FERMENTATIVE PRODUCTION OF L-SERINE
PRODUCTION OF L-SERINE FROM GLYCINE BY
CORYNEBACTERIUM GLYCINOPHILUM NOV. SP.

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A new species *Corynebacterium glycinophilum* nov. sp. was isolated from a putrefied banana, which is resistant to a high concentration of glycine and has a prominent ability of producing L-serine from glycine.

Among various substances supplemented to the medium used, nicotinamide was found to be effective for L-serine production. The L-serine production was in parallel with the consumption of glycine in the medium. Ferrous ion was found to depress L-serine production and to increase the assimilation of glycine added.

Glycine was utilized as a nitrogen source in the presence of glucose, but was not utilized as a carbon source in the absence of glucose. The addition of inorganic nitrogen increased L-serine production. Glycine was not formed from L-serine. L-Threonine was formed with L-serine from glycine, probably by serine transhydroxymethylase.

Our previous papers (1, 2) reported that some of bacteria and yeasts accumulated L-serine in the culture medium containing glycine. In these cases, the growth inhibition by glycine was more or less observed in all microorganisms, which prevented the efficient production of L-serine. It provoked the idea that potent L-serine producers might be found in microorganisms resistant to glycine.

The present paper reports the identification and characterization of a newly isolated bacterium possessing a remarkable ability of producing L-serine from glycine and the production of L-serine from glycine by it.

MATERIALS AND METHODS

Bacterium. *Corynebacterium glycinophilum* nov. sp. AJ 3170 (ATCC 21341) which was isolated from a putrefied banana was employed for L-serine production.

Medium. The following medium was employed for L-serine production.

Medium D (1): Glucose, 5%; $(\text{NH}_4)_2\text{SO}_4$, 0.2%; NH_4NO_3 , 0.3%; KH_2PO_4 , 0.1%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05%; DL-methionine, 0.03%; biotin, 0.2 $\mu\text{g/ml}$; thiamine hydrochloride, 1 $\mu\text{g/ml}$; riboflavin, 2 $\mu\text{g/ml}$; folic acid, 1 $\mu\text{g/ml}$; calcium pantothenate, 1 $\mu\text{g/ml}$; nicotinamide, 1 $\mu\text{g/ml}$; pyridoxine, 1 $\mu\text{g/ml}$; pyridoxal, 0.2 $\mu\text{g/ml}$; *p*-aminobenzoic acid, 1 $\mu\text{g/ml}$; glycine, 20 mg/ml; CaCO_3 (sterilized separately), 2.5%; pH, 6.5.

Cultivation of bacterium. A test tube containing 5 ml of medium D was sterilized and inoculated with a loopful of bacterial cells grown on nutrient agar slant. Reciprocal shaking was carried out at 30° for 72 hr.

Analytical methods. (a) For bacterial growth, the culture broth was diluted 26 times after dissolving CaCO_3 with HCl and the optical density was measured at 562 m μ (OD_{562}).

(b) Glucose was determined by the method of Fehling-Lehmann-Schoorl.

(c) Qualitative detection of serine and glycine was carried out by paper chromatography using the solvent system of dicyclohexylamine-acetone-butanol-water (2:10:10:5, by volume). Serine was detected by the ninhydrin reaction for its characteristic brown color and also by the periodate-Nessler reaction (3). L-Serine and glycine were determined quantitatively by microbiological assay with *Leuconostoc mesenteroides* ATCC 8042.

RESULTS

Isolation of L-serine-producing microorganisms from natural sources

Each piece of slightly putrefied vegetables and fruits was suspended in saline and the suspension was spread onto nutrient agar plates containing 2% of glycine. In order to detect L-serine-producing microorganisms, each colony which appeared on the plates after a 48-hr incubation at 30° (Table 1) was picked up and inoculated into 5 ml of medium D and cultivated at 30° for 72 hr.

Two microorganisms obtained from banana were found to accumulate L-serine in the culture. Of the two, a bacterium which accumulated L-serine better was employed for the following experiments. Single colony isolation was carried out for purification. Among 20 colonies tested, 17 colonies were positive for L-serine production while three were negative. One of four colonies, which gave the maximum accumulation of 8 mg/ml, was designated as AJ 3170.

Determination of AJ 3170

The characteristics of AJ 3170 were as follows:

Vegetative cells: Short rods usually single or two, variation in the forms, size 0.6~0.8 $\times$ 1.0~3.0 μ on nutrient agar at 30° for 24 hr. Non-motile.

