

© Indian Institute of Technology Delhi (IITD), New Delhi, 2014

**DEVELOPMENT AND MASS SCALE PROPAGATION OF  
HAIRY ROOTS OF *ARTEMISIA ANNUA* IN A SUITABLE  
BIOREACTOR FOR ARTEMISININ PRODUCTION**

by

**NIVEDITA PATRA**

**DEPARTMENT OF BIOCHEMICAL ENGINEERING & BIOTECHNOLOGY**

Thesis submitted

in the fulfillment of the requirements of the degree of Doctor of Philosophy

to the



**Indian Institute Of Technology, Delhi**

**February, 2014**

*Dedicated*  
*To*  
*My FAMILY...*

## ACKNOWLEDGEMENTS

*It is a moment of immense pleasure and satisfaction for me to express my sense of gratitude and sincere appreciation to my guide Prof. A.K. Srivastava, Department of Biochemical Engineering and Biotechnology, IIT Delhi, for all the enduring supervision, interest, encouragement, precious guidance, meticulous attention and constructive criticism, which was provided to me during the execution of this research work. I am thankful to him for giving me the opportunity to work in the best lab of Biotechnology and introducing me to experimental aspects of biochemical engineering. I will always be indebted to him for his invaluable suggestions and untiring attention, which he bestowed on me right from the inception till the successful completion of this endeavour. He inspired me in many ways throughout my study academically as well as personally. It has indeed been a privilege to be associated with him for so many years during which I tried to learn a lot from him. I feel lucky to have him as my guide. I express my gratitude to my co-guide Dr. Shilpi Sharma for her continuous support.*

*My sincere gratitude also goes to the members of my research committee Prof. Saroj Mishra (SRC chairperson), and Dr. D. Sundar (Internal expert), who encouraged me a lot throughout the course of this research work.*

*I am thankful to all the faculty members at DBEB, Prof. T. R. Srikrishnan (HOD), Prof. G.P. Agarwal, Prof. Sunil Nath, Prof. P.K. Roychoudhary, Prof. V.S. Bisaria, Prof. Prashant Mishra, Prof. Subhash Chand, Dr. Atul Narang and Prof S. N. Mukhopadhyay and Prof. Vikram Sahai for their support and encouragement. I am also thankful to the new faculty members at DBEB, Prof. M. N. Gupta, Dr. Ritu Kulshreshtha, Dr. Ziauddin Shiekh, Dr. Preeti Srivastava and Dr. E. Ravikrishnan for their constant efforts to develop the potentiality of the students.*

*This is the opportunity I would like to take to offer my sincere respect and thanks to all those researchers and authors who, by contributing to the knowledge of the "Hairy root cultivation", helped to enrich my knowledge and ease the path of my research work.*

*I am thankful to the staff members of all the laboratories of Biotechnology Department, for extending a helping hand whenever needed.*

*I gratefully acknowledge the help and cooperation given by all my friends and colleagues, who made the lab lively and pleasant. I am especially thankful to my seniors - Dr. Smita Srivastava, Dr. Gunjan Prakash, Dr. Saurabh Chattopadhyay and juniors whom I love a lot - Dhara, Sajjan, Swati, Sunil, Anveshika, Geeta, Guneet, Vaibhav, Sahil.*

*I will never forget the presence of Dhara, who was my roommate and best friend during my research. She was very helpful in nature and never says no for any kind of help.*

*I am also thankful to Kunal (Dhara's husband), Lalita, Vinay, Suhas, Jyoti, Papiya, Prabha, and Syamal, to name a few, for their helping hand whenever required.*

*I would also like to thank the biggest source of inspiration, support and encouragement in my life. I am grateful to my mother for the immense moral support and blessings. I would like to thank my dear father, who was a Scientist in Genetics and was my first teacher.*

*I am also grateful to my sisters Nikita and Nandita for their continuous help, support and encouragement.*

*Finally, I would thank the 'ALMIGHTY' for HIS impeccable guidance and support, which has helped me to remain focused in entire period of my journey.*

***Nivedita Patra***

## Certificate

This is to certify that the thesis entitled “**Development and mass scale propagation of hairy roots of *Artemisia annua* in a suitable bioreactor for artemisinin production**”, being submitted by **Ms. Nivedita Patra** 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.

