PRODUCTION OF AZADIRACHTIN (BIOPESTICIDE) FROM PLANT CELL SUSPENSION CULTURE OF *AZADIRACHTA INDICA* A. JUSS. (NEEM)

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

March 2006

Certificate

This is to certify that the thesis entitled **"Production of Azadirachtin (Biopesticide) from Plant Cell Suspension Culture of *Azadirachta indica* A. Juss. (Neem)"**, being submitted by **Ms. Gunjan Prakash** 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 her, which has been prepared under my 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.

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Acknowledgements

I thank Almighty for seeing over me in this journey of present research and through out all my life. Like any other journey, this journey is associated with all kinds of challenges, achievements, failures and I express my sincere regards to all those who have contributed for the completion of my aim either directly or indirectly....

I take this opportunity to express my deep sense of gratitude and indebtness to my research supervisor, ***Prof. Ashok K. Srivastava*** *for his constant training, which he has imparted throughout these years not only in research but various aspects of life as well. Day or night he never refused to be present in laboratory whenever required. I gratefully recall his presence in the laboratory till late nights during the bioreactor runs. His deep involvement, critical assessment, timely advice, valuable suggestions, motivation and encouragement have led to the completion of this work in successful manner. He is a source of never ending energy and novel ideas. His enthusiasm in each and every arena, never tiring and giving up attitude, perfection in each and every work whether so big or tiny is 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.*

I am grateful to my research committee members; ***Prof. Virendra S. Bisaria, Prof. Saroj Mishra*** *(IIT, Delhi) and* ***Dr. Amulya K. Panda*** *(National Institute of Immunology, New Delhi) for their critical comments, valuable suggestions and new ideas during the course of my work. I would also like to extend my gratitude to* ***Prof. S. S. Bhojwani,*** *Direactor Dayalbagh Univerisity, Agra (former Prof. Department of Botany, University of Delhi). Having his guidance in the initial phases of work was like a dream come true after reading his books during my M.Sc.*

I would like to extend my gratitude to both my Head of the department, ***Prof. G. P. Agarwal*** *and* ***Prof. Saroj Mishra*** *for providing all the facilities throughout my work. I am also thankful to all the faculty members for their time and suggestions, which helped me during the present work. I would like to thank Prof C. J. S. K.* ***Emmannuel,*** *Direactor, Arid Forest Research Institute, Jodhpur, India and* ***Dr. Uttar Kumar*** *(AFRI, Jodhpur) in the collection of neem seeds of different agroclimatic zones.*

The help and guidance given by my lab senior ***Dr. Saurabh Chattopadhyay*** *is gratefully acknowledged. I specially remember him for giving me the excellent lab environment. I would like to extend my thanks to* ***Dr. Rakhi Chaturvedi;*** *Assistant Professor; Indian Institute of Technology, Guwahati for teaching me the basic plant tissue culture techniques during the initial phase of my work. I would also like to thank* ***Dr. Manoj K. Upreti;*** *Scientist, Indian Oil Corporation Limited, for his suggestions and encouragement in my academics.*

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. I also thank* ***Mr. Abhishek Kumar*** *and* ***Santosh Jha*** *(Applikon, Dependable instrument) for their very fast service regarding the bioreactor related queries and problems each and every time. My work was never hampered due to delay from their side. I am also thankful to* ***Mr. Swapan Patra*** *for helping me with the computer related problems.*

I would like to thank ***Mrs. Meena Mathur, Mrs. Neera Verma, Mrs. Sunita Dang, Mr. Rajiv*** *for helping me in the administrative work. I thank* ***Mr Prashant Kumar*** *for always giving me the chemicals on time. I thank* ***Mr. V.K. Ghosh*** *and* ***Mr. S.P. Rana*** *for helping me out with the usage of lab equipments and facilities. Thanks are also due to* ***Mr. S.K. Khurana, Mr. J.A. Khan, Mr. Babu lal, Bhagvan Singh*** *for helping me at times. The assistance rendered by* ***Ranbeer*** *and* ***Hari lal ji*** *during the daily lab working is thankfully appreciated.*

