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Enzymatic Production of L-Alanine by *Pseudomonas dacunhae*

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ABSTRACT

CHIBATA, ICHIRO (Tanabe Seiyaku Co., Ltd., Osaka, Japan), TOSHIO KAKIMOTO, AND JYOJI KATO. Enzymatic production of L-alanine by *Pseudomonas dacunhae*. Appl. Microbiol. **13**:638–645. 1965.—To establish an advantageous method for the production of L-alanine, a procedure was studied for converting L-aspartic acid to L-alanine by microbial L-aspartic β-decarboxylase. A number of organisms were screened to test their ability to form and accumulate alanine from aspartic acid. *Pseudomonas dacunhae* was selected as the most advantageous organism. With this organism, enzyme activity as high as 3,910 μliters of CO_2 per hr per ml of medium could be produced by shaking the culture at 30 C in the medium containing ammonium fumarate, sodium fumarate, corn steep liquor, peptone, and inorganic salts. For the enzymatic conversion of L-aspartic acid to L-alanine, the culture broth was employed as the enzyme source. A large amount of L-aspartic acid (as much as 40% of the broth) was converted stoichiometrically to alanine in 72 hr at 37 C. Furthermore, appropriate addition of a surface-active agent to the reaction mixture was found to be highly effective in shortening the time required for the conversion. Accumulated L-alanine was readily isolated in pure form by ordinary procedures with ion-exchange resins. Yields of isolated L-alanine of over 90% from L-aspartic acid were easily attainable.

In this paper, an enzymatic procedure is presented which utilizes L-aspartic β-decarboxylase for the production of L-alanine from L-aspartic acid.

L-Aspartic acid is readily available as a result of a recent development of L-aspartic acid fermentation. Accordingly, this procedure, which enables efficient conversion of L-aspartic acid to L-alanine, is likely to become one of the most advantageous methods for the production of L-alanine.

Since the first report by Mardashev and Gladkova (1948) on L-aspartic β-decarboxylase [L-aspartate 4-carboxy-lyase (E.C.4.1.1.12)], which catalyzes β-decarboxylation of L-aspartic acid in species of *Pseudomycobacterium*, its occurrence and enzymatic properties have been reported in a number of microorganisms such as *Clostridium perfringens* (Meister, Sober, and Tice, 1951; Nishimura, Manning, and Meister, 1962), *Desulfovibrio desulfuricans* (Senez and Cattanéo-Lacombe, 1956; Cattanéo-Lacombe, Senez, and Beaumont, 1958), *Nocardia globerula* (Crawford, 1958), *Pseudomonas reptilivora* (Seaman, 1960), *Acetobacter* sp. (Cooksey and Rainbow, 1962), *Achromobacter* sp. (Wilson, 1963; Wilson and Kornberg, 1963), and *Alcaligenes faecalis* (Novogrodsky, Nishimura, and Meister, 1963; Novogrodsky and Meister, 1964). No report has appeared on the application of this enzyme for the production of L-alanine.

MATERIALS AND METHODS

Organisms. Screening tests were performed on 63 strains of bacteria, 17 strains of fungi, 7 strains of yeasts, and 4 strains of *Streptomyces*, all from the collection in this laboratory.

Screening experiments. Slant cultures of bacteria and *Streptomyces* were grown on a medium containing 0.25% peptone, meat extract, yeast extract, and 0.5% sodium chloride; cultures of fungi and yeasts were grown on malt extract medium. All organisms were grown for 24 hr at their respective optimal temperatures for growth. The screening medium, which contained 2% glucose, 0.5% peptone, 0.5% corn steep liquor, 0.2% yeast extract, 0.05% KH_2PO_4, and 0.01% $MgSO_4 \cdot 7H_2O$, was adjusted to *p*H 7 with NaOH. The medium was distributed in 3-ml amounts to test tubes and sterilized. After inoculation with test organisms from the slant cultures, shaking culture was carried out for 24 hr at 30 C. The cells were collected by centrifugation, washed with 0.9% saline, and mixed with 1 ml of 2% aspartic acid in 0.2 M acetate buffer (*p*H 4.9). The mixture was incubated for 24 hr at 30 C.

