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Production of L-DOPA from cell suspension culture of *Mucuna pruriens f. pruriens*

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Summary. Production of L-DOPA was studied in cell suspension culture of Mucuna pruriens f. pruriens. Suspension culture was established in MSI medium composed of half concentration of Murashige and Skoog's salts and 2% sucrose. A two-stage cell suspension culture was developed for enhanced accumulation of L-DOPA. In the first stage, the culture system was composed of MSI medium without $CaCl_2$ which was suitable for cell growth and in the second stage MSI medium containing 42.5 mg.l^{-1} KH_2PO_4 and 4% sucrose favoured L-DOPA production. A discernible higher production of L-DOPA was obtained in this two-stage cell suspension culture in comparison to single stage culture.

Keywords. L-DOPA : 3-(3,4-dihydroxyphenyl)-L-alanine; MS : Murashige and Skoog (1962); Kn : Kinetine; dw : dry weight.

Introduction

3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA) is receiving much attention for its use in the treatment of Parkinson's disease and as a remedy in mental disorder (British Pharma., 1973). The isolation of L-DOPA from natural sources (Guggenheim, 1913; Daxenbichler et al. 1971) and its production by chemical synthesis (Berntsson et al. 1973) and microbial transformation (Sih et al. 1969; Chattopadhyay and Das, 1990) have been reported, but an economically attractive process is yet to be achieved. Among the plant sources, seeds of Mucuna plants are rich in L-DOPA and have the possibility of being used as a commercial source. The in vitro culture of Mucuna for L-DOPA production has, therefore become of increasing interest. Brain (1976) first reported the accumulation of L-DOPA in the media of callus cultures of Mucuna pruriens, at levels of almost 1% (w/v). Mucuna deeringiana cell cultures, in contrast, did not accumulate L-DOPA but catabolised this product to stizolobic acid and CO_2 (Remmen and Ellis, 1980). Suspension-grown cells of Mucuna pruriens have been shown to synthesize and accumulate L-DOPA (Huizing et al. 1985). Wichers et al. (1985) observed that product synthesis can be manipulated by a proper selection of specified nutrients. Our initial studies on callus culture of Mucuna spp. (Chattopadhyay and Datta, 1985) revealed that the calli are capable of synthesizing trace amounts of L-DOPA characteristic of the parent plant. We report here the nutritional requirements for optimal cell growth and L-DOPA production in cell suspension culture of Mucuna pruriens f. pruriens using a two-stage culture system.

Materials and Methods

Cell and culture conditions : Initial callus of Mucuna pruriens (L.) f. pruriens was obtained and maintained following the procedure as reported earlier (Pramanik and Datta, 1986). Calli were kept in a growth chamber under controlled conditions (25±2°C, 30 μE $m^{-2}s^{-1}$. and 16h/8h day/night regime) and subcultured at 15 day intervals. Cell suspension cultures were obtained from stock callus by placing friable calli (50 mg dw/100 ml medium) in induction medium (MSI) containing half concentrations inorganic salts of MS (Murashige and Skoog, 1962) medium and 2% (w/v) sucrose. Suspension cultures were maintained in MSI medium supplemented with 2% sucrose and 0.5 mg.l^{-1} kinetin at 120 rpm on a rotatory shaker under continuous illumination. L-DOPA content was measured both from cells and medium. Data represent combined result.

Analytical procedure :
Extraction of L-DOPA : Cell cultures, cells as well as medium were acidified with 0.1N HCl for 8 h at 4°C and homogenised under cooling. The homogenate was centrifuged (10 min, 2000 rpm) followed by filtration through a 0.45 μm membrane filter. The filtrate was concentrated in vacuo. Aliquots of the concentrated filtrate were used for

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TLC and HPLC analysis of L-DOPA.

TLC analysis : TLC was performed on activated silica gel G glass plates (0.3 mm) using the solvent system of n-butanol : acetic acid : water (2 : 1 : 2) and a mixture of equal proportion of 5% $FeCl_3$ with 10% $K_3Fe(CN)_6$ as the detecting agent against the authentic sample of L-DOPA.