Spore not formed. Gram-positive. Not acid-fast.

Agar colonies: Circular, smooth, entire, rises to convex, opaque, grayish white, medium unchanged.

Table 1. Isolation of microorganisms resistant to glycine from vegetables and fruits.

The isolates were obtained from colonies which appeared on nutrient agar plates containing 2% of glycine after 48 hr of incubation at 30°.

Vegetable or fruit	Number of samples	Number of isolates	Vegetable or fruit	Number of samples	Number of isolates
Pineapple	7	35	Watermelon	2	20
Tomato	6	56	Burdock	2	14
Banana	6	34	Onion	2	13
Loquat	5	32	Orange	2	10
Broad bean	4	43	Cucumber	1	6
Potato	4	11	Peach	1	6
Lettuce	3	21	Strawberry	1	5
Apple	3	19	Pimento	1	5
Carrot	3	18	Cherry	1	4
Melon	3	18	Garlic	1	3
Radish	3	11	Eggplant	1	3
Cabbage	3	8	Lemon	1	2

Gelatin colonies: Circular, rises to convex, entire, no liquefaction, smooth, opaque.

Agar stroke: Moderate growth, filiform, glistening, grayish-white butyrous, medium unchanged.

Nutrient broth: No growth on the surface, turbid, sediment flocculent, amount of sediment scanty.

Milk: Changed to slightly alkaline.

Indole not produced.

No acid and gas from arabinose, xylose, glucose, mannose, fructose, galactose, lactose, maltose, saccharose, trehalose, raffinose, sorbitol, inositol, glycerol, salicin, α -methylglycoside, inulin, dextrin, starch, and cellulose (peptone medium).

Slightly acid but no gas from glucose aerobically, but no acid and gas anaerobically by the method of Hugh and Leifson.

Gelatin stab: No liquefaction.

Starch not hydrolyzed.

Nitrites not produced from nitrates.

Citrate not utilized.

Catalase-positive.

Aerobic.

Temperature relations: Growth at 20° to 37°, no growth at 42°.

Source: Isolated from a putrefied banana.

The bacterium grew on the medium of nutrient broth added with 6% glycine.

No species could be found in Bergey's Manual of Determinative Bacteriology, 7th Edition, which could be identified as being the same as the present microorganism. It is, therefore, considered that this isolate is a new species belonging to *Corynebacterium* and we have named it *Corynebacterium glycinophilum* nov. sp. AJ 3170 (ATCC 21341).

Isolation and identification of the substance produced by Corynebacterium glycinophilum nov. sp. AJ 3170

Corynebacterium glycinophilum nov. sp. AJ 3170 was cultured in medium D. The bacterial cells and calcium salts were separated from the culture broth by centrifugation. The amount of L-serine in the supernatant was estimated as 7.3 mg/ml. One liter of the supernatant, adjusted to pH 5.0 with HCl, was treated with active carbon for decolorization and then passed through a Dowex 50 (H⁺ type) column for the adsorption of L-serine. After washing the column with water and 0.024 N HCl, the column was eluted with 0.24 N HCl, and the eluate was further passed through an Amberlite IR-4B (OH⁻ type) column for elimination of chlorine ion. The eluate was finally concentrated *in vacuo* to obtain colorless syrup, from which 2.2 g of a crystallized compound was obtained by cooling in a refrigerator.

Paper chromatogram of the material isolated gave a characteristic brown color after the ninhydrin reaction and the material was positive to the periodate-Nessler reaction (3). Elemental analysis of the material gave following values: C, 34.27%; H, 6.81%; N, 13.24% (C₈H₇NO₃ requires C, 34.28%; H, 6.71%; N, 13.34%). The optical rotation was estimated as $[\alpha]_D^{20} = +13.85^\circ$ ($c=10$, 2 N HCl). These data agreed with those of L-serine.

Essential ingredients of the medium for L-serine production

In some microorganisms, it is known that L-serine formation from glycine requires one-carbon unit and vitamins such as tetrahydrofolic acid and pyridoxal phosphate (4-6). Therefore, the effect of glycine, vitamins, and methionine on L-serine formation in this bacterium was examined and, as indicated in Table 2, it was found that L-serine was not formed without glycine, even when inorganic nitrogen sources were present in medium D. Furthermore, the more L-serine was formed, the less the residual glycine existed. On the other hand, under the conditions which gave little L-serine formation, the consumption of glycine was small. These facts suggested that glycine was the precursor of L-serine.

