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Production of L-Proline by *Kurthia catenaforma*

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A number of organisms were screened for their ability to produce L-proline. *Kurthia catenaforma*, which we recently isolated, was selected. A serine-requiring mutant, strain 45, produced about 1.5 times the amount of this amino acid that the parent strain did. In investigations of various media, it was found that approximately 30 ml of L-proline per ml was produced in shaken culture at 30 C in a medium containing glucose, urea, corn steep liquor, casein hydrolysate, L-aspartic acid, and inorganic salts. To study the effect of L-aspartic acid on the production of L-proline, various amino or organic acids were substituted for L-aspartic acid, and the changes during fermentation were investigated. L-Aspartic acid was not replaced by the compounds tested, and this acid appeared to increase growth during the later stages of fermentation with a concurrent increase in the production of L-proline.

The production of L-proline has, in the past, been carried out by the extraction of protein hydrolysates. A direct fermentation, however, was recently reported by Suzuki et al. (3), Yamatodani et al. (4), and Yoshinaga et al. (5, 6).

During the course of the present investigation on the ability of microorganisms to produce various amino acids in medium containing L-aspartic or L-glutamic acid, a bacterium isolated by the authors and designated as *Kurthia catenaforma* by H. Iizuka was selected for the production of L-proline. Using a mutant of this organism, we investigated fermentation conditions and found that yields of L-proline were substantially increased. The present paper describes these investigations and comments on the probable role of L-aspartic acid in this fermentation.

MATERIALS AND METHODS

Organisms. Screening tests were performed with several hundred strains of bacteria, fungi, yeasts, and actinomycetes, all from the collection of this laboratory. A serine-requiring mutant, no. 45, which was derived from the bacterium *K. catenaforma* ATCC 21144, was selected for the fermentation experiments.

Screening experiments. The three screening media shown in Table 1 were distributed in 2-ml amounts to test tubes and sterilized. After inoculation, cultures were shaken for 72 hr at 30 C.

Routine identification and rough quantitative estimation of the amino acids present in the fermentation broth were made by paper chromatography.

Fermentation experiments. Unless otherwise noted, fermentations for the production of L-proline were carried out as follows. Media were distributed in 30-ml amounts to 500-ml flasks, sterilized, and inoculated with one loopful of *K. catenaforma* no. 45. Cultures were incubated at 30 C for 72 hr with reciprocal shaking (140 rev/min, 8-cm stroke). Since there was no marked difference between the yields obtained at 48 hr and at 72 hr, only the 48-hr results are reported in this paper. Each yield given is the average of three fermentation flasks.

Methods of analysis. The assay of L-proline and L-aspartic acid was carried out by microbioassay with *Leuconostoc mesenteroides* p-60. L-Glutamic acid was determined by glutamic decarboxylase (1). Glucose was estimated by use of the method of Somogyi-Nelson (2). For the estimation of growth, the fermentation broth was diluted 1:10 with saline, and optical density was measured at 660 mμ with a Hitachi photoelectric photometer (EPO-B type).

RESULTS AND DISCUSSION

Screening experiments of organisms. Several hundred strains of bacteria, fungi, yeasts, and actinomycetes were screened for their ability to produce amino acids. A bacterium recently isolated by the authors was selected as the organism producing the largest amount of L-proline in the two screening media containing L-aspartic acid. This strain was classified and designated *K. catenaforma* by H. Iizuka of Tokyo University (H. Iizuka and Furuya H., Abstr. Ann. Meeting Japan. Agr. Chem. Soc., p. 106, 1968). Because the proline-producing ability of this organism varied, a number of auxotrophic mutants were obtained by ultraviolet irradiation and by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. These mutants were tested for stability and pro-

TABLE 1. *Composition of screening media for the production of L-proline*[a]

Component	I	II	III
	%	%	%
Glucose	6.0	6.0	6.0
Urea	0.5	0.5	0.5
NH_4Cl	0.3	0.3	0.3
Corn steep liquor	0.7	0.7	0.7
Casein hydrolysate	1.1	1.1	1.1
L-Aspartic acid	2.0	2.0	0
L-Glutamic acid	0	0	2.0
K_2HPO_4	0.1	2.0	0.1
$MgSO_4 \cdot 7H_2O$	0.05	0.05	0.05
$CaCO_3$	1.0	1.0	1.0

[a] The initial pH of all media was adjusted to 7.0 with NaOH.

