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SHORT CONTRIBUTION

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Inducive effect of cresoquinone on microbiological transformation of L-tyrosine to 3,4 dihydroxy phenyl L-alanine by *Aspergillus oryzae* NG-11^{P1}

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Abstract The present work describes the inducive effect of cresoquinone on microbiological transformation of L-tyrosine to 3,4 dihydroxy phenyl L-alanine (L-DOPA) by *Aspergillus oryzae* NG-11^{P1}. Mould mycelium was used for biochemical conversion of L-tyrosine to L-DOPA because tyrosinases, β-carboxylases and tyrosine hydroxylases are intracellular enzymes. The maximum conversion of L-tyrosine to L-DOPA (0.428 mg/ml) was achieved after 60 min of biochemical reaction. To enhance the production of L-DOPA, cresoquinone was added to the reaction mixture. Best L-DOPA biosynthesis results were observed when the concentration of cresoquinone was 3.5×10^{-6} M (1.686 mg/ml L-DOPA produced with 1.525 mg/ml consumption of L-tyrosine). Cresoquinone not only increased enzyme activity but also enhanced cell membrane permeability to facilitate secretion of enzymes into the reaction broth. Comparison of kinetic parameters revealed the ability of the mutant to yield L-DOPA {$Y_{p/x}$ [i.e., mg L-DOPA formed (mg cells formed)$^{-1}$] =7.360±0.04}. When the culture grown on various cresoquinone levels was monitored for Q_p, Q_s and q_p [Q_p: mg L-DOPA produced ml^{-1} h^{-1}; Q_s: mg substrate consumed ml^{-1} h^{-1}; q_p: mg L-DOPA formed (mg cells)$^{-1}$ h^{-1}], there was significant enhancement (P<0.025) of these variables.

Introduction

L-DOPA (3,4-dihydroxy phenyl L-alanine) is the drug of choice in the treatment of Parkinson's disease and for controlling the changes in enzymes of energy metabolism in Myocardium following neurogenic injury. Parkinson's disease affects individuals worldwide, with the incidence increasing sharply with age to about 200–250 per 20 million in those over 60 years old (Rarveen and Michael 1992). L-DOPA occurs naturally in seeds, pods and beans of *Vicia faba* and *Mucana pruriens* (Rosazza et al. 1995; James and Fling 2001). L-DOPA is produced from L-tyrosine by a one-step oxidation reaction in submerged fermentation. The key enzymes responsible for biosynthesis of L-DOPA, i.e., tyrosinases, are derived from microbial (*Aspergillus*, *Rhizopus* and *Neurospora* spp.) and plant sources (*Agaricus* and *Vicia* spp.). However, in microorganisms, tyrosinase activity is generally very weak and L-tyrosine and L-DOPA are rapidly decomposed to other metabolites (Raju et al. 1993). Thus, stoichiometric formation of L-DOPA is difficult to achieve. The mycelia of fungal cultures of different *Aspergillus* species show a greater frequency of catalytic activity of L-tyrosine to L-DOPA conversion than all other mushrooms. A diagram of the biochemical reaction converting L-tyrosine to L-DOPA can be seen in Scheme 1.

According to Nofre et al. (1960), when the monophenolase tyrosinase acts upon tyrosine, the first product of the reaction is L-DOPA. The variation in production of L-DOPA can be attributed to the presence of contaminating enzymes, which oxidize L-DOPA but not L-tyrosine in the tyrosine-tyrosinase cycle. Fling (1998) worked on the recovery of L-DOPA from dopamine-containing materi-

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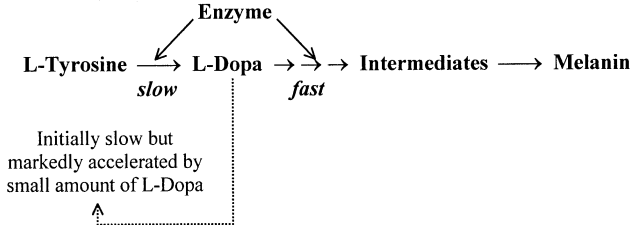

Scheme 1

als. L-DOPA was recovered from velvet bean using an HO-extraction technique. In this paper, we describe the increased biomass, and consequently the increased production of L-DOPA, of a mutant culture of *Aspergillus oryzae* NG-11^{P1}, using a submerged fermentation technique in 250 ml Erlenmeyer flasks. Tyrosinase, β-carboxylase and tyrosine hydroxylase are intracellular enzymes, therefore mould mycelium was used for biochemical conversion of L-tyrosine to L-DOPA. Different concentrations of cresoquinone were added during the course of the reaction to increase the production of L-DOPA in the reaction mixture.

