FERMENTATIVE PRODUCTION OF L-HISTIDINE*

I. PRODUCTION OF L-HISTIDINOL BY A HISTIDINE AUXOTROPH OF *BREVIBACTERIUM FLAVUM* AND FORMATION OF L-HISTIDINE FROM L-HISTIDINOL BY MICROORGANISMS

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A histidine auxotroph, AJ 3127 (ATCC 21349) derived from *Brevibacterium flavum* ATCC 14067 which is a glutamic acid-producing bacterium, produces a large amount of L-histidinol from glucose and inorganic nitrogen. About 0.15 g/liter of histidine or 40 ml/liter of soybean protein hydrolyzate gives the maximum production of L-histidinol. The amount of L-histidinol accumulated in the presence of the latter is 9.2 g/liter (as dihydrochloride).

Among various substances supplemented to the medium, organic acids such as lactic acid, succinic acid, and maleic acid are found to enhance L-histidinol production. L-Alanine, L-phenylalanine, L-tryptophan, propionic acid, adenine, and 4-amino-5-imidazolecarboxamide riboside depress L-histidinol production.

L-Histidine formation from L-histidinol is carried out by various bacteria including *Brevibacterium flavum* ATCC 14067.

Fermentative production of various amino acids by microorganisms has been carried out extensively. However, direct production of L-histidine from sugar and inorganic nitrogen by fermentation has never been disclosed. Only MOYED (1) reported extracellular accumulation of histidine by mutants of *Escherichia coli* resistant to 2-thiazolealanine.

While screening for L-histidine production by microorganisms, a histidine auxotroph derived from *Brevibacterium flavum* ATCC 14067 with X-ray irradiation was found to produce a large amount of L-histidinol in the culture medium. Moreover, L-histidinol produced was capable of being converted into L-histidine by various bacteria.

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The present paper reports the production of L-histidinol by the auxotroph and L-histidine formation from L-histidinol by microorganisms, especially the conditions for L-histidinol production, and isolation and identification of L-histidinol produced.

MATERIALS AND METHODS

Microorganisms. A histidine auxotroph, AJ 3127 (ATCC 21349) which was derived from *Brevibacterium flavum* ATCC 14067 by X-ray irradiation, was used for L-histidinol production. *Micrococcus luteus* ATCC 398, *Staphylococcus epidermidis* CCM 50, *Aeromonas punctata* IAM 1646, *Escherichia coli* ATCC 13070, *Enterobacter liquefaciens* ATCC 14460, *Pseudomonas perflurida* ATCC 490, *Flavobacterium heparinum* ATCC 13125, *Brevibacterium flavum* ATCC 14067, *Brevibacterium divaricatum* NRRL B-2312, *Corynebacterium acetoacidophilum* ATCC 13870, and *Arthrobacter citreus* ATCC 11624 were employed for L-histidine production from L-histidinol.

Media. The following media were employed.

Medium A: Glucose, 10%; KH_2PO_4 , 0.1%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04%; $(\text{NH}_4)_2\text{SO}_4$, 6%; biotin, 200 $\mu\text{g/liter}$; thiamine hydrochloride, 50 $\mu\text{g/liter}$; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001%; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.001%; L-histidine, 0.015% or "Ajeki" (a solution of amino acid mixture obtained by the hydrolysis of soybean protein, total nitrogen 2.4%. Ajinomoto Co., Inc., Tokyo), 4%; CaCO_3 (sterilized separately), 5%; pH 7.0.

Medium B: Glucose, 7%; KH_2PO_4 , 0.1%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04%; $(\text{NH}_4)_2\text{SO}_4$, 0.5%; yeast extract, 0.5%; peptone, 0.2%; L-histidinol $\cdot 2\text{HCl}$, 0.5%; CaCO_3 (sterilized separately), 2.5%; pH 7.0.

Culture methods. A 500-ml Sakaguchi flask containing 20 ml of either medium was sterilized and inoculated with a loopful of cells grown on nutrient agar slants. Reciprocal shaking was carried out at 31° for 72 hr.

Analytical methods. (a) Bacterial growth was measured by using a Hirma Electro-photocolorimeter, Model II B, by the optical density at 562 m μ (O.D._{562}) of the culture broth diluted 26 times after dissolving CaCO_3 with HCl.

(b) Glucose was determined by the methods of Fehling-Lehman-Schoorl.

(c) Qualitative analyses of histidine and histidinol were carried out by paper chromatography using the solvent systems of dicyclohexylamine-acetone-butanol-water (2 : 10 : 10 : 5, by volume), propanol-ammonia water-water (75 : 1.5 : 23.5), and butanol-acetic acid-water (4 : 1 : 1). Histidine and histidinol were detected by ninhydrin method and Pauly's diazo reaction (2). Histidine was measured by microbiological assay with *Leuconostoc mesenteroides* ATCC 8042. Histidinol was determined by spectrophotometry according to the modified method of MACPHERSON (3). A solution (0.01 ml) containing 10 to 100 μg of histidinol was spotted on the filter paper and paper chromatography was carried out using a solvent system of butanol-acetic acid-water.

