F. Isolation of total RNA

Total RNA was isolated from callus cultures following LiCl method with appropriate modifications. Concentration of total RNA in samples were quantified using UV spectroscopy.

G. MRNA

GenElute m RNA miniprep kit (M/s Sigma-Aldrich, USA) was used to isolate polyadenylated mRNA from previously isolated total RNA. The final pellet was resuspended in 10 µl RNA- free water. cDNA sysnthesis was carried out using MMuLV Reverse transcriptase following standard protocol. Sequence analysis was done using AB1-3130 genetic analyzer following standard procedures.

III. RESULTS AND DISCUSSION

The hairy roots raised in agitated batch cultures registered 12-16 fold increase in fresh wt over a period of 25 days which was complemented by product synthesis (Fig 1). Hence the concentration of plumbagin on unit dry wt basis remained the same through out the culture period. The stoichiometric relationship observed between biomass increase and product synthesis is also reported in certain other root cultures [9]. On the other hand, cell cultures as evidenced from published literature, unlike the roots and hairy roots, show a different pattern of growth often warranting use of different growth and production media for culture to increase the production rats. In fact in shake flask cultures, biosynthetic ability of the cells increase when growth decreases in late log phase. The pattern of biomass production and product synthesis, by and large remained the same during cultivation of the roots in the bioreactor also. However, at least two-three fold increases in biomass accumulation and product synthesis was observed during the scaled up process (Table-1). The concentration of plumbagin per unit biomass did not show any difference in both the culture systems. The enhanced rates of production observed in the bioreactor based cultivation might be attributed to several reasons: unrestricted nutrient availability, continuous O_2 supply in an air lift system, better rheological properties due to absence of shearing force, uniform pH maintenance and so on. Although the growth rates of even the hairy roots in culture may not be comparable to undifferentiated cell cultures, the reasonable biomass production and closely linked product synthetic patterns as observed indicated the desirability of using the bioreactor based culture of hairy roots for sustainable production of the plant specific napthoquinon. The hairy roots in culture gave the appearance of a mat without division into smaller colonies and this posed severe problems of lifting them up by the air. The major disadvantage of the hairy root culture system in shake flask or bioreactor based was the destructive harvesting of the roots after 25-40 days of growth for plumbagin extraction. Despite the difficulties in lifting the heavy mat like roots in the bioreactor, the roots were not damaged as in the case of shake flasks. It would be beneficial to devise methods to willfully divide the root mass into colonies and culture them under conditions to release the product into medium without affecting their life process.

The ointment made out of the root extract showed significant antimicrobial activities against both gram negative and gram positive bacteria. Inhibitory influence on the growth of *Candida albicans* suggested that the product possessed broad spectrum activities against both bacteria and fungi. Since it does not have any toxicity or allergic effects on the skin, this product may be useful for a range of skin infections / disorders including eczema, warts, wounds and other skin outgrowths

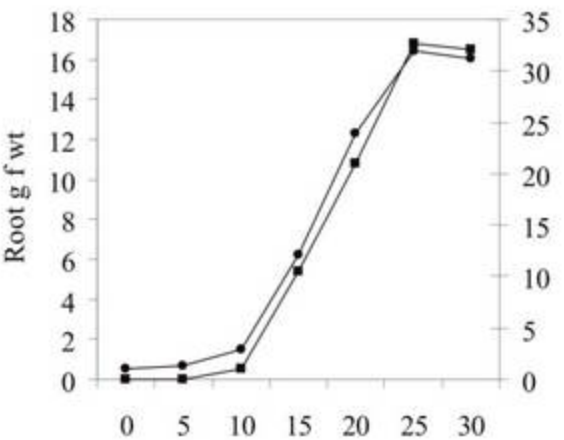

Fig.1. Relationship between hairy root growth and plumbagin synthesis in shake flask cultures

Table-1. Comparative rates of growth and product synthesis in hairy root cultures in shake flasks and bioreactor.

