DEVELOPMENT OF A SUITABLE BIOREACTOR SYSTEM FOR AZADIRACHTIN PRODUCTION BY HAIRY ROOTS OF *AZADIRACHTA INDICA*

by

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to the

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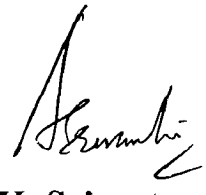
Dedicated

To

My FAMILY...

CERTIFICATE

This is to certify that the thesis entitled **"Development of a suitable bioreactor system for azadirachtin production by hairy roots of *Azadirachta indica*"**, being submitted by **Ms. Smita Srivastava** 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 and guidance in conformity with rules and regulations of the "Indian Institute of Technology, Delhi". The results described in it have not been submitted in part or full to any other University or Institute for the award of any Degree / Diploma.



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Smita

Smita Srivastava

ABSTRACT

Plants are the important source of food, fiber, color, fragrance and medicine. However yield and productivity from the natural plants are significantly low, making the plant production route uneconomical. Alternate *in vitro* production technologies like specialized plant cell/hairy root cultivation processes are being developed to mass produce the plant secondary metabolites. *In vitro* cultivations of hairy roots have a distinct advantage of rapid growth in hormone free medium. They have better genetic and biochemical stability in comparison to cell/tissue cultivations. Scientific communities have focused their attention toward its exploitation as a source of rare and valuable secondary metabolites.

Hairy root culture was developed in the present study to establish and demonstrate an alternative commercially viable production technology for Azadirachtin against extraction from the seeds of *Azadirachta indica.*

The hairy roots of *Azadirachta indica* were initiated in the present study by transformation of the plant cells by *Agrobacterium rhizogenes*. 175 hairy root lines were induced from the different explants of *in vitro* germinated seedlings from the elite high Azadirachtin yielding varieties of seeds collected from different agroclimatic regions of the country. Further, screening and selection of a single hairy root line (Az-35) was done in liquid culture on the basis of its highest Growth Index (1.75) and Azadirachtin accumulation (content) (3.8 mg/g). The integration of the T-DNA region of the Ri-plasmid of *A. rhizogenes* in the selected *A. indica* hairy root line was confirmed using the Polymerase Chain Reaction technique. The selected hairy root line was maintained on

a favorable solid medium of the following composition: MS medium major and minor salts, B5 medium vitamins and 30 g/l sucrose. The initial pH of the medium was adjusted to 5.8 and temperature was maintained at 25 ^{0}C.

Different hairy root culture medium recipe (s), growth factors and environmental conditions were studied and their optimum concentrations yielding high Azadirachtin and biomass production were derived in the shake flask. Substrate utilization, growth and production kinetics was established in the liquid culture of hairy roots under optimized medium and environmental conditions (rpm: 80, temperature: 26 ^{0}C, pH: 5.7, medium composition: (major nutrients: Sucrose – 40 g/l; Potassium dihydrogen phosphate – 0.19 g/l; Potassium nitrate – 3.1 g/l; Ammonium nitrate- 1.65 g/l; Magnesium sulphate – 0.41 g/l along with MS medium minor salts and B5 medium vitamins), Inoculum size: 3 g/l dry weight (DW) and age: 30 days, growth regulator: 1.0 mg/l Indole-3-acetic acid (IAA) and 0.025 mg/l Gibberellic acid (GA_3), permeabilizing agent: 0.5 % v/v Di-n-butyl phthalate (DNBP)). A maximum biomass of 21.3 g/l and Azadirachtin production of 78.81 mg/l in 25 days of the cultivation period (equivalent to an overall volumetric productivity of 3.15 mg/l d) was obtained with a residual sucrose concentration of 10.7 g/l. The disappearance of the nutrients was also reflected by a decrease in the medium conductivity from its initial value of 10.2 mS to 3.3 mS in 25 days of the hairy root cultivation period.

Directly and/or indirectly related biosynthetic precursors of Azadirachtin were added in the medium to enhance the Azadirachtin production in the hairy roots of *A. indica*. Maximum improvement in the Azadirachtin production (up to 70.42 mg/l) and its equivalent volumetric productivity (up to 2.81 mg/l d) could be achieved from that

obtained in control with no precursor in the medium (44 mg/l of Azadirachtin production in 25 days and its equivalent volumetric productivity of 1.76 mg/l d) when the growth medium was supplemented with 50 mg/l of Cholesterol. The Azadirachtin yield (content) in the hairy roots was found to increase from 3.3 mg/g in control to 5.82 mg/g on Cholesterol (50 mg/l) addition.

