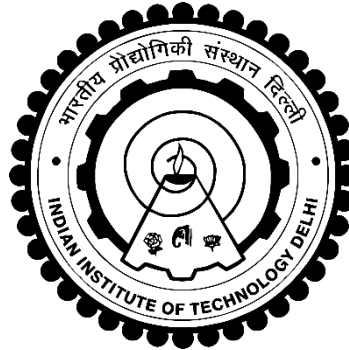


# **CULTIVATION OF HAIRY ROOTS USING INERT SOLID SUPPORTS**

**ANVESHIKA ADITYA**



**DEPARTMENT OF BIOCHEMICAL ENGINEERING & BIOTECHNOLOGY**

**INDIAN INSTITUTE OF TECHNOLOGY DELHI**

**JULY, 2018**

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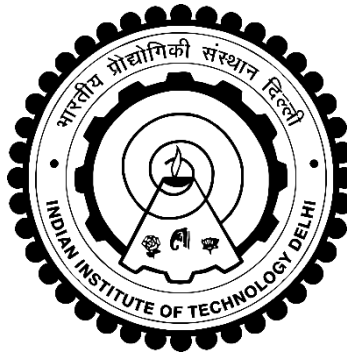
# **CULTIVATION OF HAIRY ROOTS USING INERT SOLID SUPPORTS**

by

**ANVESHIKA ADITYA**

**Department of Biochemical Engineering & Biotechnology**

*Submitted  
in fulfillment of the requirements of the degree of Doctor of Philosophy  
to the*



**INDIAN INSTITUTE OF TECHNOLOGY DELHI**

**JULY, 2018**

*Dedicated*  
*To*  
*My Parents*

## CERTIFICATE

This is to certify that the thesis entitled “*Cultivation of Hairy roots using Inert solid supports*” being submitted by **Ms. Anveshika Aditya** to the **Indian Institute of Technology Delhi**, for the award of the degree of “**Doctor of Philosophy**” is a record of the bonafide research work carried out by her under my guidance and 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 in part or full to any other university or institute for award of any degree/diploma.

Date:

**Prof. A. K. Srivastava**

Place:

Professor

Department of Biochemical Engineering  
& Biotechnology

Indian Institute of Technology Delhi

Hauz Khas, New Delhi – 110016

India

## ACKNOWLEDGEMENTS

*Like any other journey, this journey was a roller coaster ride- full of all kinds of challenges, achievements and failures. It has been a period of intense learning for me, not only in the scientific arena, but also on a personal level. I would like to thank the Almighty for looking after me not only during the PhD research but also throughout my life. I take this opportunity to express my sincere regards to all those who have helped me achieve my aim directly or indirectly.*

*At this moment of accomplishment I express my sincere gratitude to my research supervisor, Professor Ashok K. Srivastava for his enduring supervision, encouragement, precious guidance, meticulous attention and constructive criticism, not just for the thesis research work but for various aspects of life. His deep involvement, timely advice and motivation to strive for the best has led to the successful completion of this work. I thank him for all the patience and much needed support during the struggling times of this research. It has indeed been a privilege to be associated with him for so many years and I hope I have inherited some of his qualities in these years. Thank you Sir for all that I must have missed in these lines.*

*My sincere gratitude also goes to the members my Ph.D. research committee; Prof. T.R. Sreekrishnan, Prof. Atul Narang and Prof. S. K. Khare (Department of Chemistry, IIT Delhi), for their critical assessments and valuable suggestions during the course of my work.*

*I acknowledge the financial, technical and academic support provided by Indian Institute of Technology Delhi.*

*The help and guidance by my lab seniors Dr. Dhara Thakore, Dr. Nivedita Patra in teaching me the basics of plant tissue culture and patiently helping me out with my never-ending queries, especially in the beginning of this research is gratefully acknowledged. I sincerely thank Mr. Sanjay for the assistance rendered by him during the entire project. I thank Mr. Mukesh Anand for helping with HPLC troubleshooting whenever something went wrong during analysis and making sure that the HPLC analysis was done on time. I thank Mrs. Neera Verma, Mrs. Sunita Dang, Ms. Kirti, Ms. Sakshi for their help in my administrative work. Thanks, are also due to Mr. S.P. Rana and Mr. Anish Raju for helping me at times with lab equipments and facilities.*

*I also thank the IDDC, IIT Delhi staff, especially Mr. Rajaram, Mr. Joseph and Mr. Roshanlal who ensured timely fabrication of different reactor designs and actively helped whenever modifications were required.*