	Initial g fresh wt	final g fresh wt	Growth index	Plumbagin %d.wt
Bioreactor	6	238±3.89	36.16	1.11
Shake flasks	0.5	7.1±1.6	13.2	1.10

The rapid growth of the international demand for natural hypericin and their derivatives has led to several studies investigating the feasibility of using tissue culture as an alternative route to produce hypericin from *Hypericum perforatum* [10]. Some researchers also tried to find out alternative species that can compete with *H.*

Bioproduction of plant-specific molecules and product development using in vitro cultures of Indian plants

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Abstract

India figures among developing countries that have the potential biotechnological competence to add value to the natural resources, though its current share (1.6%) of the projected world market for medicinal plants and derivative products (US$ 5 trillion by the year 2010) is remarkably low. In an effort to develop appropriate technologies for bioproduction of the anti-neoplastic compound, plumbagin, hairy roots of *Plumbago rosea* L. were raised by infecting shoot cultures with *Agrobacterium rhizogenes* A4 strain and the bacteria-free roots (500mg. f.wt) established in 250ml agitated flask cultures, each flask containing 50ml Murashige-Skoog liquid medium devoid of growth regulators. The 12-16 fold increase in root biomass production and parallel synthesis of plumbagin with mean concentration of the compound (1.1% d.wt) through 25-day culture cycles indicated the stability of product synthesis and negligible differences between different hairy root clones. Cultivation of 6 gm fresh roots in 2.0 lit working volume in Infors (Labfors) airlift bioreactor through the same culture period consistently yielded 35-fold increase in root biomass without affecting plumbagin synthesis. An ointment made out of hairy root extracts in IP grade paraffin otherwise free from toxic principles was tested successfully against allergy induced skin eruptions (eczema, warts etc.). Additional tests are to be completed before a patentable product is developed. In another patented process using *in vitro* cultures, auxin mediated production of hypericin, a high value compound having antidepressant, anticancer and antiretroviral activities was achieved. Metabolic inhibitors (mevinolin, fosmidomycin and glyphosphate) at varying concentrations when fed to the shoot cultures did not inhibit synthesis of hypericin, thereby implicating mediation of polyketide synthase in the process. Attempts were made to isolate full length PKS gene(s) using RT-PCR followed by RACE analysis. 5' and 3'RLM-RACE analysis using gene specific primers designed to two RT-PCR products yielded 5'products of size range 650, 580 and 850 bp and 3' products of 750, 620 bp and 600bp. Sequence analysis of these RACE products yielded a full length cDNA having an open reading frame of 1173 bp encoding a 42 kDa protein having 390 amino acids. The deduced amino acid sequence of the of the PKS from *Hypericum* sp showed 85-99% identity with CHS-related enzymes of other plant species with highest similarity of 98-99% with *Hypericum perforatum* and *H. androsaemum*. Further, the catalytic triad (Cys 164, His 303 & Asp 336), CHS active site residues (Met 137, Gly 211, 216, Phe 215, 265 & Pro 375) and the conserved CHS residues that form the floor of the active site cavity (Thr 197, Gly 256, Ser 338 & Ile 254) are well conserved thereby indicating that the isolated gene corresponds to CHS. Isolation and characterization of other novel forms of PKS is in progress. The study reveals PKS mediated synthesis of hypericin in *Hypericum* sp. and the leads obtained hopefully result in eventual isolation and characterization of PKS gene(s) involved in the biosynthesis of hypericin.

Key words: plumbagin, hypericin.

