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TECHNIQUE FOR IMPROVED PRODUCTION OF 3,4 DIHYDROXY PHENYL L-ALANINE BY *ASPERGILLUS ORYZAE*

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Abstract

In the present study, mould mycelium of *Aspergillus oryzae* was used for biochemical conversion of L-tyrosine to L-DOPA because tyrosinase is an intracellular enzyme. The mutant *Aspergillus oryzae* IIB-6 was found to yield 3.72 fold higher production of L-DOPA than the parental strain. The comparison of kinetic parameters was also done which showed the greater ability of mutant to yield L-DOPA. When cultures grown on various incubation periods, were monitored for Qp, Qs and qp, there was significant enhancement ($p<0.0025–0.005$) in these variables by mutant strain of *A. oryzae* IIB-2 over GCB-47 on all the rates.

Introduction

L-DOPA (3,4-dihydroxy phenyl L-alanine) occurs naturally in *Vicia faba* beans and seeds of *Mucana pruriens*. Its production has been described by a number of workers (Sih *et al*., 1969; Haq *et al*., 1998; Fling & Paul, 2001). L-DOPA is produced from L-tyrosine by one-step oxidation reaction by submerged fermentation (Haneda *et al*., 1973). The optimisation of cultural conditions is necessary for the successful fermentation process. The key enzyme responsible for biosynthesis of L-DOPA is 'tyrosinase' (Rosazza *et al*., 1995; Ali & Haq, 2000). Tyrosinases are widely distributed and highly purified enzymes, derived from microbial (*Aspergillus, Rhizopus* & *Neurospora* spp.) and plant sources (*Agaricus* & *Vicia* spp.). However, in microorganisms tyrosinase activity is generally very weak and L-tyrosine and L-DOPA are rapidly decomposed to other metabolites. Thus, stoichiometric formation of L-DOPA is difficult to achieve (Kumagai *et al*., 1969). The mycelial activity of *Aspergillus oryzae* or *Aspergillus flavus* catalysing L-tyrosine to L-DOPA was observed in acidic range below pH 5.0 (Singh, 1999). In present study, the goal was to increase the biomass of *A. oryzae* and consequently the production of L-DOPA, using shake flask technique. Tyrosinase is an intracellular enzyme. So, mould mycelium was used for biochemical conversion of L-tyrosine to L-DOPA.

Materials and Methods

Organism and UV induced mutagenesis: *A. oryzae* strain GCB-47 obtained from Institute of Industrial Biotechnology, GC University, Lahore was maintained on potato dextrose agar medium. It was improved after UV-irradiation. The dose of ultraviolet irradiation was given at a rate of 1.2×10^2 J/m^2/S. One hundred ml of Vogel medium containing (g/l); trisodium citrate 2.5, NH_4NO_3 2.0, KH_2PO_4 5.0, $(NH_4)_2SO_4$ 4.0, $MgSO_4.7H_2O$ 0.2, peptone 2.0, yeast extract 1.0 at pH 5.5 with 15-20 glass beads (2mm, diameter) in 1 liter cotton wool plugged conical flask was sterilized at 15 lbs/in^2 (121°C)

for 15 minutes. A small quantity of conidia from the slant (3 to 5 days old) was aseptically transferred with an inoculating needle to the flask. The flask was incubated at 30°C in an incubator shaker at 200 rpm for 24 hours. The optical density was maintained at 1.0 with the help of a photoelectric colorimeter, using 530 nm filter. Five millilitre of the inoculum was taken in a Petri plate and then UV-treatment was given from 15 to 25 minutes, intervals following the method of Pontecarvo *et al*., (1969). The mutant cultures were incubated at 30°C for 3-4 days for maximum sporulation.

Cultivation of mycelium: Submerged culture method (Raju *et al*., 1993) was employed for cultivation of mycelium. Conidial inoculum was prepared in 10 ml of Monoxal O.T. (Dioctyl ester of Sodium sulpho succinic acid). Twenty-five ml of cultivation medium containing (% w/v); glucose 2.0, polypeptone 1.0, NH_4Cl 0.3, KH_2PO_4 0.3, $MgSO_4.7H_2O$ 0.02, yeast extract 1.0 at pH 5.0 was taken in 250 ml shake flasks. The medium was autoclaved at 15-lb/inch^2 pressure (121ºC) for 15-minutes and seeded with 1.0 ml conidial suspension. The flasks were then incubated in a rotary incubator shaker (200 rpm) at 30ºC for 48 hours. The mycelium was harvested by filtering through a funnel and washed free of adhering medium with ice-cold water. The mycelium was dried in filter paper folds and it was stored.