*It is friends who make any journey enjoyable. Coffee sessions with Priya and gossip sessions with Lovely will be fondly cherished. I thank my juniors Rabab, Jitendra, Sanjay, Navodit and M.Tech Project students Jigmet, Vaibhav, Ankita, Raghav, Isha, Archit, Ashutosh and Komal who for their fun loving nature, lightened up the mood in the lab. Special thanks are due to Archit who brainstormed with me in fabrication of large scale reactor.*

*My Family members are the four pillars of strength in my life. I can never thank enough my parents for their unconditional trust, timely encouragement and endless patience. It was their love that raised me up when I got weary. Also, the credit for sourcing and arranging different inert materials goes to them, without which this research could not have been possible. A sister is a girl's bestfriend, and the time spent in IIT after my sister Kritika joined as PhD scholar was even more special and memorable. I thank my younger brother Aloukik for keeping my mood light whenever I got tensed, sorting all my software related problems and for picking me up from metro stations whenever it got late. Without my family I could not have made it so far.*

*I thank my soulmate and husband Anurag Singhal for being a great support system without whom this Ph.D would have been a distant dream. The past five years have not been an easy ride and I thank Anurag for sticking by me right from the starting and patiently listening to my never-ending stories of experimental failures, thesis writing to what not. He always had faith in my abilities I thank him for his love and patience. Despite the long distance between us, the past few years have taught us a lot and strengthened our commitment towards each other.*

*I sincerely express my love and gratitude to my parents in law for their unfailing emotional support and heart-warming kindness. I am lucky to get into a family where I still feel the same level of care and security as I did before marriage. I would like to pay homage to my grandfather-in-law who I lost recently.*

*In the end I would like to thank all those who I have unknowingly missed out. A sincere thanks to all those people who have made me the person I am today.*

**Date:**

**Anveshika Aditya**

**Place:**

## ABSTRACT

Hairy root cultivation, induced by genetic transformation of plants with gram-negative bacterium *Agrobacterium rhizogenes*, is considered a promising alternative to conventional whole plant propagation technique for the production of important bioactive compounds due to its rapid growth and similar (or even higher) content of bioactive compounds production. Ajmalicine, one of the principle secondary metabolites found in *C. roseus*, has extensive medicinal applications in treatment of circulatory diseases and is already in use as a prescription drug (Card-Lamuran, Circolene, Duxor, Sarpan etc.) for hypertension to lower high blood pressure. Commercially, ajmalicine is extracted from the roots of *C. roseus* plant, but the content of ajmalicine in natural root systems is much lower than its demand. Therefore, alternative biotechnological production protocols such as hairy root cultivation systems are being studied to supplement the production of this valuable bioactive compound.

*Catharanthus roseus* hairy root cultures (clone CP32 var. Prabal), used in the present investigation, were induced by National Institute of Plant Genome Research (NIPGR), New Delhi. The present investigation focused on the development of suitable novel bioreactor configurations for mass cultivation of *C. roseus* hairy roots on inert solid support for maximizing the ajmalicine content in hairy roots.

Hairy root growth and ajmalicine production kinetics were established in hairy roots cultivated in two types of solidified media- agar gelled and phytagel gelled media. The maximum ajmalicine content in hairy roots was observed on 15<sup>th</sup> day on both types of solidified media. Sprinkle bioreactor, (equipped with effective custom-made media dispersal system) suitable for mass cultivation of hairy roots on inert solid supports, was designed and fabricated. Growth and ajmalicine production kinetics were established for hairy roots cultivated on selected inert

supports (saw dust and grey sand) in the custom fabricated bioreactors wherein growth associated production kinetics was observed with maximum ajmalicine content in hairy roots on 20<sup>th</sup> day with both the inert supports. Twelve different agro-industrial residues were used as inert support materials and were examined for their suitability for cultivation of hairy roots in custom fabricated reactors. Saw dust grown hairy roots exhibited maximum ajmalicine content of  $8.07 \pm 0.67$  mg/g DW out of all the inert support materials studied. Three different parameters were studied to optimize the inert bed characteristics: bed height, particle size of inert material and frequency of media addition. The complex and intriguing interaction of low and high concentrations of different effectors (inert bed parameters) and their cumulative effect on responses biomass and ajmalicine was studied using Response Surface Methodology. RSM optimized values of the three effectors (bed height, inert material particle size and frequency of media addition) predicted the ajmalicine content of 9.46 mg/g DW in the hairy roots which was experimentally validated. Hairy root cultivation was examined in five reactors having custom made inert support plate geometries; straight plate, curved plate, hemispherical plate, 40<sup>o</sup> cone and 50<sup>o</sup> truncated cone wherein high ajmalicine content ( $10.29 \pm 0.48$  mg/g DW) was observed in hairy roots cultivated in 50<sup>o</sup> truncated cone.

