INVESTIGATION ON THE PRODUCTION OF FOOD INGREDIENTS AND BIOTRANSFORMATION USING HAIRY ROOT CULTURES

A THESIS

Submitted to the

University of Mysore

For the award of degree of

Doctor of Philosophy

In

Biotechnology

By

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December 2003

DEDICATED TO .....

My beloved parents

&

To the ever green memory
of my grand mother

ACKNOWLEDGEMENTS

I would like to express my profound sense of gratitude to my mentor and guide Dr. G. A. Ravishankar, Head & Scientist, Plant Cell Biotechnology Department, CFTRI, for his constant support, encouragement, and valuable guidance. I am highly thankful to him for the freedom of thought, relaxed atmosphere and his stimulating discussions, which I enjoyed all these years.

I am thankful to Dr. V. Prakash, Director, CFTRI for providing all the research facilities during the course of my work.

I am grateful to Dr. K. S. M. S. Raghavarao, Head, Food Engineering Department for his help during my experimental work with bioreactor studies.

I would like to thank all the staff members of Plant Cell Biotechnology Department especially Dr. Sarada, Dr. Rajasekaran and Dr. Giridhar for their moral support and help during my research work.

My sincere thanks are due to Dr. K. R. S. Sambasiva Rao, Professor, Center for Biotechnology, Nagarjuna University, Guntur for his support and help.

I am thankful to all the former and present colleagues of Plant Cell Biotechnology Department specially Dr. Ramachandra Rao, Dr. Harsh Pal Bais and Dr. Usha Tripathi for their goodwill and co-operation during my research work.

I am thankful to Mr. Obul Reddy for helping with molecular techniques , Mr. Sarath Babu for his help during preparation of the manuscript, Mr. Rajesha and Mr. Chidambara Murthy for their timely help and support all these years.

I would like to thank all my friends- Sekhar, Vinod, Vanita, Indu, Raju, Naveen babu, Mohan, Praveen Reddy, Naveen Reddy, Madhu, Mouli, Venky, Dr. Rajesh, Dr. Devaraj, Subba Rao, Rajkumar, Ashok, Satya, and Janardhan for their

co-operation and support throughout my research tenure and made my stay a memorable one at CFTRI.

The co-operation and help received from the staff of Central Instrumentation Facility, Library, Administration, Glass blowing and Photography Section is gratefully acknowledged.

Thanks are due to the Department of Biotechnology (DBT) and Council of Scientific and Industrial Research (CSIR), New Delhi for the award of Project assistant ship and Senior Research Fellowship respectively.

I would like to express my heartfelt gratitude to my parents, sisters, Brother-in-law and my uncle Sri. Seshagiri Rao for their love, encouragement and constant support.

I would like to express my deep sense of gratitude to my grand mother Late Mrs. Kameswari for her love and affection for making me what I am today.

(B. Suresh)

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LIST OF ABBREVIATIONS

BCD	**β-Cyclodextrin**
CAOMT	**Caffeic acid –*O*-methyl transferase**
CTAB	**Cetyl Trimethyl Ammonium Bromide**
DOPA	**Dihydroxy phenyl alanine**
DW	**Dry weight**
FW	**Fresh weight**
HPLC	**High Performance Liquid Chromatography**
m Osmol	**Milli Osmol**
MJ	**Methyl jasmonate**
mS	**Milli Siemens**
MS	**Murashige and Skoog**
PAs	**Polyamines**
PCR	**Polymerase Chain Reaction**
PTFE	**Polytetrafluoroethylene**
Put	**Putrescine**
SAM	**S-adenosyl L-methionine**
Spd	**Spermidine**
Spm	**Spermine**
TLC	**Thin Layer Chromatography**

DECLARATION

I hereby declare that this thesis entitled "**INVESTIGATION ON THE PRODUCTION OF FOOD INGREDIENTS AND BIOTRANSFORMATION USING HAIRY ROOT CULTURES**" submitted herewith to the **University of Mysore, Mysore** for the award of degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY**, is the result of work done by me under the guidance of **DR. G. A. RAVISHANKAR**, ***Scientist & Head*** at Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore, during the period 1998-2003**.**

I further declare that the work embodied in this thesis is original and has not been submitted previously for award of any degree, diploma or any other similar title.

(B. Suresh)

Place: Mysore

Date :

Dr. G. A. Ravishankar Ph.D., FAFST., FNASc., FNAAS., FBS

Head & Scientist
Plant Cell Biotechnology Department

Certificate

I hereby certify that the thesis, entitled "**Investigation on the production of food ingredients and biotransformation using hairy root cultures"**, submitted by **Mr. B. Suresh** to the University of Mysore, Mysore for the award of degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY**, is the result of work done by him at Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore, under my guidance during the period 1998-2003.

Date: **(G. A. Ravishankar)**

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INTRODUCTION

General introduction

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1.7 Objectives of the present study

According to a report, plants make more than 100,000,000,000 tons of organic compounds and 50,000 different compounds every year (Gershenzon 2002). Secondary metabolites in plants are derived from basic photosynthates with modifications to produce simple to complex molecules (Czapek 1921). Plant secondary metabolites are classified as phenolics, terpenes, steroids and alkaloids according to their biosynthetic pathways (Harborne 1999). The secondary metabolites from plants are useful as food additives, flavours, colourants, and pharmaceuticals. It has been established that despite rapid progress in applied chemistry, 25% of the molecules used in pharmaceutical industry are of natural plant origin (Payne *et al.,* 1991). Plant secondary metabolites are generally obtained from field grown plants. However, plants originating from particular biotypes are difficult to grow outside their local ecosystems and are more prone to pathogen attack. Moreover, they are season specific and produced during specific phase of the plant's life cycle. This has led to the consideration of alternate ways of production of secondary metabolites using plant cell tissue and organ cultures (Bourgaud *et al.,* 2001). The beginning of plant tissue culture was made by Haberlandt (1902). Subsequently a number of scientists around the world explored the enormous possibility of cell and organ cultures for regeneration of plants and production of various secondary metabolites. In recent years the production of secondary metabolites using plant cells has been the subject of extensive research, wherein studies on biochemistry and molecular biology contributed a lot in better understanding of metabolic pathways and enzymology of the biosynthesis of products (Dornenburg and Knorr 1995, Bourgand *et al.,* 2001). Furthermore, cell cultures have a higher rate of metabolism than intact differentiated plants because the initiation of cell growth in culture leads to fast proliferation of cell mass and to a condensed biosynthetic cycle (Dornenburg and Knorr 1995). Compared to main molecules found in plants, these secondary metabolites are low in abundance, often less than 1% of the total carbon, or a storage usually occurring in dedicated cells or organs (Bourgaud *et al.,* 2001).

