

**PRODUCTION OF ANTICANCER DRUG
PODOPHYLLOTOXIN BY PLANT CELL CULTIVATION
OF *LINUM ALBUM***

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INDIAN INSTITUTE OF TECHNOLOGY, DELHI

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**PRODUCTION OF ANTICANCER DRUG
PODOPHYLLOTOXIN BY PLANT CELL CULTIVATION
OF *LINUM ALBUM***

by

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"We are always with you"

- MY FAMILY

"You can do it"

- Prof. S. SARAF

"Always go for the best and give your best"

- Prof. V.K. DIXIT

*"Odd time is nothing but indication of good time
waiting ahead"*

- Prof. V.S. Bisaria

"Keep it simple and straight"

- Prof. A.K. Srivastava

Dedicated to all of you.....

Certificate

This is to certify that the thesis entitled “Production of Anticancer Drug Podophyllotoxin by Plant cell cultivation of *Linum album*”, being submitted by Mr. Ashish Baldi to the Indian Institute of Technology, Delhi, for the award of the degree of “Doctor of Philosophy” is a record of the bonafide research carried out by him, which has been prepared under our supervision in conformity with rules and regulations of the “Indian Institute of Technology, Delhi”. The research reports and results presented in the thesis have not been submitted for any degree or diploma in any other University or Institutes.

Prof. V.S. Bisaria

Prof. Ashok K. Srivastava

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"It's just a new beginning"

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ABSTRACT

The discovery of podophyllotoxin, the starting compound for the semisynthetic anticancer drugs Etopophos, Tenipophos and Teniposide stimulated the research on lignans in general and alternate methods to produce these commercially important compounds at industrial scale in particular. The supply of this compound from traditional source i.e. *Podophyllum hexandrum* has become increasingly limited due to low content, intensive collection, lack of cultivation, endangered status of natural source. This supply problem along with uneconomic chemical synthesis is the main motivation for a large number of scientists to search for alternative sources of podophyllotoxin. Tissue cultures of *Podophyllum spp.* and other podophyllotoxin producing plants turned out to be quite recalcitrant, therefore plant cell cultures of *Linum album* were investigated as a source of podophyllotoxin in present study.

Various non transformed and transformed cultures of *L. album* were initiated by variation of growth regulators and *Agrobacterium* strains respectively in order to select a high podophyllotoxin yielding cell line. Cell and hairy root lines developed from *Agrobacterium rhizogenes* (NCIM 5140) mediated transformation of stem segments with podophyllotoxin contents of 4.23 mg/g and 5.12 mg/g respectively were selected for further studies. Genetic confirmation of transformed cultures indicated fragmented intergration of Ri-T DNA with incorporation of only *ags* gene in callus and of both *ags* gene and *rol* gene sequences in hairy roots.

The cell suspension culture of selected cell line was selected with podophyllotoxin productivity of 4.4 mg/L.d in comparison to hairy roots 3.68 mg/L.d. Sucrose was found to be better carbon source than glucose in suspension cultures.

Statistical optimization of medium composition and environmental conditions was done using Plackett-Burman design for selection of the significant effectors and Central Composite Design to determine optimum concentrations of key nutrients. The optimum medium and environmental conditions obtained were: sucrose: 45 g/L, inoculum: 5 g/L, ammonium: 0.28 g/L and calcium chloride: 0.5 g/L, pH: 5.7, temperature: 27 °C, rotational speed: 125 rpm and photoperiod: 16 h/8 h light/dark regime. A maximum of 23.2 g/L of biomass and 7.2 mg/g podophyllotoxin was produced with a 3.68 fold enhancement of podophyllotoxin productivity (17.13 mg/L.d).

Podophyllotoxin and 6-methoxypodophyllotoxin were isolated and identified from *L. album* cells. Cytotoxicity assay on HeLa cell lines indicated production of biologically active lignans with almost same anticancer activity.

Various precursors of phenylpropanoid pathway were incorporated in different concentrations to study their influence on lignan biosynthesis. Among the precursors tested, coniferyl alcohol (4.0 mM) resulted in highest increase in podophyllotoxin (197.40 mg/L) and 6-methoxypodophyllotoxin (9.45 mg/L) production with 34.48% and 18.2% enhancement respectively than the control cultures.

Out of various precursor availability enhancement techniques applied (cyclodextrin complexation and selective permeabilization), highest concentrations of 305.03 mg/L podophyllotoxin and 78.89 mg/L 6-methoxypodophyllotoxin were obtained, when coniferyl alcohol (4.0 mM) was added as hydroxypropyl β -cyclodextrin complex on 8th d of cultivation with a total conversion of 15%.

Addition of various abiotic and biotic elicitors was attempted to increase the lignan productivity. Individual application of 6.0 mM calcium chloride (248.32 mg/L), 20

mg/L yeast extract (268.18 mg/L) and 5% v/v *F. solani* filtered culture filtrate (255.64 mg/L) were found out to be most effective when compared to control cultures (152.08 mg/L). The increase in lignan concentrations due to biotic elicitors was found to be directly related to increase in phenylalanine ammonia lyase activity. Combined treatment of abiotic and biotic elicitors: calcium chloride (1.2 g/L), yeast extract (36 mg/L) and *F. solani* filtered culture filtrate (2% v/v), as determined by statistical design, resulted in 2.92 fold higher podophyllotoxin production (444.27 mg/L) in comparison to control cultures (152.40 mg/L).