Fig. 1. Isolation of L-histidinol·2HCl from culture broth.

Histidinol on the paper was eluted with 0.5 N HCl, then 1 ml of sulfanilic acid solution (10 g in 1 liter of 1 N HCl) and 1 ml of NaNO₂ solution (5% w/v) were added to 2 ml of the eluate. One milliliter of NaOH solution (20% w/v) was added after shaking and the optical density at 440 m μ was assayed. Amount of histidinol was calculated from the calibration curve of authentic histidinol.

RESULTS

Isolation and identification of L-histidinol produced by a histidine auxotroph AJ 3127

Brevibacterium flavum AJ 3127 (ATCC 21349) was cultured in medium A containing 4% of "Ajeki," and the bacterial cells and calcium salts were separated from the culture broth by centrifugation. The cell-free broth contained 9.2 g/liter of L-histidinol dihydrochloride. After 1.9 liters of the broth was adjusted to pH 5.0 with HCl, it was passed through an Amberlite IRC-50 column, which was eluted with 2 N NH₄OH, and the eluate brought to pH 3.5 with HCl was concentrated *in vacuo*. The compound was crystallized in a refrigerator. After separation, the crude crystals were dissolved in water and decolorized with active carbon. Dried L-histidinol dihydrochloride crystals which were obtained after recrystallization from water weighed 7.2 g. The material isolated gave a bluish-purple color after ninhydrin reaction and an orange color after Pauly's diazo reaction.

Fig. 2. Infrared absorption spectrum of L-histidinol·2HCl obtained from culture broth. The sample was measured in the solid state as KBr disc.

Fig. 3. Effect of L-histidine concentration on L-histidinol production.

Basal medium: Glucose 10%, KH_2PO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.04%, $(\text{NH}_4)_2\text{SO}_4$ 6%, biotin 200 $\mu\text{g/liter}$, thiamine hydrochloride 50 $\mu\text{g/liter}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.001%, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.001%, CaCO_3 5%, pH 7.0. Fermentation was carried out at 31° for 72 hr on a reciprocal shaker.

Elemental analysis of the material gave following values: C, 33.66%; H, 6.12%; N, 19.63%; Cl, 33.43% ($\text{C}_6\text{H}_{13}\text{ON}_3\text{Cl}_2$ requires C, 33.77%; H, 6.28%; N, 19.55%; Cl, 33.51%). The optical reaction was $[\alpha]_{\text{D}}^{20} = -3.3^\circ$ ($c=5$, H_2O). The melting point was 196–197°. Infrared absorption spectrum of the material is shown in Fig. 2. These physical data agree with those obtained by VOGEL *et al.* (4), and by AMES *et al.* (5) for L-histidinol dihydrochloride.

Table 1. Effect of carbohydrate source on L-histidinol production.

Basal medium: KH_2PO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.04%, $(\text{NH}_4)_2\text{SO}_4$ 6%, biotin 200 $\mu\text{g/liter}$, thiamine hydrochloride 50 $\mu\text{g/liter}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.001%, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.001%, L-histidine 0.015%, CaCO_3 5%, pH 7.0. Fermentation was carried out at 31° for 72 hr.

Carbohydrate (10%)	Growth (O.D. ₅₆₂)	L-Histidinol·2HCl produced (g/liter)
Glucose	0.96	5.7
Starch hydrolyzate	1.03	6.0
Fructose	0.85	3.0
Sucrose	1.00	5.5
Maltose	1.09	4.3
Galactose	0.05	0.8
Mannose	0.14	0.9
Sorbose	0.04	0.8

Table 2. Effect of ammonium sulfate concentration on L-histidinol production.

Basal medium: Glucose 10%, KH_2PO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.04%, biotin 200 $\mu\text{g/liter}$, thiamine hydrochloride 50 $\mu\text{g/liter}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.001%, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.001%, L-histidine 0.015%, CaCO_3 5%, pH 7.0. Fermentation was carried out at 31° for 72 hr.

Ammonium sulfate (%)	Growth (O.D. ₅₆₂)	L-Histidinol·2HCl produced (g/liter)
2	0.90	3.6
3	1.00	3.8
4	1.00	4.4
5	1.00	4.8
6	1.00	6.0
7	0.97	4.5

Effect of L-histidine concentration on L-histidinol production.