The basic secondary metabolite pathway is given in Figure 1.1

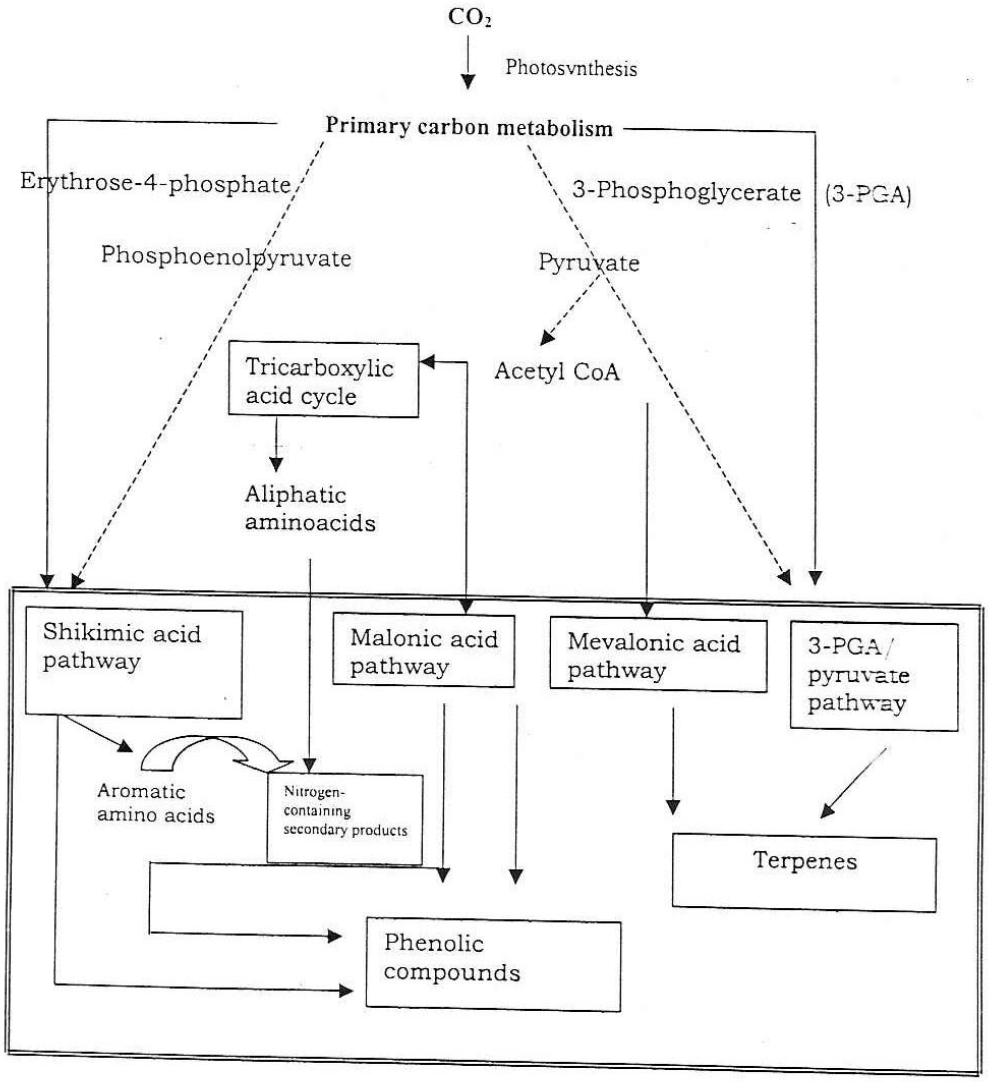


Fig 1.1: General secondary metabolite biosynthetic pathway
(Source: Ohlrogge and Benning 2000)

1.1 Secondary metabolites of food importance from plant cell cultures

Plant cell cultures in recent years have been exploited for their efficient production of various secondary metabolites of food and pharmaceutical importance. So far, only a small percentage (5-10 %) of all plants have been screened for useful compounds with the aid of modern scientific tools for the production of secondary metabolites (Schripsema *et al.,* 1996). These products include food and pharmaceutical compounds. In the food segment, they are well known as producers of colours, flavours, sweeteners, food additives etc. (Ravishankar and Venkataraman 1990). Some of the important food additives from plant cell cultures are given in the table 1.1.

Table 1.1: Secondary metabolites of food importance from plant cell cultures

Product type	Plant species	Reference
Colours		
Anthocyanins	*Vitis vinifera*	Pepin *et al*., 1995
	Daucus carota	Rajendran *et al*., 1994
	Perilla frutescens	Zhong and Yoshida, 1995
Betalaines	*Beta vulgaris*	Klebnikov *et al*., 1995
	Chenopodium rubrum	Berlin *et al*., 1986
Crocin	*Crocus sativus*	Sujata *et al*., 1990
Crocetins	*Gardenia jasminoides*	George and Ravishankar, 1995
Carotenoids	*Lycopersicon esculentum*	Fosket and Radin, 1983
Anthraquinones	*Cinchona ledgeriana*	Robins and Rhodes, 1986
	Morinda citrifolia	Kieran *et al.,* 1993
Flavours		
Vanillin	*Vanilla planifolia*	Dornenburg and Knorr, 1996
Basmati	*Oryza sativa*	Suvarnalatha *et al*., 1994
Pungent food additive		
Capsaicin	*Capsicum frutescens,*	Lindsey and Yeoman, 1984
	Capsicum annuum	Johnson *et al*., 1990
Sweeteners		
Stevioside	*Stevia rebaudiana*	Swanson *et al*., 1992
Glycyrrhizin	*Glycyrrhiza glabra*	Hayashi *et al*., 1988

(Adapted from Ramachandra Rao, 1998)

It is also reported that the cell cultures lose their genetic stability upon subculture due to somaclonal variations and other environmental effects (Dornenburg and Knorr 1995). Most of the secondary metabolites are produced during stationary phase of cell cycle rather than during early stages of growth, the reason being the distribution of carbon for primary metabolism (building of cell structures and respiration) when growth is very active. Once the growth slows, carbon is no longer needed in large quantities for primary metabolism and secondary products are more actively synthesized (Bourgaud *et al.,* 2001).

The genetic instability which is commonly associated with cell (callus) cultures and the problems of scale-up in bioreactors has driven researchers to look for alternate methods to obtain secondary metabolites of importance through organ culture.

1.2 Root cultures as sources of secondary metabolites

Roots are the most important "hidden half" of the plant system anchoring the plant, and are sources of many valuable products such as pharmaceuticals, insecticides, colours, flavours and others. They are the major sites of metabolite synthesis and storage, which exhibit indefinite growth because of the meristematic activity of root tip meristem. Many compounds that are scarcely synthesized such as coumarins, caffeic acid esters and catechins in undifferentiated cells are produced at higher levels in root cultures (Bais *et al.,* 1999, 2002 a). They are also exploited for their enormous ability to extract soil and water pollutants such as organic compounds, heavy metals, xenobiotics from the contaminated sites which is generally called as "Phytoremediation" (Raskin 1996, Salt *et al.,* 1998). Root exudates include various primary and secondary metabolites and micro and macromolecules that play an important role in the regulation of symbiotic and protective interactions with microbes (Buee *et al.,* 2000).

In recent years 'hairy root culture' gained importance as production system for secondary metabolites due to their tremendous potential exhibiting greater biomass production as well as metabolite contents. Being organized, root cultures are

amenable for scale-up in reactors hence have gained importance (Flores 1992). The following sections throw light on the developments in hairy root culture systems.