**Dr. Shilpi Sharma**

Department of Biochemical  
Engineering and Biotechnology,

IIT Delhi

New Delhi-110016

**Prof. Ashok K. Srivastava**

Department of Biochemical  
Engineering and Biotechnology,

IIT Delhi

New Delhi-110016

## ABSTRACT

Hairy roots are induced by genetic transformation of a specific plant part using *Agrobacterium rhizogenes* bacteria. These roots are capable to produce secondary metabolites comparable to parent plants and grow at a rapid rate in culture medium under controlled environmental conditions. Artemisinin is a compound of immense therapeutic importance. It is widely accepted as a drug for the treatment of various deadly forms of malaria caused due to *Plasmodium falciparum*. Commercially artemisinin is isolated from the shoots of *Artemisia annua* plant. Since the plant is seasonal the supply of this drug is much lower than its high demand. Alternative biotechnological protocols are, therefore, highly sought to supplement the supply of this drug. One such production route could be mass scale hairy root cultivations in selected bioreactor.

Hairy root culture was initiated by *Agrobacterium rhizogenes* mediated genetic transformation of *in vitro* grown plants of *Artemisia annua*. Amongst different strains of bacteria LBA 301 was found to be the most potent strain for transformation of *Artemisia annua*. The integration of the T-DNA region of the Ri-plasmid of *A. rhizogenes* in the successful rootline of *A. annua* was confirmed using PCR technique specific for the amplification of root locus gene *rolA*. Characterization of artemisinin produced by hairy root culture was done using HPLC analysis using crystalline artemisinin (Sigma, USA) as standard for estimation of artemisinin concentration and peak validation.

Optimization of hairy root cultivation conditions was essential to enhance biomass concentration and artemisinin accumulation. Different trial experiments resulted in following environmental optimum conditions – rotational speed (70 rpm), temperature (25 °C), size of inoculum (1 g/l DW), age of inoculums (8 d) and medium volume to vessel volume ratio (0.18).

The composition of culture medium was statistically optimized. Screening of different basal growth media and their different dilutions indicated MS/4 to be the best medium for growth (5.67 g/l) of hairy roots and production of artemisinin (0.12 mg/g) under *in vitro* conditions. Analysis of Plackett-Burman design protocol and literature studies revealed that all the effectors – nitrate, potassium phosphate, sucrose and GA<sub>3</sub> were either affecting biomass and / or artemisinin production in hairy root cultivation. The cumulative effect of all the effectors were therefore, studied on *Artemisia annua* hairy root cell line using Response Surface Analysis. The optimal concentration of different effectors was established by the responses with respect to artemisinin accumulation and biomass obtained in the 30 design experiments and model equations. The optimized concentrations were identified as follows NO<sub>3</sub>/NH<sub>4</sub><sup>+</sup> ratio: 3.5, KH<sub>2</sub>PO<sub>4</sub>: 0.5 mM, sucrose: 37.13 g/l and GA<sub>3</sub>: 10 µg/l in MS/4 media. Experimental verification of optimized media in shake flask was performed which indicated that the correlation between model predicted and experimental values was 99.65 % for biomass (5.7 g/l) and 86.29 % for artemisinin (1.94 mg/g).

Growth kinetics of hairy root culture was studied in detail and by using it a mathematical model was proposed and identified. The extrapolation of model to fed batch (pseudo steady state with respect to substrate) resulted in accumulation of 0.99 mg/g artemisinin.

Enhancement of artemisinin accumulation from hairy root cultures was also done by application of different yield enhancement strategies. Addition of a combination of elicitors and precursors (3 % v/v extract of *Penicillium chrysogenum*) an artemisinin content of 3.8 mg/g dry weight was obtained.

Scale-up of the hairy root cultivation was attempted in modified Stirred tank Bioreactor, Bubble column reactor, Nutrient mist bioreactor for identification of an appropriate bioreactor configuration. Hairy root cultivation in modified Stirred tank bioreactor cultivation

resulted high biomass of 18.41 g/l (132 g fresh weight) and artemisinin content of 4.22 mg/g by using optimized media, elicitors and precursors.