Co-cultures of live plant and fungal cells in suspension cultures were developed as a novel methodology for production of phytochemicals in plant cell cultures and patented. Under the optimum conditions of exposure time (24 h) and fungal concentrations, the podophyllotoxin and 6-methoxypodophyllotoxin content were enhanced by 3.76 times (628.84 mg/L) and 8.74 times (116.76 mg/L) respectively for *S. vermifera* (2.5 g/L) treated cultures and 3.4 times (570.27 mg/L) and 4.9 times (65.32 mg/L) respectively for *P. indica* (1 g/L) treated cultures than control cultures. The addition of *F. solani* at 2.5 g/L concentration resulted in maximum enhancement of podophyllotoxin and 6-methoxypodophyllotoxin of 1.96 times (329.07 mg/L) and 5.31 times (70.98 mg/L) respectively than control cultures (167.17 mg/L podophyllotoxin and 13.36 mg/L 6-methoxypodophyllotoxin). The enhancement in lignan accumulation was found to be directly associated with activity of PAL enzyme. Chitin was found to be the main molecule responsible for elicitation in co-culture experiments. The degree of deacetylation of chitosamine and β (1-4)-2-acetamido linkage were also found to exhibit significant effect on elicitation mechanism.

Different modes of cultivation (batch, fed batch and continuous) of *L. album* cells were conducted for the production of podophyllotoxin in optimized medium and cultivation conditions. Aeration rate and dissolved oxygen concentration were found to be important parameters for cell growth and lignan accumulation in batch cultivation. Aeration rate of 0.3 vvm and dissolved oxygen concentration of 30% were found optimum. Batch kinetics was also studied in centrifugal impeller and bubble column bioreactors. Higher biomass (25.9 g/L DCW) and podophyllotoxin production (243.97 mg/L) were observed in centrifugal impeller bioreactor in comparison to stirred tank bioreactor with Setric impeller (23.9 g/L, 181.97 mg/L) and bubble column bioreactor (21.2 g/L, 150.73 mg/L). Increased biomass and lignan formation rates in centrifugal impeller bioreactor were attributed to better mixing time and less shear stress.

An unstructured mathematical model was developed using the batch kinetics in stirred tank bioreactor and extrapolated to identify nutrient(s) feeding strategies for fed-batch and continuous cultivation with cell retention. For fed-batch cultivation, model based feeding strategy [sucrose (510 g/L), ammonium as ammonium sulphate (4.69 g/L) and phosphate as potassium dihydrogen phosphate (3.54 g/L)] in B5 medium with calcium chloride (0.5 g/L) at a feed rate of 0.12 L/d from 7-14th d was experimentally implemented. This strategy resulted in biomass concentration of 89.8 g/L, 597.52 mg/L podophyllotoxin and 198.9 mg/L 6-methoxy-podophyllotoxin on 14th d.

Model derived strategy for continuous cultivation in spin filter bioreactor for cell retention (feeding of B5 medium supplemented with sucrose (75 g/L), ammonium (0.94 g/L) and phosphate (0.68 g/L) for a period of 7-11 d and sucrose (55 g/L), ammonium (0.107 g/L) and phosphate (0.068 g/L) for a period of 12-15 d at a rate of 0.9 L/d) was

experimentally implemented. Biomass concentration of 76.8 g/L, 631.54 mg/L podophyllotoxin and 202.53 mg/L 6-methoxypodophyllotoxin were obtained on 15th d in this cultivation strategy.

Integrated bioprocess (normal production + product yield enhancement strategies) was then developed in batch, fed-batch and continuous cultivation. For this, the most effective precursor (coniferyl alcohol complexed with hydroxypropyl β -cyclodextrin), statistically optimized combination of elicitors (yeast extract, calcium chloride and *F. solani* filtered culture filtrate) were simultaneously added for 96 h and 48 h respectively. Batch cultivation in stirred tank and centrifugal impeller bioreactor with integrated strategy yielded podophyllotoxin accumulation of 340.46 mg/L and 464.54 mg/L respectively.

Application of integrated bioprocess in fed-batch cultivation and continuous cultivation in spin filter bioreactor by addition of most effective precursor (coniferyl alcohol complexed with hydroxypropyl β -cyclodextrin, 15.0 mM) for 96 h and Simultaneous addition of combination of elicitors (yeast extract: 135 mg/L; calcium chloride: 4.5 g/L and *F. solani* filtered culture filtrate: 7.5% v/v) for 96 h were added. Significantly higher podophyllotoxin productivities of 42.57 mg/L.d and 45.46 mg/L.d were obtained in fed-batch and continuous cultivations with integrated approach.

Co-cultivation of *P. indica* and *S. vermifera* with *L. album* cells in stirred tank bioreactor resulted in most significant enhancement of productivity to 57.42 mg/L.d and 61.75 mg/L.d respectively. A maximum enhancement in podophyllotoxin productivity of about 14 fold was achieved in this study as opposed to batch cultivation in unoptimized medium (4.4 mg/L.d).

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