1.3 Hairy root cultures- *Agrobacterium rhizogenes* mediated transformation

Agrobacterium rhizogenes, a gram- negative soil bacterium is the causative agent of 'hairy root disease', proliferating the root-like structures upon infecting the plant. Such roots can be grown axenically on plant growth hormone-free media and produce relatively large amounts of auxins and cytokinins. *Agrobacterium* attaches to plant cells in a polar manner in a two-step process. The first step is likely mediated by a cell –associated acetylated, acidic capsular polysaccharide (Reuhs *et al.,* 1997). The second step in attachment involves the elaboration of cellulose fibrils by the bacterium, which enmeshes large number of bacteria at the wound surface (Matthysse *et al.,* 1996). T-DNA transfer from *Agrobacterium* to plant cells may rapidly follow bacterial attachment. The 'Root inducing' (Ri) plasmid is the genetic determinant of virulence (two separate regions); a portion of the Ri plasmid is actually transferred to the cells that give rise to the rooty structures, which produce opines (psuedoaminoacids such as octopine, nopaline and agrocinopine) that are in turn utilized by the infecting *A. rhizogenes*. The Ri plasmid consists of a T-DNA, a border sequence and a virulence area (Fig 1.2). The T-DNA of Ri plasmid comprises of two different parts, called the T_L and T_R DNAs. The T_R -DNA encodes proteins responsible for phytohormone production (specifically auxin synthesis) and opine biosynthesis by root cells (Bevan and Chilton 1982). The Ri plasmid T_R -DNA-encoded genes are eukaryotic in nature, being expressed in transformed plant cells and not the inciting bacteria. The virulence area containing different silent *vir* genes does not enter the plant genome but are required for T-DNA transfer (Table 1.2). The first step in the transfer of T-DNA into plants is the mobilization of the T-DNA in the inciting *Agrobacterium*. This process is initiated by a number of substances such as lignin precursors and acetosyringone produced by plant cells at the site of wound (Melchers *et al.,* 1989). These inducing compounds consequently activate the expression of the genes in the *vir* region of the Ri plasmid. This activation is mediated

by two gene products, those of *vir* A and *vir* G genes. The *vir* A gene product is a membrane – spanning protein that senses external inducer compounds and in turn activates *vir* G product. Autophosphorylation of Vir A protein and the subsequent transphosphorylation of Vir G protein result in the active transcription of the remaining *vir* genes (*vir* B, *vir* C, *vir* D, and *vir* E) (Jin *et al.,* 1990). Apparently, the *vir* D gene products (Vir D1 and Vir D2) recognize the borders of the T-DNA, nick one strand of the Ri plasmid and create, by unidirectional DNA replication catalyzed by the cellular repair apparatus, a single stranded copy of the T-DNA. The Vir D1 protein appears to be the agent responsible for nicking the Ri plasmid and the Vir D2 protein becomes covalently attached to the 5' end of the T-strand, resulting in a linear, single stranded copy of the T-DNA. The Vir E2 and Vir E1 proteins bind to the T-strand as well. The net result is a distinct nucleoprotein that is apparently translocated from the bacterial cytoplasm to the plant cell nucleus (Gelvin 2000). The T_R-DNA apparently causes a phytohormone imbalance that results in the proliferation of hairy roots in infected wounds. It has been shown that the T_L-DNA of Ri plasmid could also cause hairy root production, in the absence of the T_R-DNA. This effect is not the result of T_L-DNA-directed auxin production in transformed cells but can instead be traced to the synergistic action of three genes *rol A, rol B* and *rol C*. In some plants, these individual genes can cause rooting in transformed cultures, but combinations of these genes are needed in other plants (Estruch *et al.,* 1991a, Estruch *et al.,* 1991b).

Table 1.2: Functions of *vir* genes during transformation of plants with *Agrobacterium rhizogenes*

***vir* locus**	**Function**
A	Regulatory (recognizes plant metabolites, activates *vir* G)
G	Regulatory (transcriptional activator of other *vir* loci)
D	Nicks Ri plasmid at T-DNA borders, covalently attaches to "T-strand"
C	Unknown function involved in host-range determination
E	Ss-DNA binding protein (stabilizes T-DNA during or after transfer)
B	Transfer apparatus

(Source: Winans 1992)

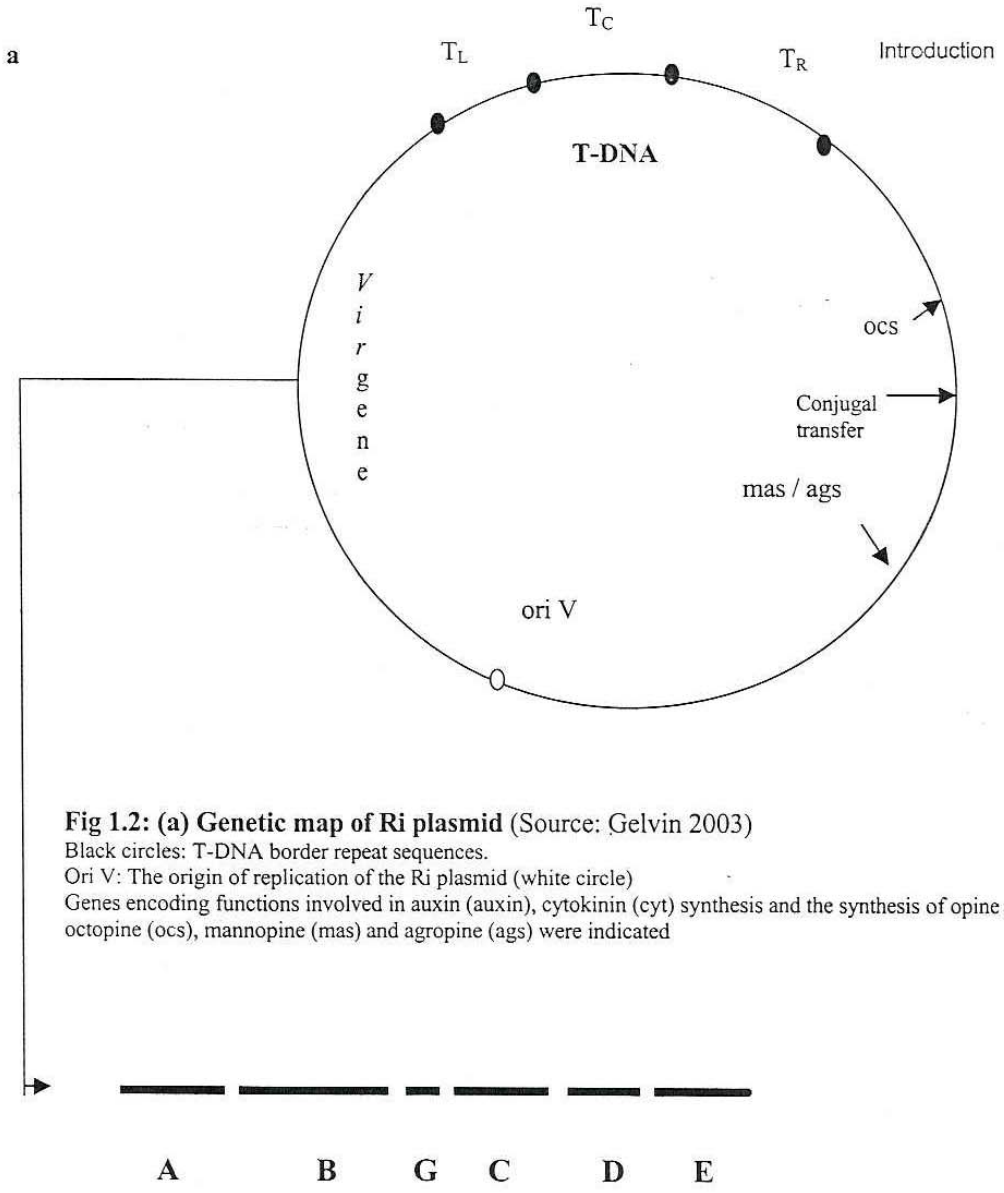
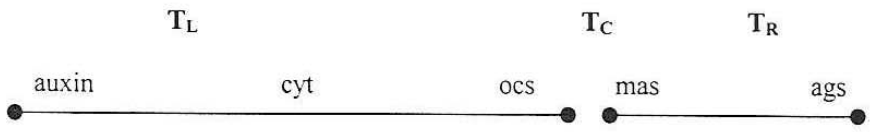