TABLE 2. *Effect of carbon source upon the production of L-proline*

Carbon source[a] (6%)	pH	Growth[b]	L-Proline	L-Aspartic acid
			mg/ml	*mg/ml*
Dextrin	7.8	.500	19	0
Maltose	8.6	.610	28	0
Sucrose	8.4	.520	12	0.5
Lactose	8.4	.380	0.5	15
Galactose	8.0	.465	8	1
Glucose	8.8	.610	28	0
Fructose	7.8	.625	16	0
Mannose	6.8	.060	0.5	20
Sorbose	6.8	.060	0.5	20
Xylose	8.6	.240	0	15
Arabinose	6.6	.055	0.5	20
Ribose	6.6	.415	6	4

[a] In addition to the above carbon source, all media contained 0.5% urea, 0.3% NH_4Cl, 0.7% corn steep liquor, 1.1% casein hydrolysate, 2% L-aspartic acid, 2% K_2HPO_4, and 0.1% $MgSO_4 \cdot 7H_2O$.

[b] Optical density.

duction. A serine-requiring mutant, strain 45, produced about 1.5 times the amount of L-proline that the parent strain did. This mutant was, therefore, used for fermentation experiments.

Effect of carbon source on fermentation for the production of L-proline. Twelve sugars were investigated at the 6% level to determine the most favorable carbon source for the production of L-proline. Maltose and glucose gave the best results (28 mg/ml), followed by dextrin and fructose (Table 2). Maximal yields were obtained at glucose concentrations of 6 to 10%, with reduced yields at 4% (Table 3).

Effect of inorganic nitrogen source on proline production. Various nitrogen sources were tested for the production of L-proline, at a concentration equal to 0.5% urea on a nitrogen basis. Urea, ammonium chloride, and ammonium nitrate gave the highest yields, 22 to 25 mg/ml (Table 4). The addition of both urea and ammonium chloride resulted in slightly better yields than the addition of either compound alone. The highest yield (28 mg/ml) was obtained in medium containing 0.5% urea and 0.3% ammonium chloride (Table 5).

TABLE 4. *Effect of nitrogen source upon the production of L-proline*

Nitrogen source[a]	pH	Growth[b]	L-Proline	L-Aspartic acid
			mg/ml	*mg/ml*
$(NH_2)_2CO$, 0.5%	8.6	.520	25	0.5
NH_4Cl, 0.89%	6.4	.635	23	0.5
$(NH_4)_2SO$, 1.1%	5.6	.535	16	0.5
$(NH_4)_2HPO_4$, 1.1%	6.2	.605	17	0
NH_4NO_3, 0.67%	6.4	.580	22	0
$NaNO_3$, 1.4%	7.2	.590	11	0

[a] In addition to the above nitrogen source, all media contained 6% glucose, 0.7% corn steep liquor, 1.1% casein hydrolysate, 2% L-aspartic acid, 2% K_2HPO_4, and 0.1% $MgSO_4 \cdot 7H_2O$.

[b] Optical density.

TABLE 3. *Effect of concentration of glucose upon the production of L-proline*

Glucose[a]	pH		Growth[b]		L-Proline (mg/ml)		L-Aspartic acid (mg/ml)		Glucose (mg/ml)	
	48 hr	72 hr	48 hr	72 hr	48 hr	72 hr	48 hr	72 hr	48 hr	72 hr
%										
0	8.4	9.0	.310	.250	0	0	8	6	0	0
4	8.6	8.8	.530	.535	19	20	1	1	0	0
6	8.0	8.8	.600	.600	30	29	0	0	0	0
8	6.6	7.2	.630	.620	31	30	0	0	0	0
10	6.2	5.8	.640	.630	31	30	0	0	27	0

[a] In addition to glucose, all media contained 0.5% urea, 0.3% NH_4Cl, 0.7% corn steep liquor, 1.1% casein hydrolysate, 2% L-aspartic acid, 2% K_2HPO_4, and 0.1% $MgSO_4 \cdot 7H_2O$

[b] Optical density.