Materials and methods

Microorganism

A. oryzae mutant culture NG-11^{P1} was used for present study. This culture was obtained from Biotechnology Research Labs, Government College, Lahore, Pakistan. It was maintained on potato dextrose agar medium and stored at 5°C in a refrigerator. The culture has already been made resistant to 2-deoxy D-glucose (Sigma, Gillingham, Dorset, UK) and improved by alternate treatments with ultraviolet irradiation (1.2 J m^{-2} s^{-1}) and *N*-methyl *N*-nitro *N*-nitroso guanidine of 24-h-old mycelial cells in Vogel's medium (Pontecarvo et al. 1969).

Mycelial cultivation

A submerged culture method in 250 ml Erlenmeyer flasks was employed for all microbial cultivations. Conidial inoculum was prepared in 10 ml Monoxal O.T. (dioctyl ester of sodium sulpho succinic acid). Cultivation medium (25 ml) containing (% w/v): glucose 2.0, polypeptone 1.0, NH_4Cl 0.3, KH_2PO_4 0.3, $MgSO_4 \cdot 7H_2O$ 0.02, yeast extract 1.0, at pH 5.5 was placed in 250 ml shake flasks. The medium was autoclaved at 15-lb/inch^2 pressure (121°C) for 15 min and seeded with 1.0 ml conidial suspension (1.5×10^6 conidia/ml). The flasks were then incubated on a rotary incubator shaker (200 rpm) at 28°C for 48 h. The mycelia were harvested by filtering through a funnel and washed free of adhering medium with ice-cold water (1°C). The mycelium was dried in filter paper folds (Whatman No. 44, Brazil) and stored at 4°C until further use.

Reaction procedure, critical phases and assay methods

The reaction for L-DOPA production from L-tyrosine was carried out in a suspension of intact mycelia in reaction mixture (Haneda et al. 1969). Acetate buffer (15 ml; pH 3.5, 50 mM) containing (mg/ml): L-tyrosine 2.5, L-ascorbic acid 5.0 and intact mycelia 15.0, was placed in a 250 ml Erlenmeyer flask. The reaction was carried out aerobically (1.0 l l^{-1} min^{-1} air supply) at 50°C for different time intervals (30–70 min) in a hotplate with magnetic stirrers (Perkin Elmer, Boston, Mass.). Samples were withdrawn, centrifuged (9,000 rpm for 10 min) and the clear supernatant was kept in the dark at room temperature for further investigation. L-DOPA and L-tyrosine were determined according to the methods of Arnow (1937). The kinetic parameters were studied according to the procedures of Pirt (1975).

Results

The effect of different incubation periods (30–70 min) on the reaction mixture for bioconversion of L-tyrosine to L-DOPA by a 2-deoxy D-glucose-resistant mutant culture of *A. oryzae* NG-11^{P1} using a hotplate with magnetic stirrers was investigated (Fig. 1). The maximum conversion of L-tyrosine to L-DOPA was achieved after 60 min of biochemical reaction. When the incubation period was increased beyond 60 min, L-DOPA production was greatly reduced. The inducive effect of cresoquinone

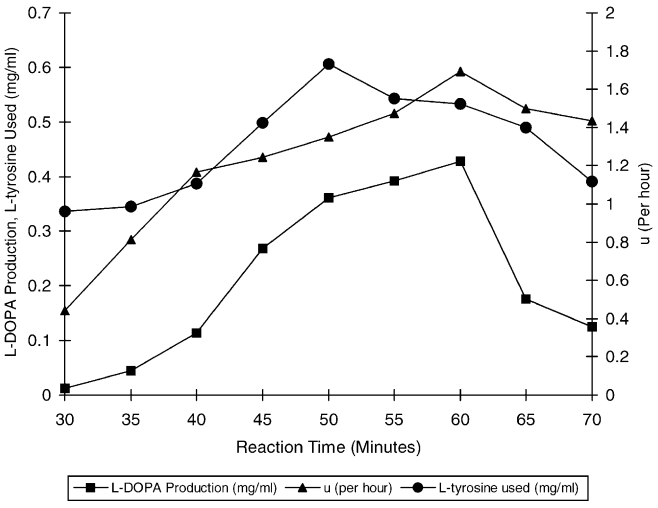

Fig. 1 Effect of incubation period (during reaction) on the production of 3,4 dihydroxy phenyl L-alanine (L-DOPA) by a stabilized mutant culture of *Aspergillus* oryzae NG-11^{P1}. Culture conditions: Initial pH 5.5, incubation temperature 28°C, fermentation period 48 h, inoculum level 1.0% (1.5×10^6 conidia/ml). All experiments were run in parallel in triplicate. Reaction conditions: L-tyrosine added 2.5 mg/ml, dry mycelial wt. 15 mg/ml, L-ascorbic acid 5.0 mg/ml and pH range 3.5, reaction temperature 50°C. μ h^{-1} Specific growth rate