Fig 1.2: (a) Genetic map of Ri plasmid (Source: Gelvin 2003)
Black circles: T-DNA border repeat sequences.
Ori V: The origin of replication of the Ri plasmid (white circle)
Genes encoding functions involved in auxin (auxin), cytokinin (cyt) synthesis and the synthesis of opine octopine (ocs), mannopine (mas) and agropine (ags) were indicated

(b) The *vir* region of Ri plasmid



(c) T – DNA structure

1.3.1 Secondary metabolite production from hairy root cultures

Hairy roots are obtained after the successful transformation of a plant with *Agrobacterium rhizogenes.* They have received considerable attention from plant biologists, for the production of secondary metabolites. They can be subcultured and indefinitely propagated on a synthetic medium without phytohormones (Tepfer 1984, Chilton *et al.,* 1982, Ramachandra Rao and Ravishankar 2002). They often display higher growth rates due to profuse lateral root growth with doubling time ranging from 1-day (Maldonado-Mendoza *et al.,* 1993) to 1-week (Arellano *et al.,* 1996). They are genetically stable with consistent growth and metabolite production, which was experimentally proved by Maldonado-Mendoza *et al.,* (1993) in more than 500 hairy root lines of *Datura stramonium*. However, some researchers also reported reduced growth rates and productivities such as that of *Duboisia* hairy roots as reported by Yukimune *et al.,* (1994). This is probably because of variation of the expression of Ri T-DNA oncogenes in the transformed roots (Durand-Tardiff *et al.,* 1985, Ooms *et al.,* 1986).

The metabolite pattern found in hairy roots is similar, if not always identical to that of plant roots (Flores and Filner 1985, Parr and Hamill 1987). A major characteristic of hairy roots is the concomitant production of secondary metabolites with growth. Hence it is possible to get a continuous source of secondary metabolites from actively growing hairy roots (Holms *et al.,* 1997). Successful results have been obtained by modifying the nutrient composition of the medium (Tone *et al.,* 1997) or applying elicitors (Rijhwani and Shanks 1998). Some of the important secondary metabolites from hairy root cultures is given in the table 1.3.

Table 1.3: Hairy root cultures as productive systems of secondary metabolites

Plant	Secondary metabolite	Reference
Aconitum heterophyllum	Aconites	Giri *et al.,* 1997
Ajuga replans var.	Phytoecdysteroids	Matsumoto *et al.,* 1991
Atropurpurea	Hydroxyecdysone	Banarjee *et al.,* 1994
Ajuga	Polyacetylenes and	Flores *et al.,* 1988

Ambrosia sp.	thiophenes	Sauerwein *et al.,* 1991
Amsonia elliptica	Indole alkaloids	Jobanovic *et al.,* 1991
Anisodus luridus	Tropane alkaloids	Taya *et al.,* 1989 a
Armoracia laphthifolia	Peroxidase, Isoperoxidase,	Babcov *et al.,* 1995
Armoracia	Fusococcin	Nin *et al.,* 1997
Artemisia absinthum	Essential oils	Jaziri *et al.,* 1995
Artemisia annua	Artemisinin	Ionkava *et al.,* 1997
Astragalus mongholicus	Cycloartane saponin	Christen 1999
Atropa belladonna	Atropine	Allan *et al.,* 1999
Azadirachta indica A. Juss	Azadirachtin	Mukundan *et al.,* 1998 a &
Beta vulgaris	Betalain pigments	Hamill *et al.,* 1986
		Flores *et al.,* 1988
Bidens sp.	Polyacetylenes and	Giulietti *et al.,* 1993
Brugmansia candida	thiophenes	Jung and Tepfer 1987
Calystegia sepium	Tropane alkaloids	Tada *et al.,* 1996
Campanula medium	Cuscohygrine	Flores *et al.,* 1988
Carthamus sp.	Polyacetylenes	Asamizu *et al.,* 1988
Cassia obtusifolia	Thiophenes	
	Anthraquinone polypeptide	Parr *et al.,* 1988
Catharanthus roseus	pigments	Davioud *et al.,* 1989
Catharanthus tricophyllus	Indole alkaloids, ajmalicine	Christen 1999
Centranthus rubber	Indole alkaloids	Constable and Towers 1988
Chaematis douglasis	Valepotriates	Bais *et al.,* 1999
Cichorium intybus	Thiarubrins	Hamill *et al.,* 1989
Cinchona ledgeriana	Esculin, Esculitin	Sasaki *et al.,* 1998
Coleus forskohlii	Quinine	Marchant 1988
Coreopsis	Forskolin	Christen *et al.,* 1989
Datura candida	Polyacetylene	Payne *et al.,* 1987
Datura stramonium	Scopolamine, Hyoscyamine	Bel-Rhlid *et al.,* 1993
Daucus carota	Hyoscyamine,	Saito *et al.,* 1990
Digitalis purpurea	Sesquiterpene	Deno *et al.,* 1987

Duboisia myoporoides	Flavonoids, Anthocyanin	Muranaka *et al.,* 1992
Duboisia leichhardtii	Cardioactive glycosides	Mukundan and Hjortso 1990 &
Echinacea purpurea	Scopolamine	Trypsteen *et al.,* 1991
	Scopolamine	Couilerot *et al.,* 1999
Fagra zanthoxyloids Lam.	Alkamides	
		Trotin *et al.,* 1993
Fagopyrum	Benzophenanthridine	Motomari *et al.,* 1995
Fragaria	Furoquinoline alanine	Ishimaru *et al.,* 1991
Geranium thubergee	Falvanol	Asada *et al.,* 1998, Li *et al.,*
Glycyrrhiza glabra	Polyphenol	2002
Gynostemma pentaphyllum	Tannins	Fei *et al.,* 1993
Hyoscyamus muticus	Flavonoids	Vanhala *et al.,* 1995
	Saponin	Halperin and Flores 1997
Hyocyamus niger	Tropane alkaloids	Jaziri *et al.,* 1988
Lactuca virosa	Hyoscyamine, Proline	Kisiel *et al.,* 1995
Leontopodium alpinum	Hyoscyamine	Hook 1994
Linum flavum	Sesquiterpene lactones	Oostdam *et al.,* 1993
	Anthocyanins & essential	
Lippia dulcis	oil	Sauerwein *et al.,* 1991
Lithospermum	Lignans (5-methoxy	Fukui *et al.,* 1998
erythrorhizon	podophyllotoxins)	Yamanaka *et al.,* 1996
Lobelia cardinalis	Sesquiterpenes,	Yonemitsu *et al.,* 1990
Lobelia inflata	hernandulcin	Carron *et al.,* 1994
Lotus corniculatus	Shikonin, Benzoquinone	Parr *et al.,* 1987
Nicotiana hesperis	Polyacetylene glucoside	Hamill *et al.,* 1986
Nicotiana rustica	Lobeline, polyacetylene	Flores and Filner 1985
Nicotiana tabacum	Condensed tannins	Bais *et al.,* 2002
Ocimum basilicum	Nicotine, Anatabine	Kitajima *et al.,* 2002
Ophiorrhiza pumila	Nicotine, Anatabine	Bais *et al.,* 2003
Oxalis tuberosa	Nicotine, Anatabine	Yoshikawa and Furuya 1987
Panax ginseng	Rosmarinic acid	Washida *et al.,* 1998