The possibility of Azadirachtin yield (mg/g) enhancement in the hairy roots of *A. indica* was investigated by the addition of potential elicitors (biotic and abiotic) in the cultivation medium. Among all the elicitors examined the addition of 1 % (v/v) of *Curvularia lunata* fungal culture filtrate resulted in highest Azadirachtin yield enhancement (up to 7.1 mg/g with respect to control value of 3.3 mg/g with no elicitor in the medium). The addition of elicitor in the medium in the beginning of the cultivation period detrimentally affected the biomass production (g/l) as a result the overall Azadirachtin production (mg/l) and its equivalent volumetric productivity (mg/l d) got affected. Hence optimization of the time of addition of the selected elicitor in the medium was done to achieve maximum Azadirachtin production (mg/l) and its equivalent overall volumetric productivity (mg/l d). During the optimization of the time of addition, the selected elicitor was proposed to be added along with the selected precursor in the medium under optimized cultivation conditions in order to have a synergistic effect on the overall Azadirachtin production (mg/l) and its equivalent volumetric productivity (mg/l d). It was found that maximum Azadirachtin production (113.4 mg/l, (with an Azadirachtin accumulation in the hairy roots of 5.4 mg/g and biomass production of 21 g/l)) and its equivalent overall volumetric productivity (4.53 mg/l d) was achieved when the combined addition was done on 15^{th} day of the growth cycle.

In order to scale-up the hairy root cultivation the conventional bioreactor designs were modified and some new custom made bioreactors were got fabricated as per the culture characteristics and requirements (of support for growth, nutrient and oxygen transfer, liquid hold up, etc). Experiments were conducted on these different bioreactor designs to select an appropriate bioreactor configuration (with simpler and economical reactor design) where maximum biomass production (g/l) and Azadirachtin accumulation (mg/g) in hairy roots could be achieved, thereby resulting in maximum Azadirachtin production (mg/l) and its equivalent overall volumetric productivity (mg/l d). In the experiments conducted on different bioreactors, following overall volumetric productivities of Azadirachtin (mg/l d) were obtained, respectively (Stirred Tank Reactor (0.33 mg/l d), Bubble Column Reactor (0.43 mg/l d), Modified Bubble Column Reactor (with PUF) (1.14 mg/l d), Rotating Drum Reactor (0.85 mg/l d), Nutrient Spray Bioreactor (0.49 mg/l d) and Nutrient Mist Bioreactor (1.09 mg/l d). It was invariably observed that the Azadirachtin productivities obtained in the different bioreactors studied were less than that obtained in the shake flask (1.76 mg/l d), presumably due to larger mass transfer limitations encountered during scale-up. Among all the bioreactor cultivation studies done, maximum Azadirachtin overall volumetric productivity (1.14 mg/l d) was achieved in Modified Bubble Column Reactor (with Polyurethane foam (PUF) as root support).

Finally, the selected hairy root line was cultivated under optimized cultivation conditions (obtained from the shake flask studies) in the Modified Bubble Column Reactor with a minor modification of incorporating a Setric impeller beneath the perforated PUF root support (for enhanced mixing of the cultivation medium and to

ensure adequate oxygen transfer). This resulted in a 5-fold increase in biomass (up to 15.2 g/l from an initial value of 3 g/l) and Azadirachtin production of 97.28 mg/l in 25 days of the cultivation period (with an Azadirachtin accumulation of 6.4 mg/g in the hairy roots).

The efficacy of the *A. indica* hairy roots as a potential biopesticide candidate was demonstrated through a bioassay carried out on the desert locust *Schistocerca gregaria.* The hairy root sample (its crude solvent extract) demonstrated a high level of antifeedant activity (Antifeedant Index (A.I): 83.5 at 0.5 mg/ml concentration of the hairy root extract in Ethanol). The Electron Spray Ionization- Mass Spectroscopy (ESI-MS) analysis of the hairy root sample (its crude solvent extract) revealed the presence of Azadirachtin (-A, -B, -I/H), Salannin, Nimbin, Isonimbinolide and Salannol acetate in the hairy roots of *A. indica*. A study was also undertaken to reduce the photodegradation of Azadirachtin present in the hairy root sample (its crude solvent extract) by the addition of selected chemicals (photostabilizers). Maximum improvement in the Azadirachtin degradation time (in terms of the Disappearance Time, DT_{50}) was achieved when Terbutylhydroquinone was used as a photostabilizer in the sample (where the Disappearance Time (DT_{50}) increased from 3.3 days in control (sample without stabilizer) to 4.5 days in the presence of the stabilizer).

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