Routine identification and rough quantitative estimation of the formed amino acids were made by use of paper chromatography of the reaction

TABLE 1. *Screening of microorganisms for the activity of alanine formation from aspartic acid*

Genus	No. of tested strains	No. of alanine-forming strains	Genus	No. of tested strains	No. of alanine-forming strains
Acetobacter	3	1	*Nocardia*	2	
Achromobacter	13	3	*Streptomyces*	1	
Aerobacter	1		*Candida*	1	
Alcaligenes	6		*Hansenula*	1	
Bacillus	3		*Pichia*	1	
Bacterium	1		*Saccharomyces*	2	
Escherichia	1		*Torula*	1	1
Micrococcus	2		*Torulopsis*	1	1
Proteus	3		*Absidia*	2	1
Pseudomonas	20	8	*Aspergillus*	4	2
Serratia	1		*Chaetomium*	1	
Staphylococcus	1		*Cunninghamella*	1	
Lactobacillus	4		*Mucor*	3	3
Streptococcus	1		*Neurospora*	1	
Leuconostoc	3		*Oospora*	1	1
Mycobacterium	1		*Penicillium*	4	

TABLE 2. *Effect of concentrations of ammonium fumarate on the enzyme formation*

Additions to medium*	*p*H	Growth (optical density)	Enzyme activity	
			CO_2 per hr per ml of medium	CO_2 per hr per mg of protein
			μliters	*μliters*
None	7.6	.070	53	344
Ammonium fumarate				
0.5%	8.8	.218	233	193
1.0%	8.8	.368	783	398
1.5%	8.6	.448	1,201	464
2.0%	8.4	.405	921	412
2.5%	8.2	.105	360	350
5.0%	7.0	.016	30	81
Aspartic acid, 1.5%	8.2	.260	552	351
Ammonium fumarate (1.5%) plus aspartic acid (0.5%)	8.6	.350	840	282

* Besides ammonium fumarate and aspartate, all media contained 0.55% corn steep liquor, 0.05% KH_2PO_4, and 0.01% $MgSO_4 \cdot 7H_2O$.

mixture. The ascending method was used with Toyo filter paper no. 50 and butanol-acetic acid-water (4:1:1).

After development, the chromatograms were sprayed with 0.2% ninhydrin in 80% ethyl alcohol.

Fermentation experiments. Unless otherwise noted, fermentation experiments for the enzyme formation were carried out as follows. The respective media were distributed in 120-ml amounts to 500-ml shaking flasks, sterilized, and inoculated with two loopfuls of the selected organism. The cultures were incubated at 30 C for 20 hr with reciprocal shaking (140 rev/min, 8-cm stroke).

Methods of analysis. The assay of decarboxylase activity was carried out by manometric measurement of CO_2 liberated from L-aspartic acid. The cells harvested by centrifugation from the broth cultured for 20 hr were washed with saline and lyophilized. The lyophilized cells were sonically treated (10 kc, 15 min) and centrifuged. The supernatant solution was used as enzyme solution. Each Warburg vessel contained 2 ml of 10 mM L-aspartic acid (20 μmoles) and 1 ml of 1 M acetate buffer (*p*H 5.5, 1,000 μmoles) in the main compartment and 0.5 ml of the enzyme solution in the side arm. After 10-min equilibration at 30 C, the contents were mixed, and liberated CO_2 was measured for 10 min.

Protein content of the enzyme solution was estimated by use of the method of Warburg and Christian (1942).

Total enzyme activity, i.e., amount of formed

TABLE 3. *Effect of nitrogen sources on enzyme formation*

Medium*	pH	Growth (optical density)	Enzyme activity	
			CO_2 per hr per ml of medium	CO_2 per hr per mg of protein
			μliters	*μliters*
Sodium fumarate (1.5%) plus				
$(NH_2)_2CO$†	9.0	.390	492	327
NH_4Cl	8.4	.298	1,026	522
$(NH_4)_2SO_4$	8.4	.254	768	427
$(NH_4)_2HPO_4$	8.4	.350	688	340
NH_4NO_3	8.4	.362	747	336
Ammonium fumarate (1.5%)	8.6	.448	1,201	464

* Besides the above constituents, all media contained 0.55% corn steep liquor, 0.05% KH_2PO_4, and 0.01% $MgSO_4 \cdot 7H_2O$.

† Concentrations of the respective nitrogen sources were equalized to 0.5% NH_4Cl on a nitrogen basis.