Panax Hybrid (P.ginseng X	Campothecin-related	
P. quinquifolium)	alkaloids	Yoshimatsu and Shimomura
Papaver somniferum	β-carbolines	1992
Perezia cuernavcana	Saponins	Arellano *et al.,* 1996
Pimpinella anisum	Ginsenosides	Santos *et al.,* 1998
Platycodon grandiflorum		Tada *et al.,* 1995
Pratia nummularia	Codeine	Ishimaru *et al.,* 2003
Rauwolfia serpentina	Sesquiterpene quinone	Sato *et al.,* 1991
Rubia peregrina	Essential oils	Lodhi *et al.,* 1996
Rubia tinctorum	Polyacetylene glucosides	Sato *et al.,* 1991
Rudbeckia sps	Polyacetylene glucosides	Flores *et al.,* 1988
Rudbeckia hitra L.	Reserpine	Uczkiewicz *et al.,* 2002
Salvia miltiorhiza	Anthraquinones	Hu and Alfermann 1993
Scutellaria baicalensis	Anthraquinone	Zhou *et al.,* 1997
Serratula tinctoria	Polyacetylenes and	Delbeque *et al.,* 1995
Sesamum indicum	thiophene	Ogasawara *et al.,* 1993
Solanum aculeatissi	Pulchelin E	Ikenaga *et al.,* 1995
Solanum lacinialum	Diterpenoid	Hamill *et al.,* 1987
Solanum aviculare	Flavonoids and	Yu *et al.,* 1996
Swainsona galegifolia	phenylethnoids	Ermayanti *et al.,* 1994
Swertia japonica	Ecdysteroid	Ishimaru *et al.,* 1990
Tagetus patula	Naphthoquinone	Arroo *et al.,* 1995
Tanacetum parthenium	Steroidal saponins	Kisiel and Stojakowska 1997
Tricosanthes kirilowii	Steroidal alkaloids	Savary and Flores 1994
maxim var. Japonicum	Steroidal alkaloids	
Trigonella foenum graecum	Swainsonine	Merkli *et al.,* 1997
Valeriana officinalis L.	Xanthons	Granicher *et al.,* 1994
valeriana	Thiophenes	Granicher *et al.,* 1995
Vinca minor	Sesquiterpene coumarin	Tanaka *et al.,* 1994
Withania somnifera	ether	Banerjee *et al.,* 1994
	Defense related proteins	

	Diosgenin
	Valepotriates
	Iridoid esters
	Indole alkaloids (vincamine)
	Withanoloides

(Modified after Giri *et al.,* 2000)

1.3.2 Improvement of secondary metabolite production

Secondary metabolite biosynthesis in hairy roots is genetically controlled but is influenced by nutritional and environmental factors. According to Rhodes *et al.,* (1988), initiating cultures from plants with a high biosynthetic capacity is particularly beneficial. The composition of the culture medium affects growth and secondary metabolite production (Giri *et al.,* 1997, Nussbbaumer *et al.,* 1998). Various precursors, elicitors as well as some surfactants have also been tried for increasing the secondary metabolite production in hairy root cultures (Bais *et al.,* 2000 a,b; Rijhwani and Shanks 1998). The various aspects affecting the secondary metabolite production are listed in the Table 1.4

Table 1.4 : Factors influencing secondary metabolite enhancement in plant cell cultures.

Strain improvement	Selection Screening Genetic modification Nutrients
Medium variation	Phytohormones Precursors Antimetabolites
Culture conditions	Inoculum size PH Temperature Light Agitation
Specialized techniques	Elicitors Immobilization Permeabilization Two-phase systems Two-stage systems

Among various elicitors and other compounds studied for this purpose, Methyl jasmonate (MJ) and polyamines (PA) have been proved to be very effective in increasing the production of secondary metabolites. Methyl jasmonate (Fig. 1.3) is a very widely studied elicitor and better known as a molecule involved in signal transduction with multifaceted effects on plant growth, development, response to stress and eliciting plant resistance to pathogens and herbivores (Thomma *et al.,* 1998, Ozawa *et al.,* 2000, Swiatek *et al.,* 2002). During elicitation, jasmonates are expected to act as chemical signal compounds resulting in *de novo* gene transcription, finally leading to enhanced biosynthesis of secondary metabolites (Stockigt *et al.,* 1995). Its role in higher production of secondary metabolites particularly with hairy root cultures makes this molecule a promising one. Methyl jasmonate has greatly enhanced the production of secondary metabolites in hairy roots of *Catharathus roseus* (Rijhwani and Shanks 1998), *Brugmansia candida* (Spollansky *et al.,* 2000), *Tanacetum parthenium* L (Stojakowska *et al.,* 2002), *Datura stramonium* (Zabetakis

et al., 1999) and normal roots of *Hyoscyamus muticus* (Singh *et al.,* 1998). Rijhwani and Shanks (1998) reported that MJ addition caused an increase in specific yields of ajmalicine (80%), serpentine (60%), lochnericine (150%) and horhammericine (500%) in dosage studies in *Cathatanthus roseus* hairy root cultures.

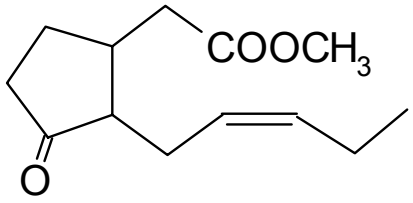


Fig.1.3. Structure of Methyl jasmonate

In plants both free and conjugate polyamines are found, which play a significant role in plant development and are associated with the physiology of flowering, metabolite synthesis in plant response to viral infections (Slocum and Galston 1985). Polyamines, such as putrescine (Put), spermine (Spm) and spermidine (Spd) are a class of aliphatic amines, are ubiquitous, non-protein, straight chain, polycationic metabolites present in both prokaryotic and eukaryotic cells. Endogenous polyamines are known to influence a variety of growth and developmental processes (Evans and Malmberg, 1989). Polyamines are shown to increase the growth as well as production in case of hairy root cultures of *Cichorium intybus* (Bais *et al.,* 1999), *Beta vulgaris* and *Tagetes patula* (Bais *et al.,* 2000 a).

Other factors such as light irradiation (Liu *et al.,* 2002), fungal cell wall compounds (Buitelaar *et al.,* 1992, Ballica *et al.,* 1993, Singh *et al.,* 1994, Bais *et al.,* 2002 b) have also been used for enhanced secondary metabolite production from hairy roots.

1.3.3 Release of secondary metabolites: down stream processing

Secondary metabolites are often stored in the cell vacuole. Various chemical and biological agents have been used for the purpose of release of secondary metabolites into the culture medium (Beaumont and Knorr 1989; Berlin *et al.,* 1988; Felix 1982). However, permeabilization of plant membranes for the release of secondary metabolites is often connected with the loss of viability of the cells treated with permeabilizing agents and methods. Cell permeabilization depends on the formation of pores in one or more of the membrane systems of the plant cell, enabling the passage of various molecules into and out of the cell (Brodelius *et al.,* 1988). Permeablizing the plant cells transiently helps in maintaining the cell viability and has short time periods of increased mass transfer of substrate and metabolites to and from the cell (Parr *et al.,* 1984, Knorr and Berlin 1987). Cell permeabilization by intermittent treatments would be beneficial as it gives sufficient recovery time to reorganize the cell membrane ultimately leading to more product efflux (Brodelius and Nilsson 1983). Table 1.5 gives various permeabilizing agents and methods used for secondary metabolite secretion in hairy root cultures.