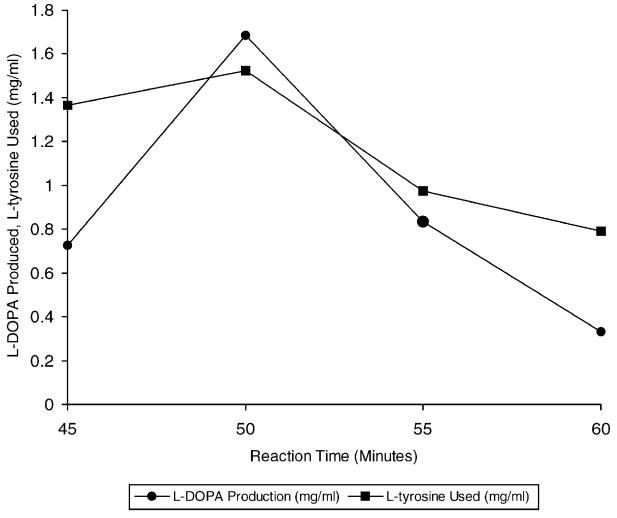

Fig. 2 Effect of cresoquinone concentration (3.5×10^{-6} M) on microbiological transformation of L-tyrosine to L-DOPA by the mutant culture of *A. oryzae* NG-11^{P1} for different incubation periods using a hotplate with magnetic stirrers. Culture and reaction conditions as in Fig. 1. All experiments were run in parallel in triplicate

Table 1 Inducive effect of cresoquinone concentration on microbiological transformation of L-tyrosine to 3,4 dihydroxy phenyl L-alanine (L-DOPA) by a mutant culture of *Aspergillus oryzae* NG-11^{P1} for different incubation periods using a hotplate with magnetic stirrers. Culture and reaction conditions as in Fig. 1

Cresoquinone concentration ($\times10^{-6}$ M)	Reaction time (min)							
	45		50		55		60	
	L-DOPA (mg/ml)	L-Tyrosine used (mg/ml)	L-DOPA (mg/ml)	L-Tyrosine used (mg/ml)	L-DOPA (mg/ml)	L-Tyrosine used (mg/ml)	L-DOPA (mg/ml)	L-Tyrosine used (mg/ml)
1.0	0.212	0.920	0.518	0.625	0.316	0.604	0.127	0.458
1.5	0.268	0.988	0.766	0.782	0.370	0.712	0.135	0.460
2.0	0.379	0.990	0.922	0.958	0.392	0.798	0.178	0.488
2.5	0.452	1.231	1.025	1.112	0.406	0.845	0.215	0.497
3.0	0.594	1.352	1.320	1.336	0.562	0.852	0.288	0.522
3.5	0.726	1.365	1.686	1.525	0.835	0.975	0.332	0.790
4.0	0.702	1.662	1.420	1.620	0.808	1.006	0.330	0.862
4.5	0.614	1.525	1.302	1.538	0.802	1.090	0.316	0.780
5.0	0.580	1.502	1.008	1.532	0.766	1.212	0.298	0.714
5.5	0.535	1.426	0.994	1.404	0.715	1.088	0.255	0.703
6.0	0.418	1.420	0.926	1.392	0.622	0.926	0.246	0.677

Table 2 Comparison of kinetic parameters of the effect of cresoquinone on the microbiological transformation of L-tyrosine to L-DOPA by the mutant culture of *A. niger* NG-11^{P1}. Each value is an average of three parallel replicates. *LSD* Least significant difference. Kinetic parameters: $Y_{x/s}$ mg cells (mg substrate utilized)$^{-1}$, Q_s mg substrate consumed ml^{-1} h^{-1}, q_s mg substrate consumed (mg cells)$^{-1}$ h^{-1}, Q_x mg cells formed ml^{-1} h^{-1}, Q_p mg L-DOPA produced ml^{-1} h^{-1}, $Y_{p/s}$ mg L-DOPA produced (mg substrate consumed)$^{-1}$, $Y_{p/x}$ mg L-DOPA formed (mg cells formed)$^{-1}$, q_p mg L-DOPA formed (mg cells)$^{-1}$ h^{-1}

Cresoquinone levels (10^{-6} M)	Kinetic parameters								LSD	*P**
	Substrate consumption rates				Product formation rates					
	$Y_{x/s}$ (mg cells/mg)	Q_s (mg/ml/h)	q_s (mg/mg cells/h)	Q_x (mg cells/ml/h)	$Y_{p/s}$ (mg/mg)	$Y_{p/x}$ (mg/mg cells)	Q_p (mg/ml/h)	q_p (mg/mg cells/h)		
2.5	0.194	0.704	0.063	0.072	0.442	4.392	0.236	0.043	0.234	S
3.0	0.208	0.767	0.080	0.074	0.940	6.620	0.248	0.072	0.248	S
3.5	0.290	0.962	0.097	0.078	0.887	7.360	0.290	0.081	0.456	HS
4.0	0.216	0.531	0.051	0.060	0.872	3.752	0.193	0.044	0.102	–
4.5	0.162	0.515	0.050	0.063	0.346	3.602	0.149	0.021	0.098	–