Reaction procedure: The reaction for L-DOPA production from L-tyrosine was carried out in a suspension of intact mycelium. The mycelium were suspended in reaction mixture (Haneda *et al*., 1973). Fifteen ml of acetate buffer (pH 3.5, 50mM) containing (mg/ml); L-tyrosine 2.5, L-ascorbic acid 5.0 and intact mycelium 75.0 were taken in 250 ml Erlenmeyer flask. The reaction was carried out aerobically at 50ºC for 60 minutes in a hot plate with magnetic stirrer. The sample was withdrawn, centrifuged (5,000/g) and supernatant was kept under dark for further investigation.

Assay methods: L-DOPA and L-tyrosine were determined colorimetrically according to the method of Arnow (1937). For determination of L-DOPA, one ml of the supernatant was taken and in it 1.0 ml of 0.5N HCl along with 1.0 ml of Nitrite molybdate reagent was added. A yellow colour appeared. Then 1.0 ml of 1.0N NaOH was added, which gave red colouration and total volume was made up to 5.0 ml. The colour intensity was read by photoelectric colorimeter (Model: AE-II, ERMA, Japan) using green Wratten filter of 530 nm and the amount of L-DOPA was determined from Arnow's standard curve of L-DOPA (Arnow, 1937). For L-tyrosine, one ml of supernatant from the reaction mixture was taken and in it 1.0 ml of mercuric sulphate reagent was added and placed in boiling water bath for 10 minutes. After which it was cooled and 1.0 ml of nitrite reagent was added. Total volume was made up to 5.0 with distilled water. It was compared in a colorimeter and the amount of L-tyrosine was determined from Arnow's standard curve of L-tyrosine (Arnow, 1937).

Kinetic parametric studies and statistical analyses: The kinetic parameters were studied according to the procedures of Pirt (1975). The statistical analyses (US-statica, version-4) were based on Duncan's multiple range and ANOVA-II design tests (Snedecor & Cochran, 1980).

Table 1. Screening of UV-irradiated mutants of *Aspergillus oryzae* for the production of L-DOPA.

***A. oryzae* mutants**	**L-tyrosine consumed (mg/ml)**	**L-DOPA produced (mg/ml)**
Parent (GCB-47)	0.92	0.32
IIB-1	0.95	0.16
IIB-2	1.21	1.28
IIB-3	1.06	0.74
IIB-4	1.35	0.30
IIB-5	0.87	0.08
IIB-6	0.94	0.82

Incubation temperature 30ºC, pH 5.5, Agitation 160 rpm.

Table 2. Biosynthesis of L-DOPA by parental (GCB-47) and mutant (IIB-2) strains of *Aspergillus oryzae*.

Incubation period (Hours)	**L-DOPA produced (mg/ml)**		**L-tyrosine consumed (mg/ml)**	
	Parent	**Mutant**	**Parent**	**Mutant**
24	0.21	0.16	0.48	0.26
36	0.34	0.68	0.62	0.48
48	0.36	1.34	0.86	0.74
60	0.32	1.26	1.10	0.80
72	0.29	1.12	1.39	0.86
84	0.24	1.06	1.78	0.96
96	0.18	0.92	1.96	1.38

L-tyrosine added 2.5 mg/ml, Incubation temperature 30ºC, Agitation intensity 160rpm, *On the basis of L-tyrosine consumed. ± indicates the standard deviation among the three parallel replicates; the values with letters differ significantly at $p < 0.005$.

Results and discussion

The production of L-DOPA by mutant cultures ranged from 0.08 to 1.28 mg/ml (Table 1). Of all the mutant cultures examined, the strain IIB-2 gave the highest yield of L-DOPA. It might be due to the fact that UV-irradiation has altered the actual structure of DNA by photolysis i.e., formation of pyrimidine dimmers. The structural change in DNA is related with the activity of enzyme tyrosinase. Thymidine-thymidine dimmers promote mycelial growth in the form of round pellets and subsequently enzyme activity, which resulted in the greater excretion of L-DOPA from the mycelial cells. Only in a few research reports such findings have been observed (Kumagai *et al*., 1969). In the present study, the mutant strain of *Aspergillus oryzae* IIB-2 was found to yield 3.72-fold higher production of L-DOPA as compared to the parent culture.