I. INTRODUCTION

Plant life of India is unique in that plants and their products have been used as medicinal agents right from pre-vedic times. As traditional exporter of raw materials rather than finished goods, India is not significantly benefited and our annual export earnings (~ Rs 3000 Crores) account for only a fraction (~1.6%) of global trade in medicinal plant products. Indian medicines are accepted more as food supplements rather than drugs as they are not products of scientific scrutiny and value addition [1]. Besides the vertical increase in demand for green medicines in very recent years and the non-availability of genuine raw materials as against the indiscriminate and unscientific harvest of medicinal plants currently followed, resulted in inevitable decline in the quality of the herbal medicines while pushing up their prices. That plant based molecules and derivatives account for 2.5% of even the modern drugs illustrate the importance and utilitarian value of herbal medicine [2]. India figures among 28 countries that lose biodiversity steadily, and injudicious exploitation of the herbal resources has resulted adulteration and substitution of non-available ingredients in medicinal preparations. In order to remedy the situation, the National Medicinal Plant Board of India recently came up with a set of recommendations for cultivation of endangered taxa, bioprocessing, standardization and value addition of plant drugs [3] . Technology intervention is also a need of the hour for prospecting the native plant resources, bioproduction of plant-specific molecules and value-added productization. It remains a fact, however, that among the comity of developing nations, India is an emerging economic and technological power and has developed necessary biotechnological competence for adding value to the natural sources. This paper deals with prospecting of plant-specific molecules and product development from certain indigenous herbs.

II. MATERIALS AND METHODS

Shoot cultures of *Plumbago rosea* (Plumbaginaceae) were raised from single node cultures [4] Aagarmedium supplemented with 2.5 mgl^{-1} BAP. Hairy roots were initiated by infecting the shoots with *Agrobacterium rhizogene*- A4 strains using scalpel method described [5]. Hairy roots emanated from the wound sites were dissected out, decontaminated and established in 250 ml flask cultures containing MS basal liquid medium. Biomass production and product (plumbagin) synthesizing abilities of the hairy roots in cultures were studied using standard methods. Plumbagin was extracted from the dried roots with chloroform and its concentration was determined in UV-VIS spectrophotometer at 423 nm against standard. Scaled-up production of roots and plumbagin was achieved in a 2.5 lit working volume air lift bioreactor (Infors-Labfors) maintained at 25 ±2°C under constant bubbling with air. The roots so raised were harvested after 5 weeks of growth, plumbagin extracted and mixed initially with IP grade paraffin for making an ointment against skin allergy (evczema), warts and related problems. Anti-microbial assays with the product were done against both gram positive and negative bacteria and also against *Candida* species.

A. Plumbagin Extraction

Plumbagin was extracted and quantified from the dried roots as described [6] and [7]. Hairy roots were harvested and dried at room temperature and powdered. Samples of root powder were extracted with chloroform for 12-h at room temperature until all the pigments were leached out of the samples. The chloroform extract was filtered and the filtrate was concentrated under vacuum in Rotavapour (Heidolph-LABOROTA-4000). The concentrated samples were separated in preparative TLC prepared with silica gel-G 60. Benzene was used as the solvent to run the TLC. The solvent front was marked and allowed to dry. The co-chromatographed authentic sample of plumbagin (Sigma Chemical Co., USA) was used to detect the presence of plumbagin on the TLC plate after the run.

The bright yellow spot corresponding to authentic sample was scraped and the compound was eluted with chloroform. The amount of plumbagin was determined by measuring absorbance at 423 nm using a Shimadzu 2100 UV-VIS spectrophotometer. Additional evidence for the purity of the compound was obtained from HPLC analysis.

B. Analysis Of Plumbagin Using Hplc

HPLC separations were done using a Gilson 321 binary gradient Liquid chromatographic system with UV-detector as described [7] Separations of the compound were performed using a C-18 Kromasil (250 x 4.6 mm) column with a mobile phase of methanol and acetic acid (80:20), buffered with trimethyl amine (pH 3.5). The flow rate was 1.0 ml min^{-1} and plumbagin was detected by UV absorbency at 254 nm. Each injection volume was 15 µl.

The compound was separated at 5.6 min (Retention Time), was co-chromatographed with the authentic compound procured from Sigma Chemicals (USA) and established the authenticity of the compound as plumbagin extracted from the hairy root cultures.

