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

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OCTOBER 2008

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by

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Thesis submitted

in fulfillment of the requirements of the degree of

Doctor of Philosophy

to the

INDIAN INSTITUTE OF TECHNOLOGY, DELHI

OCTOBER 2008

"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**

Acknowledgements

"It's just a new beginning"

It's like a walk down the memory lane, when I am specially remembering all those, who are a part of my journey of present research and my life at this juncture. Like any other journey, this journey also had its ups and downs but I really enjoyed every bit of it only because of some people, who are always with me directly or indirectly and I heartily express my sincere regards to all those……

Plant cell technology was always an area of research, which always fascinated me and it is really like a dream come true to be with ***Prof. Virendra S. Bisaria****, my research supervisor. He is like God's hand for me as this day would never have come without him. He is the man to follow in every sense. His caring attitude, deep involvement, constructive suggestions, valuable guidance, fatherly advices and perfectionist approach have helped me to overcome all challenges which came not only during this work but also in life. He is a 'Teacher' in true sense, who is a never ending source of knowledge. I really treasured every moment spent with him as I learnt some thing or the other every time I met him. Thanks Sir for everything as words can't describe my feelings for you……*

I take this opportunity to express my deep sense of gratitude and indebtness to my research supervisor, ***Prof. Ashok K. Srivastava.*** *His critical assessment, unmatched enthusiasm, 'never to give up' attitude and hard working nature are what I have tried to learn from him. His philosophy* ***'Rain or shine, the show must go on'*** *had inspired me to overcome all the hurdles and failures faced during this entire tenure and will keep inspiring me in future too. Thank you Sir for all that which I must have missed in these lines……*

I am failing in my duties if I do not recall ***Prof. Vinod K. Dixit*** *(Dept. of Pharmaceutical Sciences, Dr. H.S. Gour Univerisity, Sagar) for his faith in me. It's only because of his motivation and timely advice that I started to believe in me and aim high. He is like a sea of knowledge with very calm nature. He only taught me the basics of plant cell technology and infused a great principle of research in me i.e.'to find out solution of any problem, you have to find out the cause of it'. A special remembrance and my heartfelt gratitude is also due for* ***Prof. Shailendra Saraf*** *(Dept. of Pharmacy, Pt. Ravishakar University, Raipur) for their faith in me and for showing me the path for which even I did not think of. I would also like to thank* ***Prof. C.K. Kokate*** *(Dept. of Pharmaceutical Sciences, Kakatia University, Warangal), whose motivational words for thinking big is with me forever. Thank you all for showing me the 'right path' and being there when I don't even began to think so……*

It's only because of these teachers that I decided to choose 'Academics' as career. If I can motivate and change life of even a single student in my life, which they truly did in my case that will be my "Guru-Dakshina" for them.

I would like to extend my gratitude to both my Head of the department, ***Prof. Sunil Nath*** *and* ***Prof. Saroj Mishra*** *for providing all the facilities throughout my work. I am also thankful to all the faculty members especially members of my SRC,* ***Prof. K.D.P. Nigam****,* ***Prof. Saroj Mishra*** *and* ***Prof. T.R. Srikrishnan*** *for their invaluable time, suggestions and encouragement, which helped me during the present work.*

*The **'National Doctoral Fellowship'** awarded by All India Council for Technical Education and **'Teaching Assistantship'** awarded by Indian Institute of Technology, Delhi for this work are duly acknowledged.*

*I sincerely thank **Mr. Mukesh Anand** who always made sure that analysis of molecule of the core of this thesis is on time by HPLC. A special mention and thanks are due for **Mr. Ranbir**, **Mr. Sanjay** and **Mr. Hari Lal** for their cooperation. Many thanks are also due to **Mr. V. K. Ghosh**, **Mr. Swapan Patra**, **Mrs. Meena Mathur**, **Mrs. Neera Verma**, **Mrs. Sunita Dang**, **Mrs. Pushplata**, **Mr. Prashant**, **Mr. J. A. Khan** and **Mr. S.P. Rana** for their assistance and cooperation throughout my work.*

*It is the 'Friends' who are with you in every moment of joy and sorrow, and I thank almighty that I found 'The Fabulous Four' - **Anand, Anajli, Parul and Bhawna** during my stay in IIT. They are always there even when I am not with them physically due to my family. The time I spent with them will always bring a smile on my face. All 'Masti', tea parties, hot arguments, fruitful discussions, memorable trips and every single moment with all of you will be treasured for whole life. The simplicity of Anand, sincerity of Anjali, confidence of Parul and innocence of Bhawna made this group a complete package. No need to even thank them, I know they will be there whenever I need them.*