TABLE 5. *Effect of concentration of nitrogen source upon production of L-proline*[a]

Urea	NH_4Cl	*p*H	Growth[b]	L-Proline	L-Aspartic acid
%	%			*mg/ml*	*mg/ml*
0	0	6.4	.530	12	0
	0.3	6.6	.600	15	0
	0.9	6.4	.630	22	1
	1.8	6.0	.670	17	1
0.1	0	6.8	.580	15	0
	0.3	7.0	.610	22	0
	0.9	7.4	.650	25	0
	1.8	5.8	.560	15	0
0.5	0	8.6	.540	24	0
	0.3	8.8	.600	28	0
	0.9	8.8	.620	25	0
	1.8	5.8	.580	15	0
2.0	0	5.8	.430	13	0
	0.3	5.8	.540	16	0
	0.9	5.6	.525	15	0
	1.8	5.6	.435	13	0

[a] In addition to urea and NH_4Cl, all media contained 6% glucose, 0.7% corn steep liquor, 1.1% casein hydrolysate, 2% L-aspartic acid, 2% K_2HPO_4, and 0.1% $MgSO_4 \cdot 7H_2O$.
[b] Optical density.

TABLE 6. *Effect of organic nutrient upon the production of L-proline*

Organic nutrient[a]	*p*H	Growth[b]	L-Proline	L-Aspartic acid
			mg/ml	*mg/ml*
Corn steep liquor, 2.2%	7.4	.490	26	0
Rice liquor, 2.2%	8.6	.160	6	10
Yeast extract, 0.9%	7.0	.085	0.5	20
Meat extract, 0.9%	6.8	.030	0	20
Malt extract, 0.9%	6.8	.015	0	20
Peptone, 0.9%	7.2	.040	0	20
Casein hydrolysate, 1.1%	6.0	.280	7	5

[a] In addition to the above organic nutrient, all media contained 6% glucose, 0.5% urea, 0.3% NH_4Cl, 2% L-aspartic acid, 2% K_2HPO_4, and 0.1% $MgSO_4 \cdot 7H_2O$.
[b] Optical density.

Although a high concentration of ammonium salts has been reported to be optimal for the production of L-proline (4, 5), our fermentation yields decreased when the concentration of ammonium chloride was raised to 1.8%.

Effect of organic nutrients on proline production. Experiments were carried out in medium containing various organic nutrients, each at a concentration of 0.1% nitrogen. The physiological activity of this strain is considerably inferior to that of the other bacteria, and abundant growth and production were obtained only with corn steep liquor (Table 6). The effect of supplemental organic nutrients upon the production of L-proline was investigated in medium containing 0.7% corn steep liquor. Supplements of peptone and casein hydrolysate gave results comparable to those obtained with 2.1% corn steep liquor. Various combinations of corn steep liquor, peptone, and casein hydrolysate were, therefore, tested; there were no marked differences among these mixtures (Table 7).

Effect of L-aspartic acid on proline production. During screening experiments, L-aspartic acid was found to play an important role in fermentation for the production of L-proline. Therefore, the effect of the concentration of this acid was studied. In the absence of L-aspartic acid or in the presence of low concentrations (<1.0%), growth was poor and the production of L-proline was low

TABLE 7. *Effect of concentration of organic nutrient upon the production of L-proline*

Addition to medium[a]	*p*H	Growth[b]	L-Proline	L-Aspartic acid
			mg/ml	*mg/ml*
Corn steep liquor				
0.7%	7.2	.270	12	0.5
1.4%	7.0	.345	17	0.5
2.1%	6.8	.470	24	0
2.8%	8.4	.590	26	2
3.5%	8.4	.620	23	0.5
Corn steep liquor (0.7%) plus peptone				
0.3%	7.2	.260	14	0
0.6%	7.4	.465	24	0
0.9%	8.4	.490	23	0
Corn steep liquor (0.7%) plus casein hydrolysate				
0.3%	6.2	.380	21	0
0.7%	8.6	.480	25	0
1.1%	8.6	.600	28	0
Corn steep liquor (1.4%) plus casein hydrolyzate				
0.3%	8.4	.440	25	0.5
0.7%	8.6	.540	24	0
1.1%	8.4	.615	25	0.5

[a] In addition to the above organic nutrient, all media contained 6% glucose, 0.5% urea, 0.3% NH_4Cl, 2% L-aspartic acid, 2% K_2HPO_4, and 0.1% $MgSO_4 \cdot 7H_2O$.
[b] Optical density.