The maximum production of L-DOPA by parental GCB-47 was 0.36 mg/ml, whereas 1.34 mg/ml L-DOPA was obtained by mutant IIB-2, 48 hours after incubation (Table 2). Reduction in the formation of L-DOPA occurred when the incubation period was increased beyond 48 hours. At 96 hours of incubation period, the production of L-DOPA became very low, for both the parental (0.18 mg/ml) as well as the mutant strain (0.92 mg/ml). The decrease in L-DOPA production with the increase in incubation period might be due to the overgrowth of fungi and also an age factor. Similar findings have also been reported by Raju *et al*., (1993). Haneda *et al*., (1973) used *A. oryzae* for the conversion of L-tyrosine to L-DOPA and obtained maximum production (0.86 mg/ml), 72 hours after inoculation. Thus our finding (1.34 mg/ml L-DOPA) is more encouraging and economically significant due to greater production and decreased in incubation period.

Table 3. Comparative study of volumetric rates by parental (GCB-47) and mutant (IIB-2) strains of *Aspergillus oryzae*.

Incubation period (Hours)	Volumetric rates (mg/ml/h)			
	Qp		Qs	
	Parent	Mutant	Parent	Mutant
24	0.008 ± 0.002b	0.007 ± 0.001g	0.020 ± 0.002b	0.011 ± 0.002e
36	0.009 ± 0.001a	0.019 ± 0.001c	0.017 ± 0.002e	0.018 ± 0.002c
48	0.007 ± 0.001c	0.028 ± 0.001a	0.018 ± 0.002d	0.021 ± 0.001b
60	0.005 ± 0.001d	0.021 ± 0.002b	0.018 ± 0.002d	0.021 ± 0.001b
72	0.004 ± 0.003e	0.012 ± 0.002c	0.019 ± 0.002c	0.022 ± 0.001a
84	0.003 ± 0.001f	0.011 ± 0.001d	0.021 ± 0.002a	0.022 ± 0.002a
96	0.002 ± 0.001g	0.014 ± 0.001d	0.020 ± 0.003b	0.022 ± 0.001a

Qp = Slope of product (mg/ml) / time of fermentation (h), Qs = Slope of sustrate utilized (mg/ml) / time of fermentation (h), ± indicates the standard deviation of three parallel sets of replicates, within the column, values with letters differ significantly at $p<0.005$.

Table 4. Comparative study of specific rate constants by parental (GCB-47) and mutant (IIB-2) strains of *Aspergillus oryzae*.

Incubation period (Hours)	Specific rate constants (mg/mg/h)			
	qp		qs	
	Parent	Mutant	Parent	Mutant
24	0.00184 ±0.0001c	0.01107 ±0.0005g	0.00420 ± 0.0001d	0.00773 ± 0.0002f
36	0.00321 ±0.0002b	0.01283 ±0.003de	0.00585 ± 0.0002b	0.00905 ± 0.0001e
48	0.00270 ±0.0001d	0.03740 ±0.002a	0.00645 ± 0.0002a	0.02065 ± 0.005a
60	0.00170 ±0.0001e	0.02646 ±0.002b	0.00586 ± 0.0004b	0.01680 ± 0.005b
72	0.00117 ± 0.00005e	0.01742 ± 0.0005c	0.00560 ± 0.0002b	0.01334 ± 0.002c
84	0.00068 ± 0.00002f	0.01338 ± 0.002d	0.00509 ± 0.0005c	0.01212 ± 0.003d
96	0.00335 ± 0.0001a	0.00881 ± 0.0005f	0.00364 ± 0.0003e	0.01322 ± 0.0001cd

qp = specific L-DOPA production rate (mg/mg/h) = (μ x Yp/s), qs = specific substrate uptake rate (mg/mg/h) = (μ x Yx/s). The values differ significantly at $p < 0.0025$ while ± indicates the standard deviation among the replicates.

The comparative time course and kinetic parameters i.e., volumetric rates (Qp, and Qs in mg/ml/h) and specific rate constants (qp and qs in mg/mg/h) on the production of L-DOPA by parental (GCB-47) and mutant (IIB-2) are depicted in Tables 3 and 4. Maximum growth in terms of overall specific growth and production rate was significantly different ($p<0.005$ & $p<0.0120$, respectively) during growth of GCB-47 and IIB-2, 24, 48, 72, 96 hours after incubation. When cultures grown on various incubation period, were monitored for Qp, Qs and qp, there was significant overall enhancement ($p < 0.0025$ - 0.005) in these variables by mutant strain of *A. oryzae* IIB-2 over GCB-47 for all the rates. Other workers have also described the hyperproducability of mutants over the isolated cultures of *A. oryzae* (Sarin *et al*., 1980).

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