CHAPTER VIII
Summary

8.1 *Sida rhombifolia* Linn. ssp. *retusa* (Linn.) Borssum, collected from the Aromatic and Medicinal Plants Division of Kerala Agricultural University, was used in the present study, for ephedrine production.

8.2 Fast growing friable calli were initiated from the leaf explants of *Sida rhombifolia* Linn. on Murashige and Skoog medium supplemented with 2.5 mg l\(^{-1}\) 2,4-dichlorophenoxy acetic acid and 1 mg l\(^{-1}\) benzyl aminopurine.

8.3 Initially the process parameters including temperature, light intensity, pH, carbon source, macronutrient combination, vitamin combination, amino acid supplement and plant growth regulators were optimized in callus cultures for maximum growth and ephedrine yield.

8.4 *S.rhombifolia* Linn. callus cultures recorded maximal growth and ephedrine yield at 25°C, pH 5.8 and under 1,000 lux light intensity.

8.5 Among the various major salt formulations tried, the MS major salt formulation promoted maximal growth and ephedrine production.

8.6 Among the carbon sources tested, sucrose promoted maximal growth and ephedrine production.
8.7 A combination of MS and B5 vitamin formulations favoured maximal biomass and ephedrine yield compared to individual additions.

8.8 Among the amino acids tested, the media supplemented with L-lysine and L-glutamine enhanced the biomass and ephedrine yield.

8.9 Among the various combinations of phytohormones tested, combination of 2.5 mg l⁻¹ of 2,4-D and 1.0 mg l⁻¹ of BAP resulted in maximal biomass and ephedrine production.

8.10 MS basal medium was modified (MSSR-4) and optimized by incorporating MS macronutrients and micronutrients, (MS+B5) vitamin combinations, a phytohormonal combination of 2.5 mg l⁻¹ of 2,4-D and 1.0 mg l⁻¹ of BAP and supplemented with 1mM each of L-glutamine, glycine and L-lysine for maximal growth and ephedrine yield.

8.11 Growth analysis in callus culture indicated that the exponential growth phase is of 14 days after an initial 7 days of lag phase. This is followed by a progressive deceleration stage of 7 days before setting in of stationary phase of growth.

8.12 Ephedrine production was growth associated although maximum production was on 27th day of callus culture.
Specific growth rate ($\mu$) of 0.132/day and doubling time (td) of 5.25 days in callus cultures were observed during time course experiment.

Suspension culture was established from healthy friable callus.

Presence of *Bacillus* sp. as latent contaminant was observed.

From the antibiogram, ciprofloxacin and gentamycin were identified as the most sensitive antibiotics.

The contaminant bacteria could effectively be controlled by administering the minimal lethal concentration of gentamycin (14 $\mu$g ml$^{-1}$) and ciprofloxacin (18 $\mu$g ml$^{-1}$), to the growth medium, without adversely affecting the plant cell viability.

A pH of 5.0-5.5 was optimum for maximal biomass and ephedrine yield in suspension culture.

An inoculum concentration of 5% was optimum for maximal growth of culture.

Growth analysis in suspension culture revealed a typical sigmoid pattern with well defined lag (about 5 days), exponential (about 9 days) and stationary phases. The maximum growth was recorded on 14$^{th}$ day of culture.
While ephedrine production started at the early logarithmic phase, it got accumulated more during the late logarithmic phase. However, maximum ephedrine production was noted on the 14th day of culture.

The specific growth rate ($\mu$) was 0.173 day$^{-1}$ and the doubling time (td) was 4 days.

About 27% of the total ephedrine alone was released into the medium.

Repeated mutagenesis with both chemical (EMS & AO) and physical agents (UV) resulted in a mutant line which recorded a maximal enhanced ephedrine yield of 32%.

Among the various stress factors studied for their influence on ephedrine yield, phosphate stress was most effective for ephedrine production in suspension culture, and hence, phosphate was chosen as the limiting nutrient. In a two stage culture system, where the actively growing cells, on mid log phase (10th day of culture), were transferred to a medium lacking the limiting nutrient, the ephedrine yield was enhanced by 2.6 fold.

When the actively growing cells were transferred to a medium with half the level of nitrate concentration, 46% increase of ephedrine yield could be effected. Similarly 29% increase in ephedrine yield was obtained by transferring the cells to sucrose deficient medium.
Addition of 0.75 mM L-phenylalanine promoted an increase of 1.78 fold ephedrine yield on 18th day of culture, whereas addition of 2 mM L-methionine resulted in 1.60 fold increase of ephedrine yield on 14th day of culture.

Addition of chitosan at 5 μg ml\(^{-1}\) resulted in 56.2% release of ephedrine, without affecting the cell viability percentage.

*S*_rhombifolia* Linn. cell culture system was highly susceptible to fungal elicitor treatment and an increase of 2.65 fold ephedrine was recorded in elicitor treatment with 5% Mycelial Extract of *Pythium aphanidermatum* after a contact time of 4 days.

Mycelial Extract of *Rhizoctonia solani* at 5% (v/v) level could enhance 2.21 fold increase in ephedrine production after a contact time of 4 days.

Among the various abiotic elicitors tried, calcium effected the maximum positive influence (1.76 fold increase) followed by manganese (1.52 fold increase) and zinc (1.36 fold increase).

Initially the process parameters that influence the preparation of immobilized viable cell beads were optimized. The concentration of sodium alginate, calcium chloride, cell concentration in the beads, curing time, activation and retention periods were optimized.
It was observed that 1.5% sodium alginate, and 2 h of curing time in half MS medium with 50 mM CaCl$_2$·2H$_2$O favoured optimum gel stability and maximal ephedrine yield.

Immobilized cell beads with 40% cell concentration, yielded maximum ephedrine.

Activation period of 18 h and retention time of 10 days were recorded as optimal conditions for maximum ephedrine yield by immobilized cells.

Half life of the immobilized cells in shake flask cultures, after running 6 cycles of batch process was recorded as 3 cycles.

The NMR spectral data obtained for the isolated ephedrine correlated well with that of standard ephedrine.