TABLE 8. *Effect of concentration of L-aspartic acid upon the production of L-proline*

L-Aspartic acid[a]	pH	Growth[b]	L-Proline	L-Aspartic acid
			mg/ml	*mg/ml*
0	5.8	.260	6	0
0.1	6.0	.320	8	0
0.5	6.0	.440	14	0
1.0	7.6	.500	23	0
1.5	8.6	.520	28	0
2.0	8.8	.550	29	0
2.5	8.8	.600	28	0.5
3.0	8.6	.610	27	1

[a] In addition to L-aspartic acid, all media contained 6% glucose, 0.5% urea, 0.3% NH_4Cl, 0.7% corn steep liquor, 1.1% casein hydrolysate, 2% K_2HPO_4, and 0.1% $MgSO_4 \cdot 7H_2O$.

[b] Optical density.

TABLE 9. *Effect of concentration of dipotassium phosphate upon the production of L-proline*

K_2HPO_4[a]	pH	Growth[b]	L-Proline	L-Aspartic acid
%			*mg/ml*	*mg/ml*
0	5.4	.095	0.5	20
0.1	5.6	.100	0.5	20
0.5	6.0	.540	9	0
1.0	7.8	.640	24	0
1.5	8.8	.585	28	0
2.0	8.6	.580	28	0
2.5	8.4	.595	25	0
3.0	7.4	.570	22	0

[a] In addition to K_2HPO_4, all media contained 6% glucose, 0.5% urea, 0.3% NH_4Cl, 0.7% corn steep liquor, 1.1% casein hydrolysate, 2% L-aspartic acid, and 0.1% $MgSO_4 \cdot 7H_2O$.

[b] Optical density.

(<15 mg/ml). The addition of higher concentrations of L-aspartic acid (1.5 to 3%) significantly increased yields to approximately 30 mg/ml.

Effect of potassium ion on proline production. Dipotassium phosphate was found to exert a considerable effect upon the production of L-proline. The optimal concentration was unusually high (1.5 to 2.0%), and the level generally employed in other fermentations (0.1%) was unfavorable for growth and production in this fermentation (Table 9).

To determine whether this effect is due to potassium or phosphate ion, experiments were carried out with medium containing various potassium salts, each at a concentration of potassium ion equal to the amount present in 2.0% dipotassium phosphate. Since 26 to 28 mg of L-proline per ml was produced with all of these salts, irrespective of the anion, the effect of dipotassium phosphate was attributed to the potassium ion (Table 10).

TABLE 10. *Effect of potassium salt upon the production of L-proline*

Potassium salt[a]	pH	Growth[b]	L-Proline	L-Aspartic acid
			mg/ml	*mg/ml*
None	6.0	.520	6	0
K_2HPO_4, 2.00%	8.6	.565	28	0
K_2SO_4, 2.00%	8.4	.640	26	0
KNO_3, 2.32%	8.6	.660	27	0
KCl, 1.71%	8.4	.655	27	0.5
KBr, 2.74%	8.6	.640	26	0
KOH, 1.29%	8.6	.625	26	0.5

[a] In addition to the above potassium salt, all media contained 6% glucose, 0.5% urea, 0.37% $(NH_4)_2HPO_4$, 0.7% corn steep liquor, 1.1% casein hydrolysate, 2% L-aspartic acid, and 0.1% $MgSO_4 \cdot 7H_2O$.

[b] Optical density.

TABLE 11. *Effect of alkali metal ion upon the production of L-proline*

Alkali metal ion[a]	pH	Growth[b]	L-Proline	L-Aspartic acid
			mg/ml	*mg/ml*
$Li_2SO_4 \cdot H_2O$, 1.47%	5.6	.520	4	0.5
Na_2SO_4, 1.63%	6.0	.590	5	0
K_2SO_4, 2.00%	8.8	.640	26	0
Rb_2SO_4, 3.07%	6.4	.270	1	15
Cs_2SO_4, 4.16%	6.6	.040	0	20

[a] In addition to the above alkali metal salt, all media contained 6% glucose, 0.5% urea, 0.37% $(NH_4)_2HPO_4$, 0.7% corn steep liquor, 1.1% casein hydrolysate, 2% L-aspartic acid, and 0.1% $MgSO_4 \cdot 7H_2O$.

