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

by

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Dedicated

To

My FAMILY...

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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.

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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 amplication of root locus gene *rol*A. 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_3 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_3/NH_4^+ ratio: 3.5, KH_2PO_4: 0.5 mM, sucrose: 37.13 g/l and GA_3: 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.

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