It is the Friends who are with you in your every moment of laugher and sorrow and I thank almighty that I found ***Ritu Sareen, Keyur Raval, Shilpi Khanna, Smita Srivastava,*** *and* ***Ashish Baldi*** *during my stay in IIT. **Ritu**, meeting you again in IIT (batch mates in Banasthali) and discovering the wonderful friend as you have been was search that I could count as the 'bonus' of my time spent here. I recall you for your cheerful company, to become happy with my happiness, helping me out to overcome my sorrow and frustrations, for caring me at times of my sickness. I missed u at every moment after u left, be in the Department or hostel. I recall and cherish the 'hot arguments' and 'fruitful discussions' with 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 both of you for being so wonderful companion in research as well in life. A heartiest thanks to **Ashish** for his suggestions, discussions and help whenever required in the laboratory and being the wonderful lab companion. The time spent with my ex labmate, **Keyur** is happily remembered. It was fun sharing the lab responsibilities with you to keep our lab the best in the department. I thank **Mrs. Vartika Gupta** and **Dr. (Mrs.) Anamika Goel** my old friends for their invaluable friendship.*

*I thank the DBEB group (**Niju, Mausam, Vishal, Santosh**) with whom I spent my initial days in IIT. Later on they were always prompt in sending me the research papers available online from their present country of staying. I thank **Mrs. Roli Purwar** for sharing the standard of neem compounds with me. I remember my batch mates in IIT; **Ruchi Shukla, Rumpa Biswas, Vibha Bansal** and **Nidhi Gupta** for sharing good time together. I thank **Ms. Ushasri** whose lab happened to be the next door to mine and whose cheerful smile gave me the moments of relaxation at the time of stress. The conversation with you regarding the 'double distilled water' brings smile to me even today. I express my sincere thanks to **Dr. Sunita Farkya** for her help and sincerely checking the final draft of thesis. Thanks are due to **Seema Ahlawat** and **Parul Saxena** for extending their ever helping hand in the laboratory. I would also like to thank the co-operation received from Mili, Roopali, Shilpa, Alok, Anand, Amalendu, and Snehashish.*

*It would be undermining the contribution of **Ms. Manisha Rajvaidya, Ms. Jency Thomas, Ms. Rachna Rastogi** and **Ms. Vandana Shahi** by just saying thanks to them. They had not only opened their room for me in the hostel but also their hearts. They had taken care of me in each and every possible way during the time of my pregnancy and therefore the most critical phase of my work. I recall and cherish the night's joyful 'tea/coffee session' (always prepared by Rachna....) which would take away the overhanging of my tiredness and recharge me to write my thesis till late nights. Special thanks are due to **Jency** for checking the references very carefully. I enjoyed my second term of hostel staying in you people company and the sweet memories of these days will always remain fresh with me.*

*Family members are pillars on which you can always bank upon whether they are present in person or not. I take this opportunity to express my gratitude to my **Papa (Sr. Alok Prakash)** & **Mummy (Smt. Shubha Prakash)** for creating the virtues and values to me. My Papa always inspired me to excel in my academics and take on the Doctoral Degree. Regards are due to my mummy who despite of her sickness and great attachment to me allowed me to go away from home and pursue my dream of higher education. I would never be able to repay my gratitude to them. My elder sister **Mrs. Swati Garg** has been a source of inspiration to me since my childhood. Staying with her in the IIT Delhi hostel during her M.Tech was the stepping stone for origination of the idea of taking Doctoral degree from this premier institute. She would call me often and will pray for me whenever sensed trouble. My Jijaji **Sr. Anshul Garg** needs special mention. His perception of life had helped me to see things and situation from different angles. He would often take me to his home and always make a warm and cheerful environment. I would like to mention **Smt. Sushma Garg** for her encouragement and showering her love over me. I express my heartfelt affection to my younger sisters **Meenakashi (Mini)** & **Tanu (Tini)** for their love and bearing my absence from home so patiently. Although younger they had cared for me like anything.*