Table 1.5: Permeabilization methods employed in plant cultures for release of secondary metabolites

Treatment	Metabolite	Plant	Reference
Oxygen starvation	Betalaines	*Beta vulgaris*	Kino-Oka *et al.,* 1992
pH	Betalaines	*Beta vulgaris*	Mukundan *et al.,* 1998b
Tween-20	Hyoscyamine	*Datura innoxia*	Boitel *et al.,* 1996
XAD-2 & Liquid paraffin	Shikonin	*Lithospermum erythrorhizon*	Shimomura *et al.,* 1991
H_2O_2	Alkaloids	*A. belladona*	Lee *et al.,* 1998a
Heat treatment	Betalaines	*Beta vulgaris*	DiIorio *et al.,* 1993
CTAB	Betalaines	*Beta vulgaris*	Bhagyalaksmi *et al.,* 2002
Polyethylene glycol	Peroxidase	*Daucus carota*	Kim *et al.,* 1997
Fungal elicitor (*Phytophthora*	β-carbolines	*Oxalis tuberosa*	Bais *et al.,* 2003

cinnamoni)	Rosmarinic acid	*Ocimum basilicum*	Bais *et al.,* 2002b
Fungal elicitor (*Phytophthora cinnamoni*)	Coumarins	*Cichorium intybus*	Bais *et al.,* 2000b
Fungal elicitor & (*Phytophthora parasitica*) DMSO	Coumarins	*Cichorium intybus*	Bais *et al.,* 2001

1.4 Bioreactors for large- scale production of hairy root cultures

Higher growth rate with high metabolite production led to the studies on scale-up of hairy root cultures for commercial exploitation. The design and operation of a bioreactor is mainly determined by biological needs and engineering requirements, which often include a number of factors: efficient oxygen transfer and mixing, low shear and hydrodynamic forces, efficient control of physico-chemical environment, easy scale-up, and so on. Because some of the factors can be mutually contradictory, it is difficult to directly employ a conventional microbial bioreactor to shear-sensitive plant tissue cultures (Honda *et al.,* 2001). Hatch (1976) was of the opinion that evaluation of a bioreactor design depends on comparison of the performance ratios at the same oxygen mass transfer rate. Owing to the unique configuration of hairy roots with their branching nature, some specific engineering aspects of bioreactor design should be considered, including a support matrix to support the roots, protection from shear stress, homogenous growth distribution, and oxygen transfer enhancement in the interwoven root matrix (Whitney 1992, Doran 1997, Honda *et al.,* 2001). For better oxygen mass transfer, various modifications were adapted such as that of a modified stirred tank reactor by Whitney (1992) wherein the roots were separated from baffles by a mesh thus avoiding the wounding of the roots.

Several kinds of bioreactors, such as the bubble column, air-lift, rotating drum, stirred tank and mist bioreactors have been attempted for hairy root cultures as given in the Table 1.6

Table 1.6: Bioreactor systems used for cultivation of hairy root cultures

Bioreactor type	Plant system	Secondary metabolite	Reference
Stirred- tank	*Armoracia rusticana*	Alkaloids	Taya *et al.,* 1989 b
	Atropa belladona	Tropane	Jung and Tepfer 1987
Stirred-tank	*Atropa belladona*	alkaloids	Jung and Tepfer 1987
(impeller	*Datura stramonium*	Tropane	Hilton and Rhodes 1990
isolated)	*Duboisia leichhardtii*	alkaloids	Muranaka *et al.,* 1992
	Trigonella foenum-	Tropane	Rodriguez-Mendiola *et al.,*
Air-lift	*graceum*	alkaloids	1991
	Panax ginseng	Scopolamine	Yoshikawa and Furuya 1987
	Lippia dulcis	Diosgenin	Saurwein *et al.,* 1991
	Beta vulgaris	Saponins	Sanchez *et al.,* 2002
Bubble column	*Solanum tuberosum*	Hernandulcin	Tescione *et al.,* 1997
	Atropa belladona	Betalaines	Kwok and Doran 1995
	Catharanthus roseus	Alkaloids	Toivenon *et al.,* 1990
	Tagetes patula	Tropane	Buitelaar *et al.,* 1991
	Hyoscyamus muticus	alkaloids	Flores and Curtis 1992
	Beta vulgaris	Indole	Mukundan *et al.,* 1998 a
	Daucus carota	alkaloids	Kondo *et al.,* 1989
Acoustic mist	*Artemisia annua*	Thiophenes	Wyslouzil *et al.,* 2000
	Artemisia annua	Tropane	Kim *et al.,* 2001, Liu *et al.,*
	Beta vulgaris	alkaloids	1999
	Carthamus tinctorius	Betalaines	DiIorio *et al.,* 1992a
		Anthocyanins	-do-
		Artimisinin	
		Artimisinin	
		Betalaines	
		Alakaloids	

1.4. 1 Mass transfer effects on hairy root growth in bioreactors

In case of bioreactors wherein the hairy root cultures were cultivated in submerged state, mass transfer resistances near the liquid –solid phase boundary show dominant influence on oxygen transport (Kanokwaree and Doran 1997, Kanokwaree and Doran 1998, Hitaka *et al.,* 1997). Williams and Doran (1999) studied the mass transfer effects in a packed bed reactor that operated at high liquid velocities to remove hydrodynamic boundary layers. It was evident from their study that, even in the absence of boundary layers, the growth of the cultures was not exponential, indicating that layers of mucilage and root hairs also represent an additional, significant barrier to oxygen transfer. Thus, there is a need to look for bioreactors operated without regions of oxygen limitations.

Hilton *et al.,* (1998) have used a modified stirred tank reactor where they have used a stainless steel mesh cage that separated the roots from stirrer and acted as an immobilization matrix. Sim and Chang (1993) have used a two-phase bubble column reactor for hairy roots of *Lithospermum erythrorhizon* where they have used n-hexadecane as a solvent system for *in situ* extraction of shikonin. Similarly, two-liquid phase bioreactors were used by Buitelaar *et al.,* (1991) for *Tagetes patula* hairy roots for the extraction of thiophenes. Kwok and Doran (1995) modified the bubble column reactor to facilitate better oxygen transfer by providing spargers at different levels of reactor vessel. Kanokwaree and Doran (1998) used a polypropylene tubing as a supplementary aeration device and addition of FC-43 perflurocarbon emulsion to the medium in case of *Atropa belladonna* hairy root cultures which resulted in 32-65% more biomass production. However, till date the most successful aspect of cultivation of hairy roots in bioreactors is the scale-up of *Panax ginseng* hairy root cultures in a 20 ton bioreactor (Scheidegger 1990).

In addition to mass transfer, the main constraint in bioreactor usage is the biomass estimation. It is difficult to estimate the biomass growing in a bioreactor as the hairy roots get entangled to the support and cannot be taken out for analysis. For this purpose, various correlations have been developed by researchers to estimate the biomass indirectly using conductivity (Taya *et al.,* 1989c), sugar depletion (Monroy *et*

al., 1994), fluid dynamics (Ramakrishnan and Curtis 1994) and reactor weight after liquid drainage (DiIorio *et al.,* 1992a, Wilson 1997). Growth rates were improved by optimization of inoculum age (Weathers *et al.,* 1997) or the length of the subculture cycle (Rijhwani and Shanks 1998). As some secondary metabolites are growth associated, (Bhadra *et al.,* 1998, Bhadra and Shanks 1997), growth improvement would improve the metabolite production (Rijhwani and Shanks 1998).