[b] Optical density.

To confirm the specificity of the potassium ion, several alkali metal ions were tested at a concentration equivalent to 2.0% potassium sulfate on an alkali metal ion basis. Of the salts tested, only potassium sulfate was suitable for the production of L-proline. Although lithium sulfate and sodium sulfate supported abundant growth, these salts gave inferior yields (Table 11).

Effect of aeration on proline production. The effect of aeration was studied by varying the amount of medium in the flasks. Maximal production of L-proline was obtained with 50 ml or less in a 500-ml flask, which is equivalent to an oxygen absorption rate greater than 1.55 mmole per min (Table 12).

Effect of temperature and initial pH on proline

TABLE 12. *Effect of aeration upon the production of* L-*proline*[a]

Volume of medium per 500-ml flask	OAR[b]	*p*H	Growth[c]	L-Proline	L-Aspartic acid
ml	*mmoles*			*mg/ml*	*mg/ml*
15	4.05	8.8	.635	29	0.5
20	3.10	8.8	.600	28	0.5
30	2.00	8.6	.640	27	0.5
40	1.70	8.4	.640	27	0.5
50	1.55	8.2	.620	27	0.5
80	1.20	6.4	.540	13	0.5

[a] The medium contained 6% glucose, 0.5% urea, 0.3% NH_4Cl, 0.7% corn steep liquor, 1.1% casein hydrolyzate, 2% L-aspartic acid, 2% K_2HPO_4, and 0.1% $MgSO_4 \cdot 7H_4O$.

[b] Oxygen absorption rate (millimoles of O_2 per liter per minute).

[c] Optical density.

TABLE 13. *Effect of temperature upon the production of* L-*proline*[a]

Temp	*p*H		Growth (optical density)		L-Proline (mg/ml)		L-Aspartic acid (mg/ml)	
	48 hr	72 hr	48 hr	72 hr	48 hr	72 hr	48 hr	72 hr
C								
25	7.2	9.0	.610	.655	18	22	0	0
28	8.6	9.0	.640	.600	27	27	0.5	0.5
30	8.9	9.0	.580	.500	27	27	0.5	0
32	8.9	9.0	.560	.495	28	27	0.5	0
34	6.0	6.0	.335	.320	12	13	2	0.5

[a] The medium contained 6% glucose, 0.5% urea, 0.3% NH_4Cl, 0.7% corn steep liquor, 1.1% casein hydrolysate, 2% L-aspartic acid, 2% K_2HPO_4, and 0.1% $MgSO_4 \cdot 7H_2O$.

production. The optimal temperature for the production of L-proline was 28 to 32 C. Although abundant growth occurred at 25 C, yields were inferior to those obtained at 28 to 32 C. At 34 C, growth and production decreased markedly (Table 13).

The effect of initial *p*H was also studied, and the optimal initial *p*H for the production of L-proline was between 6.0 and 8.5 (Table 14).

Changes occurring during fermentation. An example of the chemical changes occurring during the fermentation of L-proline under optimal conditions is given in Fig. 1.

After an initial lag period of approximately 8 hr, vigorous growth of the organism occurred, accompanied by rapid consumption of glucose and L-aspartic acid. Production of L-proline paralleled growth and reached a maximum of approximately 30 mg/ml at 40 hr. Glucose and L-aspartic acid were exhausted at approximately 30 hr. During fermentation, a small amount of L-glutamic acid was temporarily formed in the medium. The *p*H of the medium was maintained on the acidic side as long as glucose remained in

TABLE 14. *Effect of initial pH upon the production of* L-*proline*[a]