TABLE 4. *Effect of the ratio of fumarate and ammonium ion on enzyme formation**

Medium				pH	Growth (optical density)	Enzyme activity	
Ammonium fumarate	Sodium fumarate	NH_4Cl	Fumarate/NH_4			CO_2 per hr per ml of medium	CO_2 per hr per mg of protein
%	%	%				*μliters*	*μliters*
1.5	0	0	3/1	8.6	.610	1,218	333
0.75	0.75	0	6/1	8.8	.610	2,110	503
0.5	1.0	0	9/1	8.6	.600	2,845	741
0.4	1.1	0	12/1	8.6	.580	1,922	473
0.3	1.2	0	15/1	8.8	.590	1,520	353
0.2	1.3	0	18/1	8.8	.600	1,285	338
0	1.5	0.36	9/1	8.6	.500	1,303	417
0	1.5	0.28	12/1	8.6	.560	1,333	381
0	1.5	0.21	15/1	8.6	.570	1,097	305
0	1.5	0.14	18/1	8.8	.620	1,068	262

* Footnotes are the same as in Table 3.

TABLE 5. *Effect of organic nutrients on enzyme formation*

Additions to medium*	pH	Growth (optical density)	Enzyme activity	
			CO_2 per hr per ml of medium	CO_2 per hr per mg of protein
			μliters	*μliters*
Organic nutrients†				
Corn steep liquor, 2.2%	8.4	.450	1,920	534
Yeast extract, 0.9%	8.2	.500	1,854	539
Meat extract, 7.1%	8.2	.310	924	420
Peptone, 0.9%	8.8	.490	2,381	665
Yeast extract, 0.45%				
Peptone, 0.45%	8.8	.510	1,645	485

* Besides the above constituents, all media contained 0.5% ammonium fumarate, 1.0% sodium fumarate, 0.05% KH_2PO_4, and 0.01% $MgSO_4 \cdot 7H_2O$.

† Concentrations of the respective organic nutrients were equalized to 0.1% nitrogen.

enzyme, was expressed in terms of microliters of liberated CO_2 per hour per extract of the cells grown in a unit milliliter of medium, and specific activity in microliters per hour per milligram of protein. No activity of aspartic β-decarboxylase was found in the filtered broth of *P. dacunhae*.

The assay of L-alanine and L-aspartic acid was carried out by ordinary microbioassay with *Leuconostoc citrovorum* 8081 and *L. mesenteroides* P-60, respectively.

For the estimation of growth of organisms, the culture broth was diluted with saline to 10 times the original volume, and optical density was measured at 660 mμ with a Hitachi photoelectric photometer (EPO-B type).

RESULTS AND DISCUSSION

Screening experiments of organisms. Alanine formation by a considerable number of microorganisms was observed (Table 1), and the highest accumulation was found among strains of *Achromobacter* and *Pseudomonas*. Of the tested organisms, *P. dacunhae* showed the highest activity and was employed for the following experiments.

Cultural conditions for formation of aspartic β-decarboxylase. To establish the most advantageous cultural conditions for the formation of aspartic β-decarboxylase, various conditions were investigated with *P. dacunhae*.

Effect of carbon sources. During preliminary experiments, it was found that the organism could not utilize ordinary sugars for growth. Therefore, utilization of various organic acids and amino acids was examined. Among the tested carbon sources, organic acids, such as fumaric acid and succinic acid, were found to be favorable for growth and enzyme formation. Thus, readily available fumarate was used as a main carbon source for subsequent experiments.

TABLE 6. *Effect of concentrations of peptone and corn steep liquor on enzyme formation**

Medium		pH	Growth (optical density)	Enzyme activity	
Corn steep liquor	Peptone			CO_2 per hr per ml of medium	CO_2 per hr per mg of protein
%	%			μliters	μliters
0.55	0	8.8	.345	1,218	508
0.55	0.45	9.0	.390	1,825	571
0.55	0.9	8.8	.430	2,114	614
0.55	1.8	8.6	.500	3,023	719
0.55	2.7	8.4	.500	2,269	567
1.1	0	8.8	.380	1,566	490
1.1	0.45	8.8	.415	2,376	699
1.1	0.9	8.8	.440	2,512	698
1.1	1.8	8.6	.520	3,260	858
2.2	0	8.8	.420	1,920	534
2.2	0.45	8.8	.470	2,251	593
2.2	0.9	8.8	.470	2,320	550
2.2	1.8	8.6	.460	1,918	507

* Footnotes are the same as in Table 5.