The fabricated sprinkle bioreactors were modified wherein three or more individual sprinkle reactors were connected to a common media reservoir, which allowed parallel operation of multiple reactor units at a time. It also minimized the chances of contamination, did not require an additional power source for intermittent delivery of sterile media to the growing hairy roots and made the operation and handling of the reactor simpler.

Custom made nutrient mist reactors were fabricated which were equipped with custom made misting unit for generation of fine mist inside the reactor. These custom fabricated reactors were simpler in design, cheaper which also featured easy operation as compared to commercially available mist reactors. *C. roseus* hairy roots were cultivated on selected inert

supports (polyurethane foam and coconut husk) in the fabricated mist bioreactor wherein high ajmalicine content was observed in hairy roots cultivated on coconut husk ( $8.46 \pm 0.45$  mg/g DW). Misting cycle featuring duration and frequency was statistically optimized using Response Surface Methodology wherein the optimized values of the misting cycle predicted the ajmalicine content of  $10.95$  mg/g DW in the hairy roots. This was validated by conducting independent experiments using the optimized misting cycle. A detailed study of prior soaking of hairy roots in nutrient media in the fabricated mist reactors was conducted wherein different combinations of soaking and misting durations were examined. The combination of 15 days misting followed by 5 days soaking exhibited maximum ajmalicine content in hairy roots ( $13.78 \pm 0.36$  mg/g DW).

Mist reactor was scaled up for large scale cultivation studies on mass propagation of hairy roots of *C. roseus* on inert solid supports as this reactor exhibited better ajmalicine content in hairy roots as compared to nutrient sprinkle reactor. Scale up was done on the basis of “misting area per unit cross-sectional area of the reactor”. The custom-made mist reactor (20 L) was equipped with a mist generation device for effective and uniform mist generation inside the reactor. The design was economical, simple in construction and operation and did not require any external pumps for mist generation. Optimized misting cycle and combination of preoptimized soaking and misting was used for large scale hairy root cultivation studies wherein high ajmalicine content ( $12.58 \pm 0.64$  mg/g DW) was observed in the harvested hairy roots, which is the best reported in literature so far.

## सार

हेयरी रूट्स, ग्राम-नकारात्मक बैक्टीरिया एग्नोबैक्टेरियम राइजोजेनेस के साथ पौधों के अनुवांशिक परिवर्तन से प्रेरित, बायोएक्टिव यौगिकों के उत्पादन की तीव्र वृद्धि और समान (या यहां तक कि उच्च) सामग्री के कारण महत्वपूर्ण जैव सक्रिय यौगिकों के उत्पादन के लिए परंपरागत पूरे पौधे प्रसार तकनीक के लिए एक आशाजनक विकल्प माना जाता है। *सी रोसियस* में पाए जाने वाले विशिष्ट सैकेन्डरी मेटाबोलाइट्स में से एक अजमलिसिन में परिसंचरण रोगों के उपचार में व्यापक औषधीय अनुप्रयोग हैं और उच्च रक्तचाप के लिए पहले से ही एक चिकित्सकीय दवा (कार्ड-लैमुरान, सर्कलीन, डक्सर, सरपैन इत्यादि) के रूप में उपयोग में है। वाणिज्यिक रूप से, अजमलिसिन *सी रोसियस* पौधे की जड़ों से निकाला जाता है, लेकिन प्राकृतिक जड़ प्रणाली में अजमलिसिन की सामग्री इसकी मांग से बहुत कम है। इसलिए, वैकल्पिक जैव प्रौद्योगिकी उत्पादन प्रोटोकॉल जैसे हेयरी रूट्स की खेती प्रणाली का अध्ययन इस मूल्यवान बायोएक्टिव यौगिक के उत्पादन के पूरक के लिए किया जा रहा है।

*कैथरैन्थस रोसियस* हेयरी रूट्स (क्लोन सीपी 32 var प्रबल), वर्तमान जांच में उपयोग की गई, नेशनल इंस्टीट्यूट ऑफ प्लांट जेनोम रिसर्च (एनआईपीजीआर), नई दिल्ली द्वारा बनाई गई हैं। वर्तमान जांच में हेयरी रूट्स में अजमलिसिन सामग्री को अधिकतम करने के लिए निष्क्रिय ठोस सहारा पर *सी रोसियस* हेयरी रूट्स की सामूहिक खेती के लिए उपयुक्त नवीन बायोरेक्टर कॉन्फिगरेशन के विकास पर केंद्रित किया गया।