The limitations associated with the use of stirred tank and other designs of bioreactors forced the researchers to think of new designs where the shear can be reduced and facilitate better oxygen transfer. Reactors that offer a great deal of liquid dispersion (nutrient mist and trickle bed) have advantages in greater intra tissue oxygen transfer and reduction of mucilage accumulation. Acoustic mist bioreactors where the liquid is the dispersed phase and with high mass transfer have gained attention (DiIorio *et al.,* 1992a, Kim *et al.,* 2001).

1.5 Hairy root cultures of *Beta vulgaris* as a source of betalaine production

1.5.1 Betalaines as food colours

The world market for food colours has been assessed to be worth more than $500 million (O'Callaghan 1993). The market for natural food colours has an annual growth rate of 4-6% compared with 1-2% for artificial colours (LePre 1994). In this regard, plant cell cultures as well as hairy root cultures gained importance as sources of secondary metabolites of food importance. Betalaines are water- soluble nitrogen-containing pigments, which comprise the red-violet betacyanins and the yellow betaxanthins (Fig.1.4). They are immonium conjugates of betalamic acid with cyclo-DOPA and amino acids or amines, respectively. Betalaines accumulate in flowers, fruits and occasionally in vegetative tissues of plants belonging to most families of the Caryophyllales and in some higher fungi such as *–Amanita muscaria* (Steglich and Strack 1990). In food processing, betalaines are less commonly used than anthocyanins and carotenoids. Although these water-soluble pigments are stable between pH 3 and 7, they are well suited for coloring low-acid food. The most important source of betalaines as colour agent is the red beet root (*Beta vulgaris*-

family-Chenopodiaceae). Kapadia *et al.,* (1996) have shown that *B. vulgaris* extract is effective against lung and skin cancer using a mice model system.

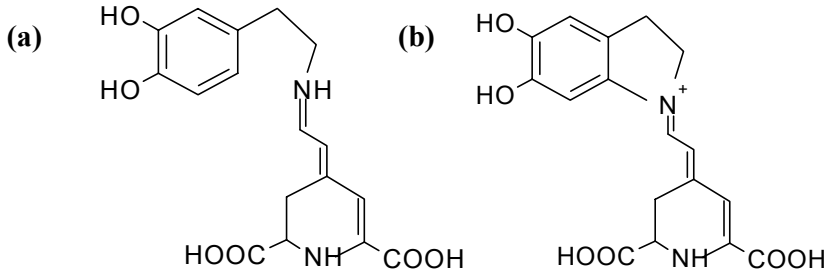


Fig. 1.4: Structure of betalaines
(a) betaxanthin (b) betacyanin

1.5.2 Biosynthesis of betalaines – L- Dihydroxyphenylalanine (L- DOPA) mediated production

The main intermediate in betalaine synthesis is L- Dihydroxyphenylalanine (L-DOPA) which is an important precursor in secondary metabolism, commonly used as a drug for Parkinson's disease, a progressive disorder associated with dopamine deficiency in the brain (Bohm and Rink 1988, Pras *et al.,* 1989). Tyrosinase and DOPA dioxygenase are the main enzymes responsible for biosynthesis of basic skeleton of betalaines. The biosynthetic pathway of betalaines is shown in Figure 1.5. Tyrosinase is responsible for the formation of DOPA and its oxidation to cyclo-DOPA by its mono and poly phenol oxidase activity where as DOPA dioxygenase catalyses the DOPA extradiol cleavage leading to the formation of betalamic acid (Steiner *et al.,* 1999, Schliemann *et al.,* 1998). The decisive step, the linkage of betalamic acid with cyclo-DOPA was suggested to be a non-enzymatic one (Schliemann *et al.,* 1999). Recently Kobayashi *et al.,* (2001) reported the formation of dopamine-derived betacyanins using hairy root cultures of *B. vulgaris.*

DOPA has been reported to be accumulated in cell cultures of *Mucuna pruriens* (Pras *et al.,* 1989)*, Vicia faba;* callus and hairy root cultures of *Stizolobium hassjoo* (Sung and Haung 2000). Chu *et al.,* (1993) reported DOPA accumulation in banana plants upon fungal infection. Phenylalanine and tyrosine have been found to

be effective in elicitation of DOPA content in callus cultures of banana (Bapat *et al.,* 2000). Biotransformations of precursors mainly tyrosine using microbial cultures such as Bacteria and Fungi were also well reported (Foor *et al.,* 1993, Tanaka *et al.,* 1974, Ul-haq and Ali 2002).

The corresponding cell cultures (Leathers *et al.,* 1992, Akita *et al.,* 2000) and hairy roots (Mukundan *et al.,* 1998a) have been attempted for the production of betalaines. Researchers at the Institute of Plant Biochemistry, Halle (Saale), Germany have done extensive studies on the biosynthetic pathways of betalaines both from plant normal roots, cell cultures as well as hairy roots.