C. Callus And Shoot Cultures Of Hypericum Spp.

Seeds of *Hypericum* sp. collected from Palni hills of the Western Ghats were germinated on MS (1962) basal agar medium and seedlings raised in 4-6 weeks were cut into nodal segments for reculture in the same medium supplemented with cytokinins to raise multiple shoot cultures. Segments of shoots (~1.0 cm) were also cultured in presence of 2,4-dichlorophenoxy acetic acid (2,4-D) or naphthalene acetic acid (NAA) in presence of cytokinins to raise callus cultures. The semi- friable callus so raised were divided and subcultured in presence of appropriate concentrations of kinetin and NAA. Sectors of proliferative mass of cells seen on certain calli were dissected out for culture in agitated (80 RPM) liquid nutrient medium containing kinetin and NAA. After 4-6 weeks of culture, aggregates of cells released into the medium were harvested and recultured in the same medium. Shoot, callus and cell cultures were regularly subcultured at 4-6 week intervals and maintained at 25±2°C under 12h photoperiod, light emanating from Philips white fluorescent tubes.

D. Extraction and estimation of Hypericin

Fresh callus, shoot and cell cultures (100 g each) were extracted in a soxhlet apparatus for 3 hrs with 50 ml diethyl ether. The residue was dried and extracted for 6 hrs with 50 ml ethyl alcohol. The extract in 5 ml ethanol was dried and the residue resuspended in 5 ml ethanol for estimation using HPLC.

E. Analysis of Hypericin using HPLC.

Standard procedures with appropriate modifications were followed for quantifying hypericin. Reverse phase column (c-18, 125X4 mm) of the HPLC system (Gilson 321 USA) was equilibrated with solvent systems comprising of (A) Acetonitrile: (B) Methanol (70:30) in the ratio (A:B) 20:80. An aliquot of 15 µl of each sample was injected into the column. Peak area of the compound was plotted against concentration for quantification.

perforatum in their active principle. Therefore it was of interest to raise *in vitro* cultures from an alternative species (Hypericum sp) from South Indian hills capable of producing substantial quantities of hypericin. That both the differentiated shoot culture and undifferentiated callus culture systems were induced the synthesis and accumulation of hypericin made the systems new and novel for bioproduction. The cell culture on the other hand proved to be less efficient for growth and bioproduction of hypericin. The concentration of hypericin recorded in both shoot and callus cultures suggested that these cultures could be scaled up for making the system commercially viable. Inhibitor studies indicated alternate pathway ie polyketide mediated synthesis of hypericin. RT-PCR followed by RACE analysis yielded a full length cDNA having an open reading frame of 1173 bp encoding a 42 kDa protein having 390 amino acids. The deduced amino acid sequence of the *Hypericum* sp PKS showed 85-99% identity with CHS-related enzymes of other plant species with highest similarity of 98-99% with *Hypericum perforatum* and *H. androsaemum*. Further the catalytic triad (Cys 164, His 303 & Asp 336), CHS active site residues (Met 137, Gly 211, 216, Phe 215, 265 & Pro 375) and the conserved CHS residues that forms the floor of the active site cavity (Thr 197, Gly 256, Ser 338 & Ile 254) are well conserved thereby indicating that the isolated gene corresponds to CHS. Attempts for isolation and characterization of novel forms of PKS are in progress.

IV. CONCLUSION

The results obtained from both *Plumbago rosea* and *Hypericum* sp tissue cultures confirm the feasibility of making value added marketable products rich in plumbagin and hypericin respectively. The development and marketing of a product for relief from depression from the *Hypericum* shoot cultures is farther away compared to the ointment rich in plumbagin for topical application to get cure from skin infection. The process of these bioproducing these chemical molecules using the tissue culture systems are already patented. It is high time that toxicity and CF dosage levels are worked out and the products are launched at the earliest. Our institute is also concurrently working on an Indian plant tissue culture-based production of a natural mosquito and honey bee repellent as an alternative to pesticides containing products currently available.

In addition to PKS I, a few other genes also have to be sequenced before using them for transforming *Hypericum* shoot cultures to make them overproducing the compound. Like the successful GenBank deposition of PKS I, PKS-II is also expected to be submitted soon. However, fruition of the metabolic engineering efforts will be realized only when the genetically enhanced transformants overproducing hypericin is obtained for economic cultivation of the species in the Western Ghats segments of India

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