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An actinomycete producing L-3,4-dihydroxyphenylalanine from L-tyrosine

C. P. SUKUMARAM, D. V. SINGH, P. D. KHEDKAR and P. R. MAHADEVAN*

Central Research Laboratory, Indian Drugs and Pharmaceuticals Limited, Rishikesh, Virbhadra 249 202

*Foundation for Medical Research, 84–A, R. G. Thadani Marg, Bombay 400018

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Abstract. An actinomycete was isolated during a soil screening programme to obtain L-3,4-dihydroxyphenylalanine producers. A mutant of this organism was isolated by chemical mutagenesis and it accumulated 1 g/litre L-dihydroxyphenyl-alanine when grown on L-tyrosine. Resting cells converted 30% of tyrosine in the reaction mixture. The use of resting cells for dihydroxyphenylalanine production is advantageous as it eliminates interfering substances which accumulate during fermentation.

Keywords. Actinomycete; L-dihydroxyphenylalanine production; fermentation.

Introduction

3,4-Dihydroxyphenylalanine (L-DOPA) is generally extensively used for treat-ment of Parkinsonism. L-DOPA is synthesised *in vivo* from L-tyrosine by the action of tyrosine hydroxylase (E. C. 1.14.3.a). Tyrosine hydroxylase from mammals, mushroom, *Streptomyces*, *Neurospora*, *Bacillus*, *Vibrio tyrosinaticus*, and *Pseudomonas* have already been studied in detail (Haneda *et al*., 1973; Yoshida *et al*., 1973, 1974a,b; Rosazza *et al*., 1974).

Economical production of L-DOPA by a microbial transformation of L-tyrosine was the focus of attention of many workers (Yoshida *et al*., 1974a,b; Tanaka *et al*., 1974). Use of L-tyrosine and its derivatives has also been studied in various micro-organisms (Yoshida *et al*., 1973).

In this paper, production of L-DOPA from L-tyrosine by micro-organisms, especially actinomycetes from soil, is reported.

Materials and methods

Media

The medium for screening consisted of 20 g glucose, 5 g $(NH_4)_2SO4$, 100 mg KH_2PO_4, 200 mg $MgSO_4.7H_2O$, 2g L-tyrosine, 500mg ferrous lactate , 20g peptone (Difco) and 20 g agar per litre with pH adjusted to 7·2.

The medium for growing the inoculum comprised of 20 g glucose, 500 mg NaCl, 5 g peptone and 5 g yeast extract per litre. The pH was adjusted to 7·2.

Fermentation medium contained 20 g glucose, 5 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 200 mg $MgSO_4 \cdot 7H_2O$, 20 g Corn steep liquor, 3 g $CaCO_3$ per litre adjusted to pH 7·2.

The isolated organisms were maintained on medium consisting of 4 g yeast extract, 1·0 g malt extract, 4 g glucose, 20 g agar per litre, pH adjusted to 7·3.

Screening of micro-organisms

Soil samples were screened, for micro-organisms producing L-DOPA from L-tyrosine, by using the specific colour reaction around the colonies which accumulate L-DOPA (Tanaka *et al.*, 1974). The plates were incubated for 2 to 3 days at 27° C until violet to black pigmentation had developed. Colonies with intense pigmentation were picked up. These isolates were further streaked on screening plates and incubated for 2 to. 3 days at 27° C and colonies were selected, based on intensity of the coloured zones. These colonies were examined for L-DOPA production. The isolated organisms were maintained on maintenance medium at 27° C.

Mutagenesis

One of the isolated organisms from soil (Dl) was selected for mutagenisation. By N-methyl-N-nitro-N′ nitrosoguanidine (NTG) treatment. The cells grown on agar slant were suspended in sterile phosphate buffer (0·05 M, pH 6·0). Two ml of the cell suspension was mixed with an equal volume of NTG solution (0·4 mg/ml in the same buffer) and incubated for 20 min at room temperature. After the incubation, cells were collected by centrifugation at 3,000 rpm for 20 min. The cells were washed three times with saline to ensure removal of the mutagen and resuspended in sterile saline. Then an aliquot (0·1) ml of a suitably diluted cell suspenion was plated on screening medium.

L–DOPA production

Isolates or mutants were transferred to the medium used for making the inoculum and grown for 24 h at 27° C. This was inoculated into fermentation medium (at 5%) and grown for 48 h at 27° C. The pH was adjusted to. 5·5 with 1 N HCl and ascorbic acid was added to make 550μg/ml. L-tyrosine and L-ascorbic acid in the ratio 4·3 : 2·75 were added every 2h thrice. The incubation was continued for 15 h at 27° C. The suspension was then centrifuged at 3000 rpm for 20 min and L-DOPA was determined in the supernatant by Arnow's method (1937).