HPLC analysis : Identification and determination of L-DOPA were performed in a Spectra Physics Model SP 8000B instrument controlled by a microprocessor which allows selection of constant pressure or constant flow for quantitation, with an autoinjector, a dual channel plotter/printer and model SP 8440 UV/Vis variable wave-length detector. The column used for HPLC was a Spectra Physics (250 x 4 mm), stainless steel column packed with octylsilane bonded to silica gel (10 µm). Phosphate buffer (pH 6.0) was used as the mobile phase under isocratic conditions at a flow rate 0.5 ml. min^{-1} and the temperature was maintained at 35°C. The UV detector was set at 280 nm. L-DOPA was conclusively detected by comparison of its retention time with that of authentic sample run under identical conditions. The final determination of L-DOPA was achieved by comparison with a calibration graph constructed by plotting peak height versus amount of L-DOPA injected. The relationships were found to be linear over 10 measurements at different concentrations (0.1-1.0 $\mu g.\mu l^{-1}$). For analysis, the extract solution (100 ml) of at least 500 mg cell biomass was used for satisfactory detection by the UV detector and the method was quantitative and reproducible. The identification of L-DOPA was confirmed by its isolation from the cell extract by preparative HPLC and recording of its mass spectrum in a Hitachi Model RMU-6L mass spectrometer at an ionization potential of 70 eV. The spectrum showed ion peaks at m/z (relative intensity) 197 (M^+) (26), 152 (M^+-COOH) (20), 123 (M^+-CH NH_2 COOH) (100), 105 (23), 77 (47), 51 (49) which are compatible with its structure and comparable with those of an authentic sample.

Result and Discussion

Mucuna pruriens f. pruriens cell suspension culture grew as a brownish homogeneous culture. The suspension culture maintained in MSI medium containing 2% sucrose and 0.5 $mg.l^{-1}$ kinetin for two years and accumulated L-DOPA at a low level, was used in the following experiments.

The effects of cytokinins e.g. kinetin and BAP and auxins e.g. NAA, IAA and 2,4-D at different concentrations (0.05-2.0 $mg.l^{-1}$) were examined on cell growth and L-DOPA yield. However, no significant variations in cell growth and L-DOPA production were observed (data not given).

Further experiments showed that accumulation of L-DOPA was significantly influenced by the presence or absence of light (Table 1). It is known that light affects the activity of hydroxylation (Griffith and Conn, 1973), and L-DOPA is considered to be synthesized via hydroxylation of L-tyrosine. In the present study L-DOPA accumulation was found to be increased with continuous illumination in comparison to 16 h photoperiod or in complete dark whereas cell growth was totally unaffected. This is in keeping with reports of other investigation (Wichers et al. 1985).

Table 1 : Effect of illumination on cell growth and L-DOPA production in cell suspension culture of M. pruriens f. pruriens.

Period of illumination (h/day)	Cell yield (g.dw/l medium)	L-DOPA content (% dw)[a]
-	-	-
8	1.0 ± 0.1	0.1 ± 0.02
16	1.5 ± 0.1	0.2 ± 0.02
24	2.0 ± 0.1	0.45 ± 0.01

[a] L-DOPA content expressed as g per 100 g of dried tissue.
Cell growth and L-DOPA for each experiment were measured from cell culture growing in MS1 medium with 2% sucrose and 0.5 $mg.l^{-1}$ Kinetin for 15 days.
The values represents the means ± S.D. of three replicate samples from three different flasks for each experiment.

The effects of decreased $CaCl_2$ concentrations were tested at 85 $mg.l^{-1}$ KH_2PO_4 (usual concentration in MSI medium) on cell growth and L-DOPA production (Fig.1). It is evident that although L-DOPA yield is marginally increased, cell growth is enhanced upto 14 g dw/l medium in the absence of this nutrient.

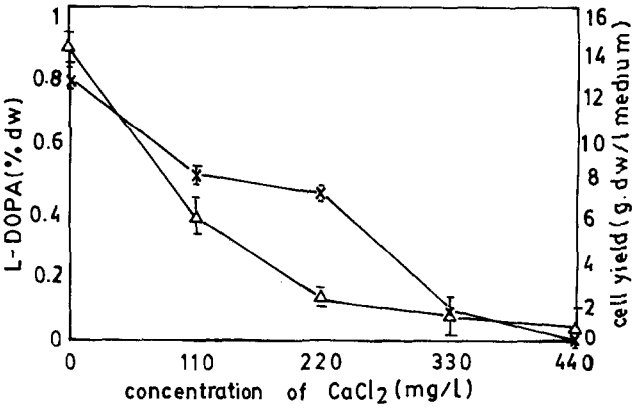

Fig.1. Effect of the $CaCl_2$ concentrations on cell growth (△---△) and L-DOPA production (X-X) in 15 days old cell suspension culture of M. pruriens f. pruriens.

However the suspension culture maintained over four years in MSI medium containing 220 mg.l^{-1} $CaCl_2$ was producing 0.45% dw L-DOPA with 2 g/l medium cell biomass.

