broth was small. The pH of the fermentation medium remained between 6 and 7 during all culture stages. Amino acid analysis of the fermentation broth cultured for 96 h indicated that it contained 6.8 mg of alanine per ml, 0.2 mg of glycine per ml, traces of leucine, and no other amino acids.

Isolation and identification of D-alanine. From the fermentation broth cultured under the optimal conditions described above, D-alanine was isolated as follows. One liter of fermentation broth containing 6.8 g of D-alanine was

TABLE 7. *Effect of corn steep liquor level on D-alanine production and optical purity*[a]

Corn steep liquor added (%)	Growth (mg/ml)	D-Alanine accumulated (mg/ml)	Total alanine accumulated (mg/ml)	Optical purity (%)
0.05	6.3	6.0	8.8	36
0.15	11.4	6.8	8.5	60
0.2	12.4	6.7	7.6	86
0.3	15.5	6.8	6.7	103
0.4	16.4	5.5	5.6	96
0.5	16.7	4.6	4.6	100

[a] Optical purity was calculated according to the following equation: [total alanine − 2× (total alanine − D-alanine)/(total alanine)] × 100%.

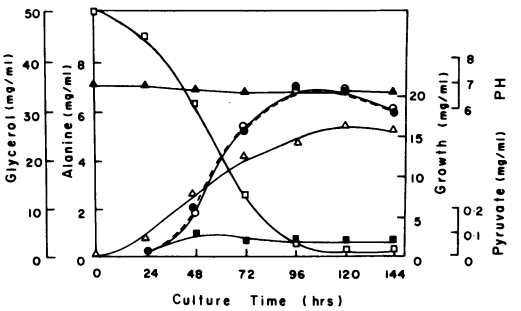

FIG. 1. *Chemical changes during D-alanine production under the optimal conditions. The medium contained 5% glycerol, 4% $(NH_4)_2HPO_4$, and 0.3% corn steep liquor. Symbols: ○, total alanine; ●, D-alanine; □, glycerol; ■, pyruvate; △, growth; ▲, pH.*

adjusted to pH 2.0 with HCl, heated, and centrifuged to remove the cells. The supernatant fluid was treated with 1 g of active charcoal and filtered. The filtrate was passed through a column packed with 500 ml of Amberlite IR120 (H^+-form) which absorbed D-alanine. After a water wash, the column was eluted with 2 N NH_4OH. The eluted solution was evaporated in vacuo, and addition of ethanol resulted in crystallization of D-alanine (yield was 6.2 g). One crystallization from aqueous methanol yielded 5.0 g of the pure product: the elemental analysis corresponded to $C_3H_7NO_2$ (calculated: C, 40.44; H, 7.92; N, 15.72; O, 35.92; found: C, 40.36; H, 7.93; N, 15.90). The optical rotary value was $[\alpha]_D^{25} = -13.6°$ (C = 2 in 5 N HCl). This value was equivalent to 93% optical purity. The IR and NMR spectra of the sample were identical to those of authentic D-alanine. Paper chromatographic analysis indicated that it contained less than 1% glycine and no other amino acids.

DISCUSSION

Alanine is the amino acid most commonly accumulated by microorganisms, and many investigators have reported on its fermentative production (4). In most cases, the DL form accumulates, explainable by the fact that alanine racemase is more widely distributed in many microorganisms than are racemases for other amino acids. In a few cases, however, accumulation of L-alanine has been reported (14, 15). Consequently, it has been accepted that the optical configuration of alanine produced by microorganisms is the L or DL form. Furthermore, there has been no report on the extracellular accumulation of a large amount of a D-amino acid by microorganisms, although the presence of D-amino acids in bacterial cell walls, antibiotics, and tumor proteins is well known (12). Therefore, it is very interesting that a large amount of D-amino acid is produced by a microorganism.

The amount of extracellular D-alanine accumulated by *C. fascians* varies with cultural conditions, especially with the composition of the fermentation medium. A simple combination of glycerol, $(NH_4)_2HPO_4$, and corn steep liquor was found to be advantageous for production of D-alanine. Although yields of total alanine increased in a medium containing a higher concentration of glycerol and $(NH_4)_2HPO_4$, practical optimal levels are considered to be 5% glycerol and 4% $(NH_4)_2HPO_4$. The level of corn steep liquor was very important for the production of optically pure D-alanine. Accumulation of total alanine was enhanced by decreasing the level of corn steep liquor, but the optical purity was decreased, indicating contamination with L-alanine. Although the experiments on the mechanism of accumulation of D-alanine are in progress, we postulate that L-alanine is formed first and remains inside the cells where it is converted to DL-alanine by racemase. Subsequently, only D-alanine leaks out stereospecifically through the cell membrane. In this process, it seems that the trace components in corn steep liquor, such as vitamins and minerals, affect the stereospecific permeability of the cell membrane. Under the optimal condition, accumulation of D-alanine was raised to almost 7 g per liter and practically pure D-alanine could be easily recovered.