# CONTENTS

<b>Title</b>	<b>Page No.</b>
<b>List of Figures</b>	<b>i – iii</b>
<b>List of Tables</b>	<b>iv - v</b>
<b>List of Abbreviations</b>	<b>vi-viii</b>
<b>List of Symbols</b>	<b>xi</b>
<b>CHAPTER 1. INTRODUCTION AND OBJECTIVES</b>	<b>1-9</b>
1.1 Introduction	1
1.2 Objectives	9
<b>CHAPTER 2. LITERATURE REVIEW</b>	<b>10-36</b>
2.1 Artemisinin: the medicine for cerebral malaria	10
2.1.1 Introduction	10
2.1.2 Chemistry	11
2.1.3 Main plant sources for artemisinin production	12
2.2 Plant cell/ tissue culture for <i>in vitro</i> production of secondary metabolites	14
2.3 Hairy Root Cultivation	18

2.3.1 Mechanism of hairy root induction	19
2.3.2 Advantages of hairy root culture	19
2.3.3 Mass propagation of hairy roots in bioreactors	20
2.3.3.1 Bioreactor configurations available for plant cell cultivation	20
2.3.3.1.1 Liquid-phase reactors	22
2.3.3.1.2 Gas-phase reactors	26
2.3.3.2 Construction of a bioreactor for plant cell cultivation	28
2.3.3.3 Bioreactors available for hairy root cultivation	31
2.3.3.4 Bioreactors used for mass production of plant hairy root systems	32
2.3.3.5 Two-phase bioreactor cultivations	33
2.3.4 Modelling of growth of hairy roots	34
2.3.5 Scale up studies for hairy root cultures in bioreactor	36
<b>CHAPTER 3. MATERIALS AND METHODS</b>	<b>37-74</b>
3.1 Chemicals	37
3.2 Equipments	38
3.3 Experimental methods	39
3.3.1 <i>Agrobacterium rhizogenes</i> based transformation of <i>Artemisia annua</i>	39
3.3.1.1 Plant material – maintenance and growth	39
3.3.1.2 Preparation of potent <i>Agrobacterium</i>	39

<i>rhizogenes</i> culture	
3.3.1.3	Induction of hairy roots 40
3.3.1.4	Selection of the hairy root line 41
3.3.2	Confirmation of transformed status of hairy root 41
	using PCR
3.3.2.1	Primers used for PCR 41
3.3.2.2	PCR Analysis protocol 42
3.3.2.3	Analysis of PCR products using agarose gel 42
	electrophoresis
3.3.3	Optimization of hairy root cultivation conditions in 42
	shake flask
3.3.3.1	Effect of shear stress 42
3.3.3.2	Effect of temperature 43
3.3.3.3	Effect of size of inoculum 43
3.3.3.4	Effect of age of inoculum 44
3.3.3.5	Optimization of medium volume to total shake 44
	flask volume ( $V_m/V_f$ ) ratio
3.3.4	Media optimization 45
3.3.4.1	Screening of basal media 45
3.3.4.2	Statistical optimization for development of 46
	medium recipe
3.3.4.2.1	Plackett Burman media design 46

3.3.4.2.2	Central Composite Design	48
3.3.4.2.3	Experimental validation of optimized media recipe in shake flask cultivation	50
3.3.5	Artemisinin enhancement strategies	51
3.3.5.1	Elicitation studies	51
3.3.5.2	Precursor studies	52
3.3.5.3	Study of effect of fungal elicitors	54
3.3.5.4	Combined addition of elicitor and precursor	54
3.3.5.5	Effect of day of elicitation and time of exposure	56
3.3.5.6	Effect of Gibberelic acid (GA <sub>3</sub> )	56
3.3.5.7	Effect of addition of permeabilizing agents	57
3.3.5.8	Study of gas phase composition	58
3.3.5.9	Effect of oxygen vectors	59
3.3.6	Development of mathematical model for growth and artemisinin production and experimental verification of model	60
3.3.6.1	Batch kinetic studies in shake flasks	60
3.3.6.2	Study of substrate inhibition kinetics	60
3.3.6.3	Analysis of batch kinetic data and determination of kinetic parameters	61
3.3.6.4	Extrapolation of batch kinetic data for fed-batch cultivation	62