AJ 3127 requires L-histidine for its growth, as shown in Fig. 3. This graph indicates the relation between L-histidine concentration in medium A and the amount of L-histidinol produced. L-Histidinol production by AJ 3127 is greatly influenced by histidine concentration in the medium and 0.15 g/liter of L-histidine in the medium is optimal for the maximum production of L-histidinol. Growth of AJ 3127 increases with the increase of L-histidine concentration in the medium.

Effect of various substances on L-histidinol production

(a) *Carbohydrates.* As shown in Table 1, glucose, sucrose, and starch

Table 3. Effect of biotin and thiamine concentration on L-histidinol production.

Basal medium: Glucose 10%, KH_2PO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.04%, $(\text{NH}_4)_2\text{SO}_4$ 6%, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.001%, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.001%, L-histidine 0.015%, CaCO_3 5%, pH 7.0. Fermentation was carried out at 31° for 72 hr.

Thiamine ($\mu\text{g/liter}$)	Biotin ($\mu\text{g/liter}$)	Growth (O.D. ₅₆₂)	L-Histidinol·2HCl produced (g/liter)
5	5	0.96	2.1
	50	1.05	4.8
	200	1.05	5.2
	500	1.07	5.4
50	5	0.93	1.9
	50	1.03	6.4
	200	1.03	6.0
	500	1.04	5.4
200	5	0.94	2.0
	50	1.05	5.6
	200	1.09	5.6
	500	1.06	5.3
1000	5	0.95	2.3
	50	1.07	5.3
	200	1.09	5.2
	500	1.06	5.4

hydrolyzate, obtained by sulfuric acid hydrolysis of starch, are suitable as the carbon source for L-histidinol production. Fructose and maltose are utilized by AJ 3127, but galactose, mannose, and sorbose are scarcely utilized for its growth.

(b) *Ammonium sulfate as the inorganic nitrogen source.* Ammonium sulfate is the most effective as the inorganic nitrogen source and 6% of ammonium sulfate is favorable for the maximum production of L-histidinol (Table 2). Ammonium chloride, ammonium phosphate, and ammonium carbonate are unfavorable for the production of L-histidinol.

(c) *Biotin and thiamine.* Both biotin and thiamine in addition to L-histidine are essential factors for the growth of AJ 3127. As seen in Table 3, low concentration of biotin (5 $\mu\text{g/liter}$) gives production less than one-half the maximum production of L-histidinol, while it serves to produce a large amount of extracellular glutamic acid. The concentration of biotin more than 50 $\mu\text{g/liter}$, and various concentrations of thiamine do not give a remarkable effect on L-histidinol production.

(d) *Soybean protein hydrolyzate.* Soybean protein hydrolyzate, "Ajieki," was used as the substitute for L-histidine added to the medium and 40 ml of

Fig. 4. Effect of soybean protein hydrolyzate on L-histidinol production.

Culture conditions were essentially the same as described in Fig. 3 except that the basal medium contained soybean protein hydrolyzate instead of L-histidine.

soybean protein hydrolyzate, which contains 100 mg of histidine, in 1 liter of the medium gives the maximum production of L-histidinol (Fig. 4).

(e) *Amino acids*. As shown in Fig. 5, none of amino acids added increases L-histidinol production. Additions of L-alanine, L-phenylalanine, and L-tryptophan are rather inhibitory on L-histidinol production.

(f) *Nucleic acid-related compounds*. The effect of supplementation of purine bases, pyrimidine bases, and 4-amino-5-imidazolecarboxamide riboside (AICA-R) is shown in Fig. 6. Guanine, hypoxanthine, and xanthine do not influence L-histidinol production, while addition of pyrimidine bases, such as uracil and cytosine, reduces L-histidinol production slightly. The effect of adenine, and AICA-R on L-histidinol production is worth noting: they decrease L-histidinol produced by more than 50%.

(g) *Organic acids*. Among the organic acids listed in Fig. 7, lactic acid, succinic acid, and maleic acid are slightly effective for L-histidinol production. The addition of propionic acid resulted in a marked decrease in L-histidinol production because of the poor growth of AJ 3127.

(h) *Vitamins*. Supplementation of vitamins is ineffective on L-histidinol production (Fig. 8).

Time course of L-histidinol production

As shown in Fig. 9, growth of AJ 3127 began to increase exponentially after about 8 hr and reached the stationary phase at 24 hr, when accumulation of L-histidinol commenced and then it increased linearly till 56 hr. The

Fig. 5. Effect of amino acids on L-histidinol production.

Each amino acid was added at the concentration of 0.5%. Culture conditions were essentially the same as described in Fig. 4. The concentration of soybean protein hydrolyzate was 4%.

amount of L-histidinol produced finally was 9.2 g/liter (as dihydrochloride), when glucose was almost completely assimilated.