हेयरी रूट्स की वृद्धि और अजमलिसिन उत्पादन कीनेटिक्स की स्थापना के लिए हेयरी रूट्स की दो प्रकार के ठोस मीडिया-एंगर जैल और फाईटजेल जैल मीडिया में खेती की गई थी। दोनों प्रकार के ठोस मीडिया पर 15 वें दिन हेयरी रूट्स में अधिकतम अजमलिसिन सामग्री देखी गई थी। निष्क्रिय ठोस सहारा पर हेयरी रूट्स की सामूहिक खेती के लिए उपयुक्त छिडकाव बायोरेक्टर, (प्रभावी विशेष रूप से निर्मित मीडिया फैलाव प्रणाली से सुसज्जित) का डिजाइन और निर्माण किया

गया था। विशेष रूप से निर्मित बायोरेक्टरों में चयनित निष्क्रिय सहारा (लकड़ी का बुरादा और भूरे रेत को देखा) पर खेती हेयरी रूट्स के विकास और अजमलिसिन उत्पादन कीनेटिक्स की स्थापना के लिए की गई थी जिसमें दोनों निष्क्रिय ठोस सहारा में विकास से जुड़े उत्पादन कीनेटिक्स 20 दिनों में हेयरी रूट्स में अधिकतम अजमलिसिन सामग्री के साथ देखी गई। बारह विभिन्न कृषि-औद्योगिक अवशेषों को निष्क्रिय ठोस सहारा सामग्री के रूप में उपयोग किया गया था और विशेष रूप से निर्मित रिएक्टरों में हेयरी रूट्स की खेती के लिए उनकी उपयुक्तता की जांच की गई थी। अध्ययन की सभी निष्क्रिय ठोस सहारा सामग्री में से लकड़ी का बुरादा पर खेती की हुई हेयरी रूट्स में अधिकतम अजमलिसिन सामग्री  $8.07 \pm 0.67$  मिलीग्राम/ग्राम डीडब्ल्यू प्रदर्शित हुई। निष्क्रिय बिस्तर विशेषताओं को अनुकूलित करने के लिए तीन अलग-अलग पैरामीटर का अध्ययन किया गया: बिस्तर की ऊंचाई, निष्क्रिय सामग्री का कण आकार और मीडिया डालने की आवृत्ति। विभिन्न प्रभावकों (निष्क्रिय बिस्तर पैरामीटर) के निम्न और उच्च स्तरों की जटिल और दिलचस्प परस्पर क्रिया और प्रतिक्रिया बायोमास और अजमलिसिन पर उनके संचयी प्रभाव का अध्ययन रिस्पॉस सरफेस मेटाडोलोजी (आरएसएम) का उपयोग करके किया गया था। आरएसएम ने तीन प्रभावकारों (बिस्तर की ऊंचाई, निष्क्रिय सामग्री कण आकार और मीडिया डालने की आवृत्ति) के मूल्यों को अनुकूलित किया और हेयरी रूट्स में  $9.46$  मिलीग्राम/ग्राम डीडब्ल्यू की अजमलिसिन सामग्री की भविष्यवाणी की गई जिसे प्रयोगात्मक रूप से मान्य किया गया था। विशेष रूप से निर्मित रिएक्टर की ठोस सहारा प्लेट पांच ज्यामिति में निर्मित की और हेयरी रूट्स की खेती की गई: सीधे प्लेट, घुमावदार प्लेट, गोलाकार प्लेट, 40 डिग्री शंकु और 50 डिग्री छिद्रित शंकु जिसमें उच्च अजमलिसिन सामग्री ( $10.29 \pm 0.48$  मिलीग्राम/ग्राम डीडब्ल्यू) 50 डिग्री छिद्रित शंकु में खेती की हेयरी रूट्स में देखी गई थी।

निर्मित किए हुए छिड़काव बायोरेक्टरों को संशोधित किया गया था जिसमें तीन या अधिक व्यक्तिगत छिड़काव रिएक्टर एक समान मीडिया जलाशय से जुड़े थे, जिसमें एक समय में कई रिएक्टर इकाइयों का संचालन किया जा सकता था। यह दूषितकरण की संभावनाओं को भी कम करता है, हेयरी रूट्स को जीवाणुरहित मीडिया के अंतराल वितरण के लिए अतिरिक्त बिजली स्रोत की आवश्यकता नहीं होती है और रिएक्टर के संचालन को सरल बना दिया जाता है।