TABLE 7. *Effect of shaking on enzyme formation*

Amt of medium*	Oxygen absorption rate (O_2 per liter per min)	pH	Growth (optical density)	Enzyme activity	
				CO_2 per hr per ml of medium	CO_2 per hr per mg of protein
ml	mmoles			μliters	μliters
50	1.55	9.0	.650	1,534	384
100	1.00	9.0	.630	2,706	677
120	0.82	8.6	.550	2,999	769
150	0.77	8.6	.520	2,206	580
250	0.48	8.4	.370	1,020	340
350	0.30	8.0	.100	108	54

* The medium contained 0.5% ammonium fumarate, 1.0% sodium fumarate, 0.55% corn steep liquor, 1.8% peptone, 0.05% KH_2PO_4, and 0.01% $MgSO_4 \cdot 7H_2O$.

To investigate the most effective fumarate concentration, the experiments were carried out in media containing varied amounts of ammonium fumarate. The highest total and specific activities were obtained at 1.5% concentration (Table 2).

The effect of aspartic acid was also investigated, but the use of this amino acid as the carbon source was not as advantageous as ammonium fumarate. Also, the addition of aspartic acid to the medium containing ammonium fumarate showed no acceleration of enzyme formation.

Effect of nitrogen sources. It is possible that the formation of the enzyme was influenced by the kind of nitrogen sources employed. To choose the most favorable source, media which contained ammonium chloride, ammonium sulfate, ammonium nitrate, secondary ammonium phosphate, ammonium fumarate, and urea were compared at a concentration equalized to 0.5% ammonium chloride on a nitrogen basis (Table 3). Ammonium chloride and ammonium fumarate gave favorable results for the enzyme formation.

Effect of ratio of fumarate and ammonium ion. The above experiments revealed the advantage of fumarate as the carbon source, and ammonium chloride or ammonium fumarate as the nitrogen source. The ratio of fumarate and ammonium ion was examined. A fumarate-ammonium ion ratio of 1:9, i.e., 0.5% ammonium fumarate and 1.0% sodium fumarate, was found to be most favorable (Table 4). The advantage of ammonium

fumarate over ammonium chloride was also confirmed by this experiment.

Effect of organic nutrients. To investigate the most effective organic nutrient, experiments were carried out on media containing corn steep liquor, yeast extract, meat extract, or peptone, each at a concentration of 0.1% nitrogen (Table 5). Peptone gave the most favorable result, followed by corn steep liquor.

The effect of the concentration of these nutrients was examined and reported in Table 6. Maximal enzyme formation was observed in a mixture of 1.8% peptone and 0.55% or 1.1% corn steep liquor, and there was no marked difference between them.

Effects of shaking and temperature. In preliminary experiments, shaking cultures gave much better results than stationary cultures. The effect of shaking was studied by varying the amount of media in the flasks. Maximal enzyme formation was attained in the case of 120 ml of medium in a 500-ml flask, at an oxygen absorption rate of 0.82 mmole per liter per min (Table 7).

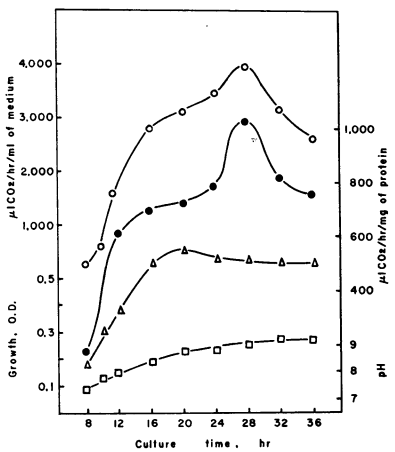

FIG. 1. *Changes during fermentation for enzyme formation. Symbols*: ○, *microliters of* CO_2 *per hour per milliliter of medium*; ●, *microliters of* CO_2 *per hour per milligram of protein*; △, *growth (optical density)*; □, *pH.*

The effect of temperature was also studied at 25, 30, and 37 C. The optimal temperature for enzyme formation was 30 C. Neither growth nor enzyme formation occurred at 37 C. Although growth occurred at 25 C, the enzyme activity was inferior to that observed at 30 C.