Conversion of L-histidinol to L-histidine by microorganisms

In order to convert L-histidinol to L-histidine, a large number of microorganisms in addition to those listed in Table 4 were tested using medium B, which contained 0.5% of L-histidinol dihydrochloride obtained from the culture filtrate of AJ 3127. Various bacteria were able to produce L-histidine from L-histidinol. Among them, *Brevibacterium flavum* ATCC 14067, which is a glutamic acid-producing bacterium and is the parent strain of AJ 3127, had potent activity. Molar efficiency of the conversion by this microorganism is 49%, when 1.8 g/liter of L-histidine is formed.

DISCUSSION

In order to find the microorganisms which are able to produce L-histidine from sugar and inorganic nitrogen by aerobic fermentation, various micro-

Fig. 6. Effect of nucleic acid-related compounds on L-histidinol production.

Each compound was added at the concentration of 0.1%. Culture conditions were essentially the same as described in Fig. 4. The concentration of soybean protein hydrolyzate was 4%.

Fig. 7. Effect of organic acids on L-histidinol production.

Each organic acid was added at the concentration of 0.5%. Culture conditions were essentially the same as described in Fig. 4. The concentration of soybean protein hydrolyzate was 4%.

Fig. 8. Effect of vitamins on L-histidinol production.

Each vitamin was added at the concentration of 1 mg/liter. Culture conditions were essentially the same as described in Fig. 4. The concentration of soybean protein hydrolyzate was 4%.

Fig. 9. Time course of L-histidinol production.

Culture conditions were essentially the same as described in Fig. 4. The concentration of soybean protein hydrolyzate was 4%.

Table 4. Conversion of L-histidinol into L-histidine by microorganisms.

Basal medium: Glucose 7%, KH_2PO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.04%, $(\text{NH}_4)_2\text{SO}_4$ 0.5%, yeast extract 0.5%, peptone 0.2%, L-histidinol 2HCl 0.5%, CaCO_3 2.5%, pH 7.0. Fermentation was carried out at 31° for 72 hr.

Microorganisms	L-Histidine produced (g/liter)
<i>Micrococcus luteus</i> ATCC 398	1.0
<i>Staphylococcus epidermidis</i> CCM 50	0.9
<i>Aeromonas punctata</i> IAM 1646	1.0
<i>Escherichia coli</i> ATCC 13070	0.1
<i>Enterobacter liquefaciens</i> ATCC 14460	0.8
<i>Pseudomonas perlurida</i> ATCC 490	0.9
<i>Flavobacterium heparinum</i> ATCC 13125	0.7
<i>Brevibacterium flavum</i> ATCC 14067	1.8
<i>Brevibacterium divaricatum</i> NRRL B-2312	1.2
<i>Corynebacterium acetoacidophilum</i> ATCC 13870	1.1
<i>Arthrobacter citreus</i> ATCC 11624	0.6

organisms were screened, but none of them could produce L-histidine. Among the auxotrophs tested, however, a histidine auxotroph derived from *Brevibacterium flavum* ATCC 14067 was found to produce L-histidinol. Moreover, L-histidine formation from L-histidinol obtained according to the above method was carried out successfully by various microorganisms.

As the biosynthesis of L-histidine in microorganisms is presumed to be regulated by endogenous histidine, probably through inhibition of the condensation of ATP and phosphoribosylpyrophosphate (PRPP) (6), extracellular histidine will not be produced. Meanwhile, L-histidine is considered not to inhibit the activity of histidinol dehydrogenase which converts L-histidinol to L-histidine. On the other hand, as the precursors of L-histidine, L-histidinol (4, 5, 8), L-histidinol phosphate (7), imidazoleacetole (8), imidazoleacetole phosphate (7), imidazoleglycerol (8), and imidazoleglycerol phosphate (7) were detected in the culture broth of histidine auxotrophs of *Escherichia coli*, *Neurospora crassa*, *Staphylococcus aureus*, and *Salmonella typhimurium*. An L-histidinol-producing strain AJ 3127 which requires L-histidine for its growth may be deficient in histidinol dehydrogenase activity.

L-Histidinol production by AJ 3127 is found to be remarkably depressed in the presence of exogenous adenine or AICA-R in addition to L-histidine. Biosynthesis of L-histidinol is known to proceed via biosynthesis of purine nucleotides, to which both adenine and AICA-R belong as one of the members. Therefore, purine nucleotides which will be formed from adenine or AICA-R may depress purine nucleotide synthesis, and subsequently L-histidinol production.

Soybean protein hydrolyzate increases L-histidinol production by about

50% when it is used instead of L-histidine, a substance required by AJ 3127. This suggests that something in soybean protein hydrolyzate other than histidine is effective for L-histidinol production by AJ 3127.

Further investigation will be carried out to clarify these aspects.

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