To overcome the limited solubility of L-tyrosine and to ensure a stable pH of 5·5 during fermentation, equal volumes of an acidic solution and an alkaline solution of tyrosine were added (Yoshida *et al.*, 1973).

Identification of L–DOPA

L-DOPA and L-tyrosine in culture broth were separated by descending paper chromatography on Whatman No. 1, using *n*-butanol-acetic acid-water (4:1:2 v/v) as solvent system. Spots were identified by spraying with ninhydrin and

also by a reagent consisting of equal volumes 0·1 M $FeCl_3$ and 0·1 M $K_3Fe(CN)_6$. The amount of L-DOPA was determined by Arnow's method.

Results

Initial screening

Seven isolates were obtained on screening of soil for the production of L-DOPA. These isolates converted L-tyrosine to L-DOPA at 10·4, 4·0, 4·0, .2·0, 2·4, 2·8 and 3·6 % efficiency. Among these, the isolate D1 showing highest conversion was taken up for further improvement.

Several mutants of D1 were isolated after NTG treatment. Ten such isolates when studied for their fermentation of L-tyrosine to L-DOPA showed improvement over the parent strain (table 1). One of the isolates M9 showed three-fold increase in efficiency. It converted 32·5 % of L-tyrosine to L-DOPA.

Table 1. L-DOPA production by wild type and mutant of soil actinomycete.

Mutant		L-DOPA (mg/ml)	% L-DOPA formed
Parent	D1	0·5	11·0
	M4	0·8	18·6
	M6	0·7	16·2
	M8	0·8	18·6
	M9	1·4	32·5
	M11	0·7	16·2
	M12	0·8	18·6
	M18	0·8	18·6
	M19	0·8	18·6
	M21	0·7	16·2
	M22	0·7	16·2

The ratio of L-tyrosine and L-ascorbic acid was 4·3 to 2·75 mg/ml.

Studies with M9

The fermentation conditions for the optimum conversion of L-tyrosine to L-DOPA by M9 were determined. Maximum yield of L-DOPA was obtained at 27° C.

Studies with restining cells

Using resting cells, maximum amount of L-DOPA was formed at 20h (table 2). Optimum concentration of L-tyrosine for maximum production was determined (table 3). Maximal conversion (28·6%) was observed at 2 mg per ml L-tyrosine.

Table 2. Effect of various incubation periods on L-DOPA formation by resting cells.

Incubation period (h)	Conversion of L-tyrosine to L-DOPA (%)
8	10·4
12	16·1
16	21·0
20	28·4
24	20·6

The concentrations of L-tyrosine and L-ascorbic acid were 50 and 25 mg in 50 ml of reaction mixture. Cells were collected from 75 ml fermented broth after 72 h growth at 27° C by centrifugation at 3000 rpm for 20 min. The cells were suspended in phosphate buffer (0·05'M) at pH 5·5 and L-ascorbic acid and L-tyrosine were added and incubated at 27° C For various periods.

Table 3. Effect of tyrosine concentrations on L-DOPA formation by resting cells.

L–tyrosine (mg/ml)	Conversion of L-tyrosine to L-DOPA (%)
0·5	24·8
1·0	28·4
1·5	28·5
2·0	28·6
2·5	22·7
3·0	16·6

The concentrations of L-tyrosine and L-ascorbic acid in this experiment were 100 and 50 mg respectively in 50 ml of reaction mixture.

Identification of product

The identity of the product was established by isolation and characterization using paper and thin layer chromatography with authentic samples.

Discussion

Production of L-DOPA from L-tyrosine was reported earlier from this laboratory by a *Pseudomonad* mutant 101 (Singh *et al.*, 1973). The yield was not very significant due to the presence of high DOPA decarboxylase activity of the mutant. However, better yields could be obtained by using formyl-L-tyrosine.

Earlier attempts to produce L-DOPA by fermentation of L-tyrosine was vitiated by the presence of enzymes catabolising the substrate and the product, and thus required the use of specific enzyme inhibitors (Rosazza *et al.*, 1974; Singh *et al.*, 1973).

In our attempts to obtain new organisms which may be able to produce L-DOPA from L-tyrosine economically, an actinomycete is obtained, which on mutation, yielded a mutant which can convert 33% L-tyrosine to L-DOPA in the fermentation system described. By using an intact cell system and avoiding an elaborate and complex fermentation, a method for production of DOPA from L-tyrosine was developed.

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