Typical changes during fermentation. The data for a typical fermentation for enzyme formation under optimal conditions are given in Fig. 1. A 500-ml flask containing 120 ml of the medium composed of 0.5% ammonium fumarate, 1.0% sodium fumarate, 0.55% corn steep liquor, 1.8% peptone, 0.05% KH_2PO_4, and 0.01% $MgSO_4 \cdot 7H_2O$ (*p*H 7.0) was inoculated with two loopfuls of the organism, and shaking culture was carried out at 30 C.

Maximal enzyme formation and highest specific activity were attained at 26 to 28 hr, after growth of the organism entered into stationary phase. Therefore, addition of aspartate at this period is considered to be advantageous for conversion to alanine.

Throughout the culture period, the enzyme activity was found in the cells and not in the filtered broth, indicating that the enzyme is intercellular.

Maximal activity, Q_{CO_2} = 1,028 μliters of CO_2 per hr per mg of protein and 3,910 μliters of CO_2 per hr per ml of medium, observed in this culture of *P. dacunhae* is much higher than that

TABLE 8. *Effect of temperature and agitation on alanine formation*

L-Aspartic acid	Temp	Agitation	L-Alanine					
			24 hr		48 hr		72 hr	
			Amt	Per cent*	Amt	Per cent*	Amt	Per cent*
%	C	*rev/min*	*g*		*g*		*g*	
30	30	0	4.6	23.0	9.0	45.0	12.0	60.0
		80	4.4	22.0	11.8	59.0	15.0	75.0
	37	0	8.9	44.5	16.1	80.5	20.8	104.0
		80	10.5	52.5	13.7	68.5	14.5	72.5
40	30	0	7.2	26.9	11.8	44.0	14.2	53.0
		80	8.1	30.2	11.6	43.3	14.5	54.1
	37	0	9.6	35.8	21.2	79.1	26.8	100.0
		80	12.2	45.6	18.4	68.7	21.7	81.0

* Figures represent conversion ratio from aspartate.

TABLE 9. *Effect of surfactants on alanine formation**

Main chemical component	Surfactant†	Conversion rate with surfactant at concn of	
		0.01%	0.5%
		%	%
None		39.9	43.5
POE‡ sorbitan monoalkylate	TL-10	59.4	100.0
	TP-10	46.4	76.1
	TS-10	45.7	76.8
	TO-10	52.9	86.2
POE sorbitol alkylate	GL-1	58.0	85.1
	GS-6	41.6	51.6
	GO-4	42.7	56.6
POE alkylate	MYS-25	46.0	76.8
	MYS-40	59.4	74.6
	MYS-45	43.5	70.7
	MYS-55	47.5	73.6
	MYL-10	60.5	55.8
	MYD-10	45.9	66.7
POE lanolin derivative	TW-10	46.4	76.8
	TW-20	45.0	56.9
	TW-30	40.9	53.6
POE castor oil derivative	CO-60TX	47.5	72.5
	HCO-40	38.8	65.2
	HCO-50	39.9	58.3
	HCO-80	37.7	55.8
	HCO-100	36.2	59.4
Sorbitan alkylate	SL-10	54.5	100.0
	SP-10	42.7	59.1
	SS-10	38.4	56.3
	SO-10	50.9	66.7
Glyceryl monoalkylate	MGS-A(SE)	42.3	51.6
	MGS-B	35.8	53.4
	MGS-D	46.6	54.8
	MGO	50.9	71.3
Propylene glycol monoalkylate	PMS-1C	39.7	51.6
POE sorbitol bee's-wax derivative	GBW-26	46.0	53.4

* Enzymatic reaction was carried out by incubating a mixture containing 2 g of L-aspartic acid, 5 ml of culture broth, and respective amounts of various surfactants for 24 hr at 37 C.

† All surfactants are designated commercially as "Nikkol" plus their respective code letters.

‡ POE = polyoxyethylene.

of *P. reptilivora* reported previously (Seaman, 1960).

Effects of temperature and agitation on enzyme formation of L-alanine. The effects of temperature and agitation on the enzymatic conversion of aspartic acid to alanine were investigated. A large quantity of aspartic acid (i.e., 30 or 40% of the broth) was added in crystalline form to the broth, and the suspension was incubated at 30 or 37 C with or without agitation. Aspartic acid at 40% concentration was converted to L-alanine by incubating without agitation for 72 hr at 37 C (Table 8). Although the enzymatic reaction appeared to be somewhat accelerated during the early stage of agitated incubation, the conversion slowed down, and no advantage over stationary incubation was found.