Addition of vitamins was effective for L-serine production, although biotin was found to be ineffective. If the vitamin mixture was omitted, the amount of L-serine produced was only 45% of that in the complete system. Methionine had no apparent effect. It may be that one-carbon unit of L-serine is derived from some carbon source other than methionine.

Table 2. Essential ingredients for L-serine production.

Medium D was employed as the basal medium for L-serine production. Cultivation was carried out at 31° for 72 hr.

Ingredient omitted	Growth (OD ₅₆₂)	pH	L-serine produced (mg/ml)	Residual glycine (mg/ml)	Residual glucose (mg/ml)
None	0.80	7.8	7.9	4.0	2.4
Glycine	0.57	6.6	0.0	0.0	15.0
Biotin	0.84	6.9	7.0	8.3	2.8
Vitamins (except biotin)	0.64	6.7	4.3	10.1	15.3
DL-Methionine	0.75	7.6	7.4	6.4	1.2

Table 3. Effect of vitamins on L-serine production.

Basal medium: Glucose 5%, (NH₄)₂SO₄ 0.2%, NH₄NO₃ 0.3%, KH₂PO₄ 0.1%, MgSO₄·7H₂O 0.05%, DL-methionine 0.03%, biotin 0.2 µg/ml, glycine 2%, CaCO₃ 2%, pH 6.5. Cultivation was carried out at 31° for 72 hr.

Vitamin added ($\mu\text{g/ml}$)		Growth (OD_{562})	pH	L-Serine produced (mg/ml)	Residual glycine (mg/ml)
Thiamine·HCl	2.5	0.42	7.3	1.3	17.0
Riboflavin	5.0	0.34	7.4	1.4	17.0
Folic acid	2.5	0.34	7.3	1.5	11.4
Ca-pantothenate	2.5	0.51	7.2	2.1	16.4
Nicotinamide	2.5	0.71	5.8	6.7	8.5
Pyridoxine	2.5	0.38	7.3	2.2	16.1
Pyridoxal	0.5	0.45	7.3	2.8	15.7
<i>p</i> -Aminobenzoic acid	2.5	0.36	7.3	2.7	15.4
Mixture ^a		0.76	7.0	8.1	4.4
None		0.42	7.3	2.8	16.0

^a Mixture: vitamins contained in medium D.

Effect of vitamins on L-serine production

As the effect of vitamin mixture proved excellent on L-serine production, an attempt was made to determine which component in the mixture was effective and its result is indicated in Table 3. Among eight components, nicotinamide was effective for L-serine production. It also stimulated the growth of AJ 3170 and increased the consumption of glycine. The addition of nicotinamide alone gave about 80% of L-serine production in the case of the addition of vitamin mixture. *p*-Aminobenzoate and thiamine were somewhat effective. Folate and pyridoxal were little effective, although their

Table 4. Effect of amino acids on L-serine production.

Medium D exclusive of DL-methionine was employed as basal medium for L-serine production. Cultivation was carried out at 31° for 72 hr.

L-Amino acid added (0.5 mg/ml)	Growth (OD ₅₆₂)	pH	L-Serine produced (mg/ml)	Residual glycine (mg/ml)
Cysteine·HCl	0.73	6.8	4.1	10.3
Glutamic acid	0.74	6.6	3.0	12.6
Methionine	0.72	7.2	5.4	8.5
Phenylalanine	0.68	7.0	1.6	12.5
Proline	0.75	6.9	3.0	12.8
Threonine	0.73	7.0	2.8	14.1
Valine	0.84	7.8	4.7	7.9
Glutamic acid plus methionine plus valine	0.75	7.7	10.1	6.6
None	0.70	7.0	3.5	12.3

derivatives, tetrahydrofolate and pyridoxal phosphate, were considered necessary for serine transhydroxymethylase reaction (4-6).

Effect of amino acids on L-serine production

Table 4 shows that, among the amino acids supplemented to medium D, L-valine or L-methionine gave a remarkable formation of L-serine from glycine. On the other hand, DL-methionine was ineffective for L-serine production (Table 1).

Addition of L-cysteine was slightly effective. L-Alanine, L-aspartic acid, L-arginine, L-isoleucine, L-leucine, L-lysine, L-tryptophan, L-tyrosine, and L-histidine, likewise L-phenylalanine or L-threonine, were rather inhibitory on L-serine production. Simultaneous addition of valine, methionine, and glutamate gave an excellent production of L-serine. The amount of L-serine produced was 10 mg/ml.