3.3.6.5	Fed-batch cultivation of <i>Artemisia annua</i> hairy roots using constant feed rate	62
3.3.6.6	Fed-batch cultivation involving pseudo-steady state with respect to substrate	63
3.3.7	Batch cultivation in different gas/liquid bioreactors	64
3.3.7.1	Modified bubble column bioreactor	65
3.3.7.2	Nutrient mist bioreactor	66
3.3.7.3	Modified nutrient mist reactor	66
3.3.7.3.1	Effect of addition of elicitor(s) in the modified nutrient mist reactor	67
3.3.7.4	Cultivation in Stirred Tank Reactor	67
3.3.7.5	Cultivation of hairy roots in Modified stirred tank reactor	68
3.3.7.5.1	Effect of addition of elicitor in modified stirred tank reactor	70
3.3.7.5.2	Fed-batch cultivation involving pseudo-steady state w.r.t. substrate in modified stirred tank bioreactor	70
3.3.8	Analytical methods	71
3.3.8.1	Estimation of biomass, residual Sugar, Phosphate and Nitrogen and Cell Viability Analysis	71
3.3.8.2	Estimation of artemisinin	72

<b>CHAPTER 4. RESULTS AND DISCUSSION</b>	<b>75-146</b>
4.1 <i>Agrobacterium rhizogenes</i> mediated genetic transformation of <i>Artemisia annua</i> .	75
4.1.1 Plant material - maintenance and growth	75
4.1.2 Preparation of potent <i>Agrobacterium rhizogenes</i> culture	76
4.1.3 Induction of hairy roots	80
4.1.4 Selection of the most desirable rootline on the basis of artemisinin content	82
4.2 Confirmation of transformed status of rootline	83
4.3 Optimization of shake flask cultivation conditions	85
4.3.1 Effect of shear stress	85
4.3.2 Effect of temperature	86
4.3.3 Effect of size of inoculum	86
4.3.4 Effect of age of inoculum	87
4.3.5 Optimization of medium volume to total shake flask volume ( $V_m/V_f$ ) ratio	88
4.4 Media optimization	90
4.4.1 Screening of basal media	90
4.4.2 Statistical optimization for development of medium recipe	92
4.4.2.1 Plackett Burman media design	92

4.4.2.2	Central Composite Design	93
4.4.2.3	Experimental validation of optimized media recipe in shake flask cultivation	99
4.5	Artemisinin enhancement strategies	99
4.5.1	Elicitation studies	101
4.5.2	Precursor studies	104
4.5.3	Study of effect of fungal elicitors	105
4.5.4	Combined addition of elicitor and precursor	107
4.5.5	Effect of day of elicitation and time of exposure	110
4.5.6	Effect of Gibberelic acid (GA <sub>3</sub> )	110
4.5.7	Effect of addition of permeabilizing agents	111
4.5.8	Study of gas phase composition	113
4.5.9	Effect of oxygen vectors	115
4.6	Development of mathematical model for growth and artemisinin production and experimental verification of model	117
4.6.1	Growth and artemisinin Production Kinetics in Shake Flask Hairy Root Cultivation	117
4.6.2	Development of batch kinetic model	118
4.6.3	Effect of substrate on growth and metabolite production	119
4.6.4	Fed-batch cultivation of <i>Artemisia annua</i> hairy roots using constant substrate feed rate	121

4.6.5 Fed-batch cultivation involving pseudo-steady state with respect to substrate	124
4.7 Batch cultivation in bioreactor	127
4.7.1 Modified bubble column bioreactor	127
4.7.2 Nutrient mist bioreactor	129
4.7.3 Modified nutrient mist reactor	131
4.7.3.1 Effect of addition of elicitor(s) in the modified nutrient mist reactor	133
4.7.4 Stirred Tank Reactor	133
4.7.5 Modified stirred tank reactor	134
4.7.5.1 Study of substrate utilization kinetics of <i>Artemisia annua</i> hairy root culture in modified stirred tank bioreactor to elucidate their key role on artemisinin accumulation	137
4.7.5.2 Effect of yield enhancement study on hairy root culture in modified STR	142
4.7.5.3 Fed-batch cultivation involving pseudo-steady state with respect to substrate in modified stirred tank bioreactor	144
<b>CHAPTER 5. CONCLUSIONS AND RECOMMENDED FUTURE STUDIES</b>	<b>148-157</b>
5.1 Summary	148
5.2 Conclusions	153

5.3 Future Recommended Studies	156
<b>REFERENCES</b>	<b>158-168</b>
<b>APPENDICES</b>	<b>169-172</b>
<b>RESUME OF THE AUTHOR</b>	
<b>LIST OF PUBLICATIONS</b>	

---