My dream would have never been fulfilled if I would have not got such supportive, loving and encouraging in laws. No words can express my regards and affection to my mother-in-law, ***Smt. Shashi Bala*** *for her constant encouragement, wholehearted support, love and care. She never let me affected by any adverse situation & always inspired me to concentrate in my work. The silent support of my Father-in-law;* ***Sr. Prabhakar Swaroop Manglik*** *from behind the curtain is deeply acknowledged. The care and concern shown by my Brother-in-law;* ***Mr. Vipul Manglik*** *cannot be repay by any means. Without their love, patience, understanding, and support my life would have never been so beautiful and this thesis would probably have never been finished. I would also like to acknowledge* ***Sr. Rajiv Manglik*** *and* ***Smt. Archna Manglik*** *for their kind support during the course of work.*

And finally I owe this day to my beloved husband ***'Rahul'*** *who stood behind me at all the good and odd times, never missing a single moment. This present endeavor is the outcome of his support in the form of constant encouragement, suggestions, patient hearing to my cries, bearing all my frustrations, taking me to lab whenever I asked be it in night, weekends or holidays, managing his office schedule to provide ample time to finish my experiments, sleepless tiring nights during bioreactor runs along with me, bearing my absence from home without any complain and what not.... Without you 'Rahul' 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....*

At this moment I could not stop myself remembering my seven & half month old miscarriaged baby whose memories would always remain with me...

Gunjan Prakash

ABSTRACT

Plants are the source of important agrochemical and pharmaceutical compounds. The neem tree (*Azadirachta indica* A. Juss, family Meliaceae) is one of the most valuable, multipurpose trees of present time. Azadirachtin is the principal secondary metabolite of neem seed extract and has been established as an efficient biopesticide worldwide. Currently, Azadirachtin is isolated by solvent extraction of the seeds of *A. indica*. There are various limitations in obtaining Azadirachtin from plants, majorly due to its limited availability/short shelf life of seeds, degradation during storage and considerable genotypic/environmental variation in its content from different sources. Therefore, plant cell and tissue cultivation have been investigated as an alternative source for the production of Azadirachtin.

In the present study, the seeds of *A. indica* were collected from different parts of India and callus cultures were initiated in order to select a high Azadirachtin producing cell line. A cell line with high initial yield of Azadirachtin (1.9 mg/g Dry Cell Weight) originating from Trivandrum seed kernels (Kerela, India) was selected.

Suspension cultures were developed from the selected cell line on Murashige & Skoog medium with 30 g/L Sucrose + NAA (2.0 mg/L) + BA (1.0 mg/L). In order to select the effective Carbon and Nitrogen source, different concentrations of Carbon (Sucrose/Glucose) and Nitrogen (NO_3^-/NH_4^+ ratio) sources were studied in *A. indica* suspension culture respectively in shake flask. Glucose and Nitrate turned out to be a

better Carbon and Nitrogen sources respectively yielding high Biomass and Azadirachtin content.

Plackett-Burman design was adopted to select the most important nutrients influencing the growth and Azadirachtin accumulation in shake flask suspension culture. After identifying effective nutrients, Central Composite Design was used to establish the optimum concentrations of the key nutrients for higher growth and Azadirachtin production in shake flask. A maximum of 15.0 g/L Biomass and 3.0 mg/g Azadirachtin was produced using optimum nutrient(s) concentration and cultivation conditions (Glucose: 37.5 g/L; Phosphate: 1.0 mM; Nitrate: 56.2 mM, Inoculum level: 5 g/L Dry Cell Weight, pH: 5.8, temperature: 27.0 °C). Nimbin (2.4 μg/g; 0.03 mg/L) and Salanin (1.2 μg/g; 0.01 mg/L) were also identified in the suspension culture grown cell extract along with Azadirachtin. Among different growth regulators studied, Indole Butyric Acid and Benzyl Adenine in the ratio of 4:1 yielded the highest Biomass (15.2 g/L) and Azadirachtin (48.7 mg/L) accumulation in shake flask. Maximum cell viability (>95 %) was obtained at 125 rpm.