Effect of metal ions on L-serine production

Supplementation of metal ions to medium D gave almost a negative effect on L-serine production as indicated in Table 5. Especially, ferrous ion decreased L-serine production remarkably in spite of increasing the growth of the bacterium. A higher concentration of the ion (40 ppm) caused a drastic reduction in L-serine production as well as a slight reduction of residual glycine, suggesting that it may be a promoting factor for decomposition of glycine or serine produced. With regard to other ions, the higher concentration of 40 ppm more or less caused the reduction of both the bacterial growth level and the assimilation of sugar, as well as the production of L-serine.

Table 5. Effect of metal ions on L-serine production.

Medium D was employed as basal medium for L-serine production. Cultivation was carried out at 31° for 72 hr.

Cr, Cu, Fe, Pb, and Mn were added as $K_2Cr_2O_7 \cdot 2H_2O$, $Cu(CH_3COO)_2 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$, $Pb(CH_3COO)_2$, and $MnSO_4 \cdot 4H_2O$, respectively.

Metal ion added (ppm)	Growth (OD ₅₆₂)	pH	L-Serine produced (mg/ml)	Residual glycine (mg/ml)	Residual glucose (mg/ml)
Cr (IV) 2	0.85	7.3	6.1	7.6	6.7
40	0.77	7.1	5.0	11.8	18.0
Cu (II) 2	0.86	7.9	7.2	7.2	2.0
40	0.77	7.1	5.6	11.4	16.4
Fe (II) 2	0.86	8.4	2.4	5.8	3.8
40	0.96	8.3	0.7	5.1	9.4
Pb (II) 2	0.81	7.6	5.6	8.7	9.2
40	0.76	6.7	4.7	11.4	10.5
Mn (II) 2	0.85	7.3	4.5	11.5	11.6
40	0.43	6.7	3.0	14.8	29.8
None	0.83	7.3	6.0	9.7	8.9

Table 6. Effect of pH on L-serine production.

Medium D was employed as basal medium for L-serine production. Cultivation was carried out at 31° for 72 hr.

pH of the medium	Duration of cultivation					
	24 hr		72 hr			
	Growth (OD ₅₆₂)	pH	Growth (OD ₅₆₂)	pH	L-Serine produced (mg/ml)	Residual glycine (mg/ml)
5.0	0.22	7.0	0.81	7.1	5.0	8.7
5.5	0.22	6.7	0.77	7.0	5.5	5.5
6.0	0.23	7.0	0.75	7.1	5.5	6.4
6.5	0.27	6.9	0.80	7.5	7.1	4.4
7.0	0.29	6.6	0.76	7.1	5.9	5.4
7.5	0.33	6.5	0.75	7.2	4.7	7.0
8.0	0.34	6.7	0.89	7.2	5.6	6.3

Effect of pH on L-serine production

As shown in Table 6, the growth level of the bacterium in the early stage (24 hr) increased as initial pH of the medium was raised. The optimal initial pH for L-serine production was 6.5.

Table 7. Effect of inorganic nitrogen sources on L-serine production.

Medium D was employed as basal medium for L-serine production. Cultivation was carried out at 31° for 72 hr.

Inorganic nitrogen source		Glucose (%)	Growth (OD ₅₆₂)	pH	L-Serine produced (mg/ml)	Residual glycine (mg/ml)	Residual glucose (mg/ml)
(NH ₄) ₂ SO ₄ (%)	NH ₄ NO ₃ (%)						
0	0	0	0.00	7.7	0.0	19.7	—
0	0	5	0.40	6.9	5.2	12.8	12.8
0.2	0	5	0.53	7.1	7.8	7.9	8.5
0	0.3	5	0.50	7.2	7.3	9.0	9.1
0.2	0.3	5	0.56	7.2	7.1	9.6	6.9
0.4	0.6	5	0.48	6.9	6.1	10.7	9.1
0.6	0.9	5	0.44	6.9	5.4	12.4	10.5

Effect of inorganic nitrogen sources on L-serine production

Table 7 shows that AJ 3170 utilized glycine as a nitrogen source for L-serine production in the presence of glucose. Without any inorganic nitrogen compound, a considerable amount of L-serine was produced from glycine. However, additional supplementation of ammonium sulfate and/or ammonium nitrate increased L-serine formation, although excess additions caused reduction of L-serine formation as well as that of bacterial growth. Glycine was not utilized as a sole source of carbon.