विशेष रूप से निर्मित धुंध बायोरेक्टर का निर्माण किया गया था जिसमें रिएक्टर के अंदर धुंध के फैलाव के लिए विशेष रूप से निर्मित मिस्टिंग इकाई बनाई थी। ये विशेष रूप से निर्मित रिएक्टर डिजाइन में आसान थे, सस्ता जो वाणिज्यिक रूप से उपलब्ध धुंध रिएक्टरों की तुलना में आसान संचालन भी दिखाता है। *सी रोसियस* हेयरी रूट्स को निर्मित धुंध बायोरेक्टर में चयनित निष्क्रिय ठोस सहारा (पॉलीयूरेथेन फोम और नारियल भूसी) पर खेती की गई थी जिसमें नारियल भूसी ( $8.46 \pm 0.45$  मिलीग्राम/ग्राम डीडब्ल्यू) पर खेती की हेयरी रूट्स में उच्च अजमलिसिन सामग्री देखी गई थी। मिस्टिंग चक्र-अवधि और आवृत्ति रिस्पॉस सरफेस मेथडोलोजी का उपयोग कर सांख्यिकीय रूप से अनुकूलित किया गया था जिसमें मिस्टिंग चक्र के अनुकूलित मूल्यों ने हेयरी रूट्स में  $10.95$  मिलीग्राम/ग्राम डीडब्ल्यू की अजमलिसिन सामग्री की भविष्यवाणी की थी। यह अनुकूलित मिश्रित चक्र स्वतंत्र प्रयोगों का संचालन करके मान्य किया गया था। धुंध रिएक्टरों में पोषक मीडिया में हेयरी रूट्स के पूर्व भिगोने का एक विस्तृत अध्ययन किया गया था जिसमें भिगोने और धुंध फैलाव अवधि के विभिन्न संयोजनों की जांच की गई थी। 15 दिनों के धुंध फैलाव के बाद 5 दिनों तक भीगी हुई हेयरी रूट्स में अधिकतम अजमलिसिन सामग्री ( $13.78 \pm 0.36$  मिलीग्राम/ग्राम डीडब्ल्यू) प्रदर्शित हुई।

धुंध रिएक्टर को *सी रोसियस* हेयरी रूट्स के बड़े पैमाने पर खेती के अध्ययन के लिए बढ़ाया गया था, क्योंकि छिडकाव बायोरेक्टर की तुलना में इस रिएक्टर ने हेयरी रूट्स में बेहतर अजमलिसिन सामग्री प्रदर्शित की थी। "रिएक्टर के प्रति यूनिट क्रॉस-सेक्शनल एरिया" के आधार पर स्केल अप किया गया था। विशेष रूप से निर्मित धुंध रिएक्टर (20 ली०) के अंदर प्रभावी और समान धुंध उत्पादन के लिए धुंध उत्पादन उपकरण से लैस था। डिजाइन आर्थिक और सरल था, निर्माण और संचालन में सरल था और धुंध पीढ़ी के लिए किसी भी बाहरी पंप की आवश्यकता नहीं थी। अनुकूलित मिस्टिंग चक्र और भिगोने और धुंध फैलाव के अनुकूलित संयोजन बड़े पैमाने पर हेयरी रूट्स की खेती के अध्ययन के लिए इस्तेमाल किया गया था जिससे हेयरी रूट्स में उच्च अजमलिसिन सामग्री ( $12.58 \pm 0.64$  मिलीग्राम/ग्राम डीडब्ल्यू) देखी गई थी, जो अब तक साहित्य में सबसे अच्छी रिपोर्ट की गई है।

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## LIST OF ABBREVIATIONS

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ANOVA	Analysis of variance
BTBB	Balloon type bubble bioreactor
CCD	Central Composite Design
CR	Cathenamine reductases
DAD	Diode Array Detector
DMAPP	Dimethylallyl diphosphate
DW	Dry weight
FDA	Food and Drug Administration
FW	Fresh weight
G10H	Geraniol 10-hydroxylase
IPAP	Internal phloem associated parenchyma
IPP	Isopentenyl diphosphate
L/D	Light/dark
MEP	Methyl-erythritol phosphate
NIPGR	National Institute of Plant Genome Research
OVAT	One variable at a time
PP	Polypropylene
Ri	Root inducing
RSM	Response Surface Methodology
SAP	Super Absorbent Polymer
SGD	Strictosidine $\beta$ -D-glucosidase

TDC	tryptophan decarboxylase
T-DNA	Transfer DNA
TIA	Terpene indole alkaloids
w/v	Weight by volume