In recent years, D-alanine has been in commercial demand as a biochemical and as a starting material for peptide synthesis. Until today, D-alanine has been prepared by optical resolution of the chemically synthesized DL form. In contrast to these methods, the present one-step fermentative method has a definite advantage and is suitable for industrial application.

ACKNOWLEDGMENT

We thank Nobuhiko Izuo in our laboratory for the screening test.

LITERATURE CITED

1. Brumby, P. E., and V. Massey. 1968. D-Amino acid oxidase from pig kidney, p. 29-41. *In* W. E. M. Lands (ed.), Biochemical preparations, vol. 12. Biochemical Preparations Inc., New York.
2. Carito, S. L., and M. A. Pisano. 1966. Production of alanine by *Fusarium moniliforme*. Appl. Microbiol. **14**:39-44.
3. Dulaney, E. L., C. A. Jones, and D. D. Dulaney. 1963. Amino acid accumulation, principally alanine, by auxotrophs of *Ustilago maydis*. Develop. Ind. Microbiol. **5**:242-249.
4. Dulaney, E. L. 1967. Microbial production of amino acids, p. 308-343. *In* H. J. Peppler (ed.), Microbial technology. Reinhold Publishing Corp., New York.
5. Friedemann, T. E., and G. E. Haugen. 1943. Pyruvic acid. II. The determination of keto acids in blood and urine. J. Biol. Chem. **147**:415-442.
6. Hirose, Y., and K. Yamada. 1961. Studies on the amino acids fermentation of pentose materials. II. Formation of amino acids from pentoses and hexoses by *Brevibacterium pentoso-aminoacidicum* nov. sp. Agr. Biol. Chem. **25**:410-416.
7. Iizuka, H., and K. Komagata. 1960. Amino acid accumulation and bacteria. J. Agr. Chem. Soc. Jap. **34**:27-32.
8. Iwai, Y., S. Omura, and T. Hata. 1971. The determination of glycerol in the broth of an antibiotic producing fermentation culture of *Streptomyces* sp. J. Ferment. Technol. **49**:842-846.
9. Kinoshita, S., S. Udaka, and M. Shimono. 1957. Studies on the amino acid fermentation. I. Production of L-glutamic acid by various microorganisms. J. Gen. Appl. Microbiol. **3**:193-205.

10. Kitai, A., H. Tone, K. Sasaki, and N. Miyachi. 1961. Studies on the mechanism of alanine fermentation. Part I. Fermentation mechanism of DL-alanine in *Corynebacterium geratinosum* no. 7183. J. Agr. Chem. Soc. Jap. **35**:862–867.
11. Matsumoto, T., T. Yano, and K. Yamada. 1967. Studies on DL-alanine formation by thermophilic bacteria. Agr. Biol. Chem. **31**:1381–1388.
12. Meister, A. 1965. The natural amino acids. E. Natural D-amino acids, p. 113–118. *In* Biochemistry of the amino acids, vol. 1. Academic Press Inc., New York.
13. Ogawa, C., M. Oide, and Y. Midorikawa. 1959. Studies on alanine and glutamic acid fermentation by *Brevibacterium alanicum* nov. sp. Part I. Amino acids, no. 1, 45–55.
14. Perry, J. J. 1967. Production of L-alanine by biotin-deficient *Micrococcus sodonensis*. J. Bacteriol. **94**:1249–1250.
15. Samejima, H., T. Nara, C. Fujita, and S. Kinoshita. 1960. L-Alanine fermentation. Part I. L-Alanine fermentation by no. 483 strain. J. Agr. Chem. Soc. Jap. **34**:832–838.
16. Sauberich, H. E., and C. A. Baumann. 1949. A microbiological determination of alanine. J. Biol. Chem. **177**:545–551.
17. Yamada, K., and Y. Hirose. 1960. Studies on the amino acids fermentation of pentose materials. I. Isolation of strains capable of producing amino acids from pentose and their taxonomical studies. Bull. Agr. Chem. Soc. Jap. **24**:621–627.