Interconversion of glycine and serine

The interconversion of glycine and serine has been reported by a number of investigators (4–6). As shown in Table 8, it was found that glycine was not produced from L-serine, although L-serine was produced from glycine. Most of L-serine added remained intact in the medium. This suggests that glycine formation from L-serine may occur under different conditions, or the bacterium cannot form glycine from L-serine.

Time course of L-serine production

As shown in Fig. 1, L-serine production began to increase with growth of the bacterium and reached the stationary phase at 72 hr when the maximum production of L-serine was accomplished and glucose was almost completely assimilated. After that, L-serine seemed to be decomposed much more than was formed and a greater part of the residual glycine remained unchanged. Glucose deficiency might decompose L-serine because it is utilized as a carbon source.

Table 8. Interconversion of glycine and L-serine.

Medium D was employed as basal medium for L-serine production. Cultivation was carried out at 31° for 72 hr.

Glycine or L-serine added (%)	Growth (OD ₅₆₂)	L-Serine produced (mg/ml)	Residual glycine (mg/ml)	Glycine produced (mg/ml)	Residual L-serine (mg/ml)
Glycine	1	0.64	5.1	1.7	—
	2	0.70	8.2	5.0	—
	3	0.66	8.7	16.9	—
	4	0.71	9.4	27.4	—
L-Serine	1	0.55	—	—	0.0
	2	0.61	—	—	0.0
	4	0.53	—	—	0.0

Fig. 1. Time course of L-serine production.

Medium D was employed for L-serine production. Cultivation was carried out at 31°.

Other amino acids accumulated in culture medium

The production of amino acids other than L-serine was examined by paper chromatography and microbioassay. After 72 hr of cultivation in medium D, the amino acids (in mg/ml) produced by AJ 3170 were as follows: Alanine, 0.53; L-glutamic acid, 0.11; L-isoleucine, 0.08; L-lysine, 0.88; L-serine, 8.10; L-threonine, 0.40; L-valine, 0.14; and residual glycine, 3.73.

Among them, L-isoleucine was probably formed from L-threonine which might be formed from exogenous glycine by serine transhydroxymethylase, the same enzyme catalyzing the conversion of glycine to L-serine (7, 8).

DISCUSSION

Our previous papers (1, 2) showed that some of bacteria and yeasts produced L-serine from glycine but they were subjected to growth inhibition by glycine. Therefore, efficient production of L-serine from a high concentration of glycine was difficult. Screening of microorganisms resistant to glycine gave an excellent L-serine-producing bacterium which was named *Corynebacterium glycinophilum* nov. sp.

A number of investigators reported growth inhibition of microorganisms by glycine. In some bacteria, glycine seems to inhibit their growth or to form a spheroplast by the inhibition of cell wall formation (9, 10, 11, 12). The fact that this bacterium forms L-serine only in the presence of glycine shows that glycine is incorporated intracellularly. It is interesting that the growth of this bacterium, unlike other L-serine-producing microorganisms (1, 2), is not inhibited by glycine. Further investigation will be carried out to clarify this point.

Conversion of glycine to L-serine requires introduction of a one-carbon unit. As its source, glycine (13), formate (14), formaldehyde (15), methionine (16), choline (17), and sarcosine (18), are known. From each of them, N¹⁰-hydroxymethyltetrahydrofolate is formed with tetrahydrofolate. Meanwhile, pyridoxal phosphate is necessary for the activation of glycine (8, 19). Hydroxymethyl group of N¹⁰-hydroxymethyltetrahydrofolate is transferred to activated glycine to form L-serine. In the present work, the addition of folate or pyridoxal to medium D was ineffective for L-serine production. Simultaneous addition of both gave a similar result. However, effect of nicotinamide on L-serine production was remarkable. Therefore, activities of enzymes catalyzing L-serine formation from glycine in AJ 3170 are not likely to be influenced by exogenous pyridoxal and folate. This shows that AJ 3170 cannot synthesize sufficient amount of pyridoxal phosphate and tetrahydrofolate. Otherwise, the bacterium may have the enzymes independent of known characteristics, because L-serine production is increased by exogenous nicotinamide and glycine is not formed from L-serine.

AJ 3170 also forms L-threonine from glycine. This reaction is probably due to the action of serine transhydroxymethylase, the same enzyme catalyz-

ing the conversion of glycine to L-serine (7, 8). Although the experimental conditions used in this study gave a low level of L-threonine formation, further investigation may give informations on larger formation in the presence of glycine.

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