Initial *p*H	*p*H		Growth (optical density)		L-Proline (mg/ml)		L-Aspartic acid (mg/ml)	
	48 hr	72 hr	48 hr	72 hr	48 hr	72 hr	48 hr	72 hr
5.5	6.8	6.8	.340	.440	12	16	3	0
6.0	7.0	9.0	.565	.635	26	28	0	0
6.5	8.0	9.0	.600	.560	28	27	0	0
7.0	8.6	9.0	.600	.600	28	27	0.5	0
7.5	8.4	9.0	.595	.600	28	27	0.5	0.5
8.0	8.8	9.0	.605	.600	28	29	1	0.5
8.5	8.6	9.0	.600	.565	27	27	0.5	0.5
9.0	8.4	7.0	.050	.500	0	13	20	1

[a] The medium contained 6% glucose, 0.5% urea, 0.3% NH_4Cl, 0.7% corn steep liquor, 1.1% casein hydrolysate, 2% L-aspartic acid, 2% K_2HPO_4, and 0.1% $MgSO_4 \cdot 7H_2O$.

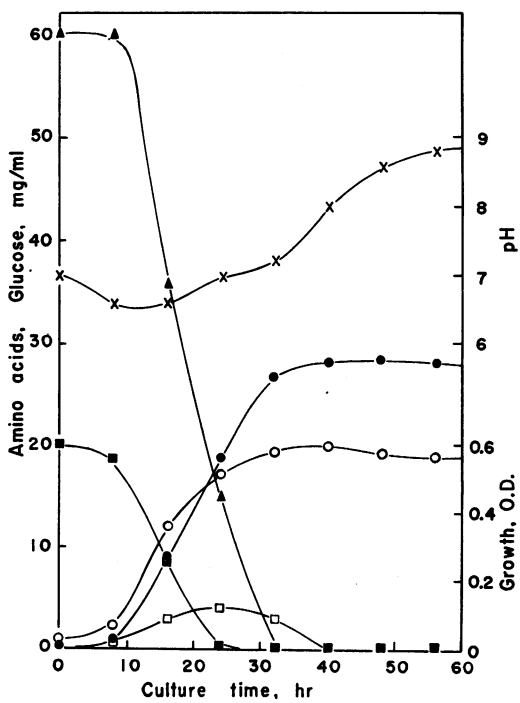

FIG. 1. *Changes during the fermentation of* L-*proline. The medium contained 6% glucose, 0.5% urea, 0.3% NH_4Cl, 0.7% corn steep liquor, 1.1% casein hydrolysate, 2% L-aspartic acid, 2% K_2HPO_4, and 0.1% $MgSO_4 \cdot 7H_2O$. Symbols: ●, L-proline; ■, L-aspartic acid; □, L-glutamic acid; ▲, glucose; ○, growth (optical density); ×, pH.*

TABLE 15. *Effect of amino acid and organic acid upon the production of L-proline*

Addition to medium[a]		pH	Growth[b]	L-Proline	L-Aspartic acid
Compound	L-Aspartic acid				
	%			mg/ml	mg/ml
None	0	5.6	.210	4	0
	1	7.6	.500	23	0
L-Aspartic acid, 2.00%	0	8.4	.570	28	0
	1	8.6	.630	26	3
L-Glutamic acid, 2.21%	0	6.0	.273	5	0
	1	8.4	.560	24	2
L-Alanine, 1.34%	0	5.8	.235	4	0
	1	7.0	.520	24	0
Fumaric acid, 1.74%	0	5.6	.230	3	0
	1	7.4	.580	21	0.5
Malic acid, 2.02%	0	5.6	.260	3	0
	1	8.0	.620	22	0.5
Succinic acid, 1.77%	0	5.8	.230	4	0
	1	7.6	.580	23	0

[a] In addition to the above compound, all media contained 6% glucose, 0.5% urea, 0.3% NH_4Cl, 0.7% corn steep liquor, 1.1% casein hydrolysate, 2% K_2HPO_4, and 0.1% $MgSO_4 \cdot 7H_2O$.

[b] Optical density.