Addition of elicitor(s) was attempted in shake flask to enhance growth and product formation. The highest increase in Azadirachtin was obtained with individual addition of elicitors; Chitosan (8.9 mg/g at 50 mg/L), Salicylic acid (8.2 mg/g at 70 mg/L) and Jasmonic acid (7.7 mg/g at 10 mg/L) as compared to the control cultures (3.2 mg/g). Combined addition of elicitors; Chitosan, Salicylic Acid and Jasmonic Acid (optimum concentration; 16.5, 137.3 and 2.9 mg/L respectively by statistical design) resulted in 5-fold enhancement of Azadirachtin (15.9 mg/g) as compared to non-elicited cultures (3.2 mg/g).

Different precursors of Azadirachtin biosynthetic pathway were added in culture medium to study their influence on Azadirachtin accumulation in shake flask. Among all the precursors added, Sodium Acetate (100 mg/L) resulted in highest increase in Azadirachtin accumulation (9.6 mg/g) in shake flask.

Effect of the addition of different concentrations of organic solvents (*n*-Hexadecane, Di *n*-Butyl Phthalate and Decanol at 2, 5, 10 and 15 %) was studied for the release of Azadirachtin in the shake flask culture medium. *n*-Hexadecane at 5 % concentration resulted in 13 % Azadirachtin release in the medium with no reduction in cell viability.

Different modes of cultivation (batch, fed batch and continuous) of *A. indica* cells were conducted for the production of Azadirachtin in optimized medium and cultivation conditions. Batch kinetics was studied in Stirred Tank (with low shear Setric and Centrifugal Impeller) and Bubble Column Bioreactor. Cultivation with Setric Impeller resulted in 15.5 g/L Biomass and 50 mg/L Azadirachtin production in 10 days. A higher Biomass of 18.7 g/L and 72.1 mg/L of Azadirachtin production were obtained in Centrifugal Impeller Bioreactor in similar cultivation period. Azadirachtin accumulation was further increased (82 mg/L in 12 days) when *A. indica* cells were cultivated in Bubble Column Bioreactor. Batch cultivation in Stirred Tank Bioreactor (Setric Impeller) with elicitor(s) addition (Chitosan: 16.5 mg/L; Salicylic acid: 137.3 mg/L and Jasmonic Acid: 2.9 mg/L on 8th day) resulted in more than 3 folds higher Azadirachtin accumulation (161.1 mg/L) than without elicitor addition (50 mg/L) in 10 days of cultivation.

A mathematical model was developed by using the batch kinetics (with Setric Impeller) and extrapolated to identify suitable feeding strategies for fed-batch and continuous cultivation with cell retention.

For fed batch cultivation, model based feeding strategy (MS salts with Glucose (500 g/L) + Phosphate (1.0 g/L) from 8-12 days and Nitrate (35.0 g/L) + Phosphate (0.5 g/L) from 13-14 days of cultivation @ 0.05 L/d) was experimentally implemented. A maximum of 20.0 g/L Biomass and 82 mg/L Azadirachtin accumulation was obtained in fed-batch cultivation in 14 days.

Model derived offline optimized continuous cultivation with cell retention (feeding of Glucose (75 g/L), Nitrate (10 g/L) and Phosphate (0.5 g/L) at a feed rate of 0.5 L/d ($D = 0.33\ d^{-1}$) resulted in an improvement in cell growth (88.2 g/L) and Azadirachtin content (280 mg/L) in 26 days of cultivation.

Application of a cell retention continuous cultivation process (from 8-40 days) involving precursor addition (Sodium Acetate: 100 mg/L on 2nd day), nutrient(s) and elicitor(s) renewal (MS salts with Glucose: 75 g/L; Nitrate: 10 g/L; Phosphate: 0.5 g/L; Chitosan: 16.5 mg/L; Salicylic Acid: 137.3 mg/L; Jasmonic Acid: 2.9 mg/L @ 0.5 L/d from 8-40 days) and permeabilization (*n*-Hexadecane: 5 % v/v on 40th day) in Stirred Tank Bioreactor (with Setric Impeller) resulted in 61.4 g/L Biomass and 751.9 mg/L Azadirachtin production with ~14 % release (105.0 mg/L) in the culture medium.

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