*Significance level on the basis of probability. The values differ significantly at $P<0.025$. *HS* Highly significant, *S* significant

concentration on microbiological transformation of L-tyrosine to L-DOPA by mutant *A. oryzae* NG-11^{P1} was investigated (Table 1). The concentration of cresoquinone ranged from 1.0×10^{-6} to 6.0×10^{-6} M (added at the start of the reaction). However, best L-DOPA synthesis results were observed when the concentration of cresoquinone was maintained at 3.5×10^{-6} M (1.686 mg/ml L-DOPA produced with 1.525 mg/ml consumption of L-tyrosine), as shown in Fig. 2.

The data in Table 2 highlight the comparison of kinetic parameters for the inducive effect of cresoquinone addition on the bioconversion of L-tyrosine to L-DOPA. At different concentrations of cresoquinone, all values of $Y_{x/s}$, $Y_{p/s}$ and $Y_{p/x}$ [$Y_{x/s}$ = mg cells (mg substrate utilized)$^{-1}$, $Y_{p/s}$ = mg L-DOPA produced (mg substrate consumed)$^{-1}$, $Y_{p/x}$ mg = L-DOPA formed (mg cells formed)$^{-1}$] were significantly improved over the control (without cresoquinone addition, Table 1). Maximum growth in terms of specific growth rate, Q_x (mg cells formed ml^{-1} h^{-1}) was only marginally different during bioconversion at 3.0×10^{-6} compared to 3.5×10^{-6} M. However, when the cultures were monitored for Q_p and q_s [Q_p = mg L-DOPA produced ml^{-1} h^{-1}, q_s = mg substrate consumed (mg cells)$^{-1}$ h^{-1}] there was significant enhancement ($P<0.025$) in these variables in the presence of 3.5×10^{-6} M cresoquinone. This indicated that the mutant exhibits maximum enzyme activity at this level of cresoquinone addition to the reaction mixture. The value of q_p [mg L-DOPA formed (mg cells)$^{-1}$ h^{-1}] (i.e., specific productivity) at 3.5×10^{-6} M is highly significant. Succinic acid and glutamic acid were also produced in the reaction mixture but their highest volumetric productivities were only 0.014 and 0.01 mg ml^{-1} h^{-1}.

Discussion

L-DOPA is produced from L-tyrosine by a one-step oxidation reaction using submerged fermentation as the method of choice (Haneda et al. 1973). The production of L-DOPA is dependent not only on the culture conditions and type of strain employed but also on the addition of

specific additives to the reaction mixture. In the time course study of L-DOPA production, the maximum conversion rate (0.428 mg/ml) was obtained 60 min after beginning the biochemical reaction. However, after 60 min the production of L-DOPA gradually decreased. This might be due to the fact that L-DOPA and residual L-tyrosine were converted into other metabolites such as dopamine or melanin. Sih et al. (1969) achieved 0.12 mg/ml L-DOPA in a 90-min biochemical reaction. Hence, our finding, i.e., 0.428 mg/ml L-DOPA after 60 min of incubation (μ=0.593 h^{-1}) is more encouraging. The production of L-DOPA, substrate utilization and time of reaction were completely altered with cresoquinone addition to the reaction mixture.

Cresoquinone increased not only the enzyme activity of tyrosinases, β-carboxylases and tyrosine hydroxylases but might also have increased the permeability of the cell membrane to facilitate enzyme secretion. This is important for the catabolism of L-tyrosine to L-DOPA under controlled conditions. Singh (1999) attempted to overexpress L-DOPA by addition of 0.16 µg vermiculite during the reaction and achieved 0.55 mg/ml of the actual product required. In the present study, maximum $Y_{p/s}$, $Y_{p/x}$, Q_p and q_p were several-fold improved over those of some other *Aspergillus* or *Cellulomonas* spp. (Haneda et al. 1973; Paul 1974; Raju et al. 1993). Achieving maximum production of L-DOPA from L-tyrosine depends on medium composition, fermentation design, physical properties of the reaction broth and mycelial morphology of *A. oryzae* used. L-DOPA is a high cost but low yield product. Optimization of adipic acid and thiophene addition might make this mutant (*A. oryzae* NG-11^{P1}) suitable for commercial exploitation of L-DOPA production.

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