*It was like a walk back to college days with **Nishtha**, **Pragya** and **Nadeem**, discovering the wonderful friends as you have been was search that I could count as the 'bonus' of my time spent here. I recall you for your lively and cheerful company and a lot more than words can describe. I have a faith that distance can't make us apart. 'Homely' Nishtha, 'Smiling' Pragya and 'Chill' Nadeem, I miss all of you a lot.*

***Dr. Gunjan Prakash,** my lab senior and friend, needs a special mention not only her practical guidance but also for the discussions about every aspect of life. I recall and cherish the 'hot arguments' and 'fruitful discussions' with her, **Dr. Shilpi** and **Smita,** regarding the present work that helped me to be more analytical in my approach. Their company has provided me the opportunity to learn from their success and failures and thus overcome the failures of my work. I wholeheartedly thank all of you for being so wonderful companion.*

*The time spent with my ex lab-mate, **Gaurav**, is happily remembered. He was very helpful and never said 'no' for anything and did everything I asked for, even though it might have caused problems and discomfort to him. I fondly remember time spent with lab mates **Dr. Sunita**, **Seema**, **Parul**, **Abhishek** and **Nishant**. Working with them and having discussions about principles involved in plant cell technology had developed my skills and knowledge a lot. Moral support and encouragement offered by **Ranjita** and **Punita** at the time of final checking needs a special mention and heartfelt thanks.*

*I thank **Raju**, **Ruhi**, **Richa** and **Shweta**, whose warm and cheerful smiles will always be in my memories. Moments with **Amlendu**, **Rajib**, **Sanjay** and **Lalit** will also be cherished. Special thanks are also due to **Ramesh**, **Zia** and **Mukesh** for their kind help.*

***Manisha**, **Alok**, **Saurabh**, **Sandeep** and **Payal** are treasured friends, who are just a phone call away and don't need any formality. Words are not enough to express the love and emotions I have for them.*

*I thank **Ashish**, **Mukesh**, **Deependra**, **Satyaendra**, **Nirmal** and **Shailesh**,, my old friends for their invaluable friendship. A special remembrance is also due for my old seniors, **Mr. Umesh K. Patil** and **Mr. N.P. Yadav** for their guidance and cordial support.*

*Sometimes you don't need words to show how much you care about someone and it is really true with my supportive and encouraging in laws. The joy and proud they felt in every success of mine inspire me to perform best from the rest. The silent support of my Father-in-law (**Sr. Vinod Jajoo**) and Mother-in-law (**Smt. Nirmala Jajoo**) from behind the curtain is deeply acknowledged. I express my heartfelt affection to my sister-in-law (**Anisha Jajoo**) for her unexpressed care. Without their love, patience, understanding, and support my life would have never been so beautiful. My other family members especially Tauji (**Sr. Jagdish Baldi**) needs a special mention for his unsaid affection and care towards me.*

*And I finally owe this to my family and especially my beloved wife, **Rachana**, without whom I am really incomplete in every sense. She stood with me in every moment of my life. She is a 'cushion' that has absorbed all my failures, frustrations, worries and tension with her endless love, steadfast support and firm belief in my capabilities. Words fail me to express my appreciation for the many sacrifices she has made to support me in undertaking my doctoral study. Without you 'Rachana' it would have been impossible for me to arrive at this stage. Your love and support is my strength and having you in my life is God's greatest blessing over me……*

*My 'Little angel', **Aashana**, is "Gift of God" for me wrapped with sweetness and innocence. World seems altogether different when I am with her. It's really difficult to imagine my life without her smiles and kisses. Everything seems lesser than hearing 'Papa' from her with a big smile on the face. It's really nice to be a child again with her and helped me to forget the 'lab' once I get back to home.*

*If God has a face, it must be of my **Papa** (**Sr. Om Prakash Baldi**) & **Mummy** (**Smt. Asha Baldi**) for me. Their unconditional support, faith and encouragement always inspire me to achieve my 'goals'. Special regards are due to my mummy who despite of her loneliness and great attachment to me allowed me to go away from home and pursue my dream of higher education. No words are sufficient to express my love and gratitude to them as what so ever I am; it's because of the values they inculcated in me. I would never be able to repay my gratitude to them.*

*Most importantly, I would like to thank the almighty **'God'**, for it is under his grace that we live, learn and flourish.*

Ashish Baldi

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 became 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 ^{0}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 phenylapropanoid 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 in2.92 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 obatined on 15^{th} 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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