Moreover, the effects of decreased KH_2PO_4 concentration at 220 mg.l^{-1} $CaCl_2$ (usual concentration in MSI medium) on cell growth and L-DOPA production are shown in Fig.2. It was observed that improvement of cell growth was not significant but L-DOPA content was increased upto 2.5% dw by lowering the concentration of KH_2PO_4 to half of that of the MSI medium. Absence of KH_2PO_4 resulted in the decrease in L-DOPA production as well as cell biomass. Similar observation was also reported for Stizolobium hassjoo (Obata-Sasamoto and Komamine, 1983). Thus it was evident that media suitable for propagative purposes have a different composition than those suitable for productive purposes.

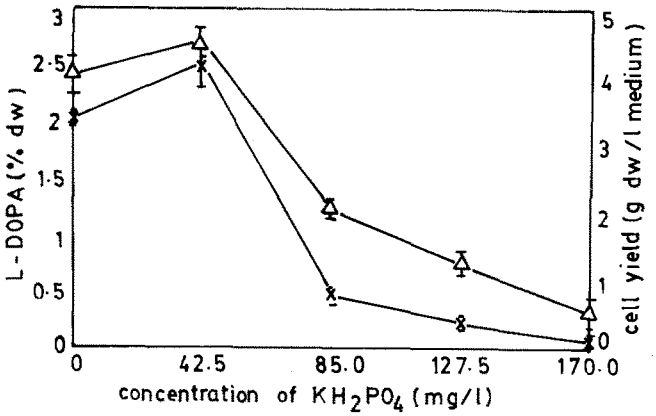

Fig.2. Effect of KH_2PO_4 concentrations on cell growth (△--△) and L-DOPA production (X--X) in 15 days old cell suspension culture of M. pruriens f. pruriens.

The influence of sucrose at a range of concentrations were studied and 4% (w/v) concentration was observed as the optimum level for the production of L-DOPA (Table 2). On the other hand 2% sucrose containing medium was found to be most effective for cell growth. Other carbon sources have no significant effect on either L-DOPA production or cell growth (Results not shown). This increase in L-DOPA content was the highest among the results obtained in any tested combination of medium nutrients, culture conditions and sucrose.

In accordance with the above results, a two-stage culture method (Yamada and Fujita, 1983) was developed for optimal output for production of L-DOPA. In the first stage, cells were grown in MS1 medium without $CaCl_2$ containing 2% sucrose and in the second stage, the resulting cells were cultured in MS1 medium containing 42.5 mg.l^{-1} KH_2PO_4 and 4% sucrose. Both stages were cultured under continuous illumination. The results are shown in Table 3. The growth rate attained nearly 14-fold increase after

Table 2 : Effect of sucrose concentrations on cell growth and L-DOPA production in 15-days old cell suspension culture of M. pruriens f. pruriens

Sucrose (% w/v)	Growth medium[a]		Production medium[b]	
	Growth Index[c]	L-DOPA content (% dw)	Growth Index	L-DOPA content (% dw)
2	7.0 ± 0.2	0.8 ± 0.03	2.2 ± 0.2	2.5 ± 0.4
3	6.0 ± 0.1	0.8 ± 0.04	2.1 ± 0.1	2.8 ± 0.4
4	5.8 ± 0.1	1.0 ± 0.03	2.0 ± 0.1	3.2 ± 0.4

[a] MS1 - $CaCl_2$ + 2% sucrose (other nutrients remained unchanged).
[b] MS1 containing 42.5 mg.l^{-1} KH_2PO_4 + 4% sucrose (other nutrients remained unchanged).
[c] Growth Index = (Harvest dry wt./Inoculum dry wt.)
The values represents the means ± S.D. of three replicate samples from three different flasks for each experiment.

Table 3 : Effect of two-stage culture method on cell growth and L-DOPA production

Stage	Medium	Culture Period (Days)	Growth rate[a] (GI)	L-DOPA content (% dw)
I Cell growth	MS1-$CaCl_2$ + 2% sucrose	15	7.0	0.8
II L-DOPA production	MS1 + 4% sucrose, + 42.5 mg.l^{-1} KH_2PO_4	15	2.0	3.2
Total		30	14	4.0

[a] GI = Harvest dry wt./Inoculum dry wt.

culture for 30 days in the two-stage culture system. The results also revealed that the intracellular accumulation of L-DOPA in the suspension culture of M. pruriens f. pruriens requires a selective salt-limited medium for the maximum release of the product to increase L-DOPA content in culture. The present study, therefore,

strongly indicates that this two-stage culture method has great potential for improvement of L-DOPA productivity. However, the validity of the two-stage concept in the production of L-DOPA on a large scale must still be confirmed in bioreactor experiments with large culture volumina.

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