Acceleration by surfactants. Four anionic surfactants, five cationic surfactants, six ampholytic surfactants, and 61 nonionic surfactants were added to the reaction mixture at 0.01 and 0.5% concentrations. After 24 hr of incubation at 37 C, rates of conversion to alanine were compared. Effective agents were of the nonionic type, especially derivatives of aliphatic acid esters (Table 9). Among the nonionic agents, ether derivatives such as polyoxyethylene alkylethers and polyoxypropylene polyoxyethylene cetylethers showed the tendency to retard the reaction. Almost all anionic, cationic, and ampholytic surfactants showed an inhibitory effect. Cationic surfactants such as cetyl pyridinium chloride, cetyl trimethyl ammonium bromide, and stearyl trimethyl ammonium chloride completely inhibited

TABLE 10. *Effect of concentrations of the surfactant on alanine formation**

Polyoxyethylene sorbitan monolaurate concn	Conversion rate	
	20 hr	44 hr
%	%	%
0	33.6	60.1
0.005	45.5	69.0
0.01	52.2	76.5
0.05	91.0	99.3
0.1	96.3	100.0
0.5	100.7	
1.0	95.5	100.7

* Enzymatic reaction was carried out by incubating a mixture containing 2 g of L-aspartic acid, 5 ml of culture broth, and polyoxyethylene sorbitan monolaurate at various concentrations at 37 C.

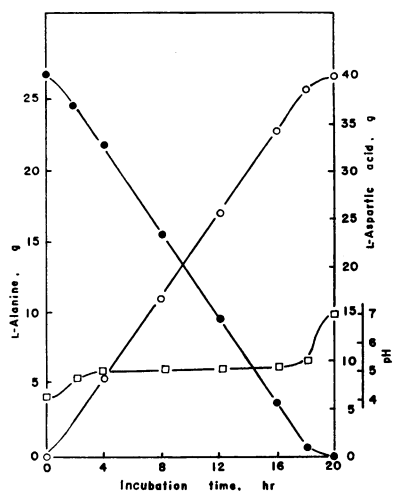

FIG. 2. *Changes during the enzymatic reaction. Symbols*: ○, L-*alanine*; ●, L-*aspartic acid*; □, *pH*.

the reaction at 0.01% concentration. The effect of the most favorable surfactant, polyoxyethylene sorbitan monolaurate (TL-10), was examined at different concentrations. The addition of 0.5% was most effective (Table 10). L-Aspartic acid at 40% concentration was completely converted to alanine within 20 hr. The period of enzymatic reaction was one-third that without its addition.

Alanine formation under optimal conditions. A typical alanine formation is illustrated in Fig. 2. An experiment was conducted by incubating a mixture of 100 ml of the broth cultured under optimal conditions as enzyme source, 40 g of L-aspartic acid, and 0.5 g of polyoxyethylene sorbitan monolaurate at 37 C. Alanine increased linearly with the consumption of aspartic acid. During the reaction, the *p*H was maintained at around 5, which is optimal for the enzymatic reaction. When aspartic acid was consumed at the end of the reaction, the *p*H of the mixture rapidly rose to 7. In this enzymatic procedure, neither formation of amino acids other than alanine nor decomposition and racemization of accumulated L-alanine occurred during prolonged incubation.

Thus, the formed L-alanine is easily isolated by an ordinary procedure. In this step, the reaction mixture was adjusted to *p*H 4.0, heated, and centrifuged to remove the cells. The supernatant solution was passed through a column packed with Amberlite IR-120 (H^+ form). The column was washed with water and eluted with 5% aqueous ammonia. The eluant was concentrated in vacuo, and methanol was added. The separated crude crystals were collected by filtration, and were recrystallized from aqueous methanol to yield colorless needles of L-alanine: $[\alpha]_D^{25} = +14.3°$ (c = 4 in 6 N HCl). Analysis: $C_3H_7O_2N$; calculated: C, 40.44; H, 7.92; N, 15.72; found: C, 40.57; H, 7.59; N, 15.63. Yields of L-alanine of over 90% from aspartic acid were obtained. No amino acid other than L-alanine was detected in the product obtained by paper chromatography. The product showed no O_2 uptake by the action of D-amino acid oxidase (Chibata, Tosa, and Sano, 1964).

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