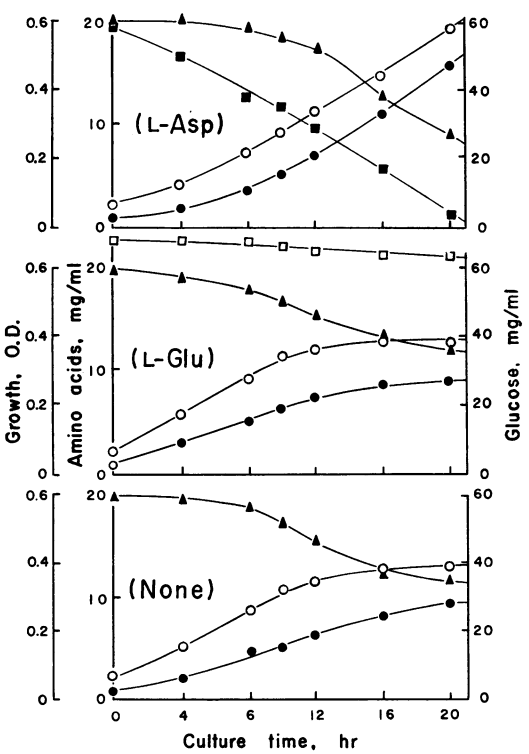

FIG. 2. *Comparison of changes during incubation on medium containing L-aspartic acid or L-glutamic acid. In addition to 2% L-aspartic acid or 2.2% L-glutamic acid, all media contained 6% glucose, 0.5% urea, 0.3% NH_4Cl, 0.7% corn steep liquor, 1.1% casein hydrolysate, 2% K_2HPO_4, and 0.1% $MgSO_4 \cdot 7H_2O$. Symbols: ●, L-proline; ■, L-aspartic acid; □, L-glutamic acid; ▲, glucose; ○, growth (optical density).*

the medium, and shifted gradually to the alkaline side with the exhaustion of glucose.

The chemical changes occurring during fermentation with the parent strain were compared with those of the serine-requiring mutant, strain 45, under the same conditions shown in Fig. 1. There was no significant difference in the course of fermentation, except that the maximal production of L-proline by the parent strain was only 18 mg/ml.

The L-proline in the fermentation broth was isolated by ordinary separation procedures, employing ion-exchange resins, and was purified as the picrate. The L-proline obtained from the picrate was recrystallized from isopropyl alcohol. $[\alpha]_D^{20} = -85.3°$ (c = 4 in water). Analysis: $C_5H_9O_2N$; calculated: C, 52.16; H, 7.88; N, 12.17; found: C, 52.31; H, 7.81; N, 12.24. The infrared spectrum of the product was identical with that of authentic L-proline, and no other amino acid was detected by paper chromatography.

Role of L-aspartic acid. As described above, a high concentration of L-aspartic acid is required for the maximal production of L-proline. To establish the role of L-aspartic acid in this fermentation, other amino acids or organic acids concerned in the metabolism of L-aspartic acid were substituted. Experiments were carried out with media containing these compounds at a concentration equivalent to 2.0% L-aspartic acid, both in the presence and in the absence of 1% L-aspartic acid. All compounds tested were without effect upon growth or the production of L-proline in either the presence or the absence of

L-aspartic acid, indicating that L-aspartic acid cannot be replaced by these compounds (Table 15).

This suggests that L-aspartic acid exerts an influence upon the fermentation of L-proline by increasing growth. To confirm this effect, the chemical changes during fermentation were compared in media containing 2.0% L-aspartic acid, 2.2% L-glutamic acid, or no additive. The cells having high L-proline-producing activity were obtained by shaking the culture for 15 hr under the optimal conditions described in Fig. 1. After being harvested by centrifugation, cells were washed twice with saline and mixed with 30 ml of the respective medium. The mixture was incubated both with and without shaking. Without shaking, no growth occurred. Figure 2 shows the changes occurring during incubation with shaking in these three media.

During the early stages of fermentation, there were no marked differences in growth, glucose consumption, or production of L-proline. Later, both growth and yield reached a plateau in the absence of L-aspartic acid, i.e., in medium with L-glutamic acid and in medium with no addition. In medium with L-aspartic acid, however, the production of L-proline linearly increased in parallel with the continued growth of the organism, even during the later stages of fermentation. Although L-aspartic acid was readily utilized by the organism, L-glutamic acid was scarcely consumed. This accounts for the changes that occurred during fermentation in the presence of L-glutamic acid and that were the same as the changes occurring with no addition.

These results suggested that L-aspartic acid does not play the role of precursor of L-proline, but that it exerts its effect by promoting growth during the later part of fermentation. This concept is currently under investigation with the use of labeled L-aspartic acid.

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