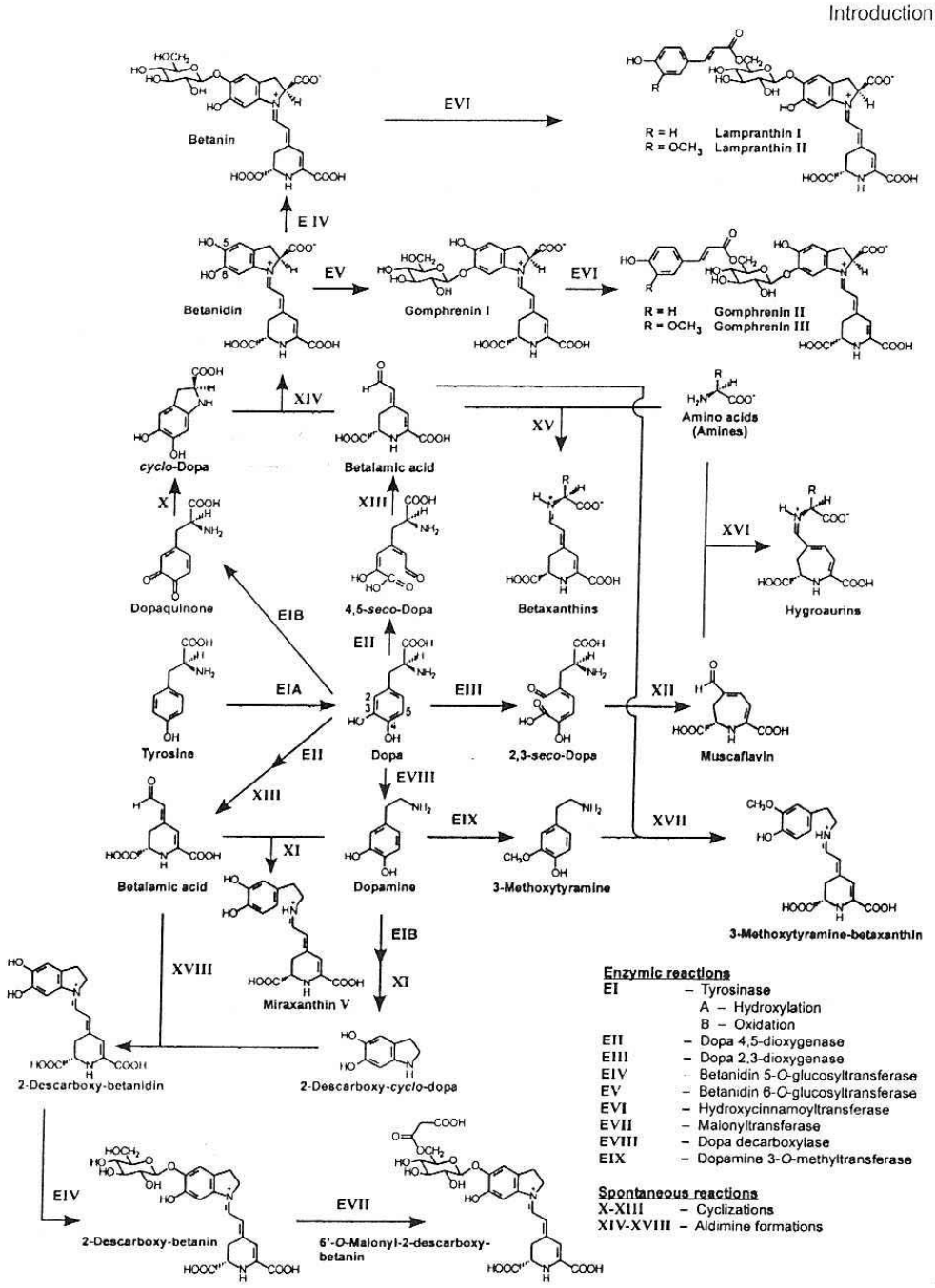


Fig 1.5: Betalaine biosynthetic pathway
(Source: Strack *et al.*, 2003)

1.6 Biotransformation as a tool for the production of high value metabolites

Biotransformation is a process wherein the functional groups of substrates/precursors are modified either sterio or regio specifically to products of interest by living cell cultures, entrapped enzymes or permeabilized cells. Biotransformations by biological catalysts *viz*. enzymes and whole cells have been well reported (Armstrong *et al.,* 1993, Rhodes *et al.,* 1994, Berger 1995, Cheetham 1995, Dornenburg and Knorr 1996, Meyer *et al.*, 1997, Scragg 1997, Krings and Berger, 1998). Plant cultures can also bioconvert externally added compounds to various valuable products, such as aromatic compounds, steroids and alkaloids (Pras *et al.,* 1995, Johnson *et al.,* 1996, Ramachandra Rao 1998, Morgan and Shanks 2000). The biotransfromation reactions by plant cultures are not just limited to compounds, which are intermediates in the endogenous pathways, but extend even to externally added compounds (Goel *et al.,* 1997). Biotransformations in particular are useful in production of novel products, which are generally not produced by chemical synthesis and they are important to elucidate the biosynthetic pathways. Precursor addition is a well-studied phenomenon which not only increases the secondary metabolite accumulation but also provides valuable information on the biosynthetic pathways (Ramachandra Rao 1998, Giri *et al.,* 2001).

1.6.1 Vanilla flavour production through biotransformation

Vanillin is the main flavour ingredient of vanilla beans and is used as a food flavour. The cost of natural vanillin is 1200 $ per Kg where as that of synthetic is 12 $ per Kg, showing its importance for production in large scale. Production of vanilla flavour by biotransformations using microbial cultures (Lesage-Messen *et al.,* 1996, Yoshida *et al.,* 1997), plant cell cultures (Romagnoli and Knorr 1988, Johnson *et al.,* 1996) and immobilized cell cultures (Ramachandra Rao 1998) has been well reported from various precursors. Plant cultures exhibit various biochemical reactions such as reduction, oxidation, hydroxylation, acetylation, esterification, isomerization, methylation, and demethylation. Vanillin formation through biotransformations by microbes, plant cells has been given in Table 1.7

Table 1.7: Vanillin production by biotransformations mediated by microbial and plant cultures

Organism	Substrate	Reference
Microbial		
Aspergillus niger	Ferulic acid	Lesage-Messen *et al.,*
Pseudomonas fluorescens	Ferulic acid	1996
Corynebacterium glutamicum	Ferulic acid	Andreoni *et al.,* 1995
Pseudomonas sps.	Eugenol	Labuda *et al.,* 1993
Enterobactor sps	isoeugenol	Rabenhorst, 1996
Aspergillus niger	Vanillylamine	Rabenhorst, 1991
Pencillium simplicissimum	Vanillyl alcohol	Yoshida *et al.,* 1997
Pycnoporus cinnabarinus	Vanillic acid	Fraaije *et al.,*1997
Plant cell cultures		Stentelaire *et al.,* 2000
Vanilla planifolia	Ferulic acid	
		Romagnoli and Knorr
Capsicum frutescens	Ferulic acid	1988 &
Spirulina platensis	Isoeugenol	Funk and Brodelius
(Blue green alga)	Vanillylamine	1990a,b
	Protocatechuic acid	
		Ramachandra Rao 1998
Immobilized cultures		
Capsicum frutescens	Ferulic acid	
Spirulina platensis	Isoeugenol	
	Vanillylamine	
	Protocatechuic acid	
Haematococcus pluvialis	Ferulic acid	Ramachandra Rao 1998
(Green alga)		
		Usha *et al.,* 2002

(Source: Ramachandra Rao and Ravishankar 2002)

1.6. 2 Improvement of biotransformations

Many a times, plant cell cultures exhibit lesser bioconversion rates when fed with precursors as most of the precursors, especially organic compounds are less/sparingly soluble in medium (aqueous phase). A novel approach to solve this problem is to use cyclodextrins along with precursors. Cyclodextrins are cyclic oligosacchardides of six (α), seven (β), or eight (γ)-1,4 linked glucose units. They have an outer hydrophilic layer and inner lipophilic cavity (Figure 1.6). They form stable inclusion complexes with lipophilic substances and make them more soluble in the culture medium, making the substrate more available to the cell culture for efficient bioconversion. Through complexation, the physical properties of the compounds are changed including their solubility in aqueous media (Szejtli 1986, van Uden *et al.,* 1994, Qi and Hedges 1995). They have been used in food, pharmaceutical industries and recently in cleaning up of hazardous wastes (Lipkowitz and Anderson 1994). Precursor addition along with β-cyclodextrin (BCD) has been employed for various studies as given in the Table 1.8

Table 1.8: β-cyclodextrin mediated biotransformations in plant cell cultures

Plant system	Precursor	Product	Reference
Mucuna pruriens	β-estradiol	4-(OH) β-estradiol	Woerdenbag *et al.,* 1990 a
Podophyllum hexandrum	Coniferyl alcohol	Podophyllotoxin	Woerdenbag *et al.,* 1990 b
Capsicum frutescens	Ferulic acid & isoeugenol	Vanillin	
	Digitoxin		Ramachandra Rao, 1998
Capsicum frutescens		Digoxin	
			Ramachandra Rao *et al.,* 2002

1.6.3 Scale-up of biotransformations

Scale-up of secondary metabolite production using bioreactors has received increasing attention in recent years. However, very few reports are available on application of bioreactors for scale-up of biotransformations, such as that of methyl digitoxin to methyl digoxin by *Digitalis lanata* cells using air-lift reactors (Kreis and Reinhard 1990, 1992), and for production of alkaloids by precursor feeding in stirred tank reactors in *Holarrhena antidysentrica* suspension cultures (Panda *et al.,* 1992).

1.6.4 Capsaicin and vanillin production using cell cultures of *Capsicum frutescens* through biotransformation

1.6.4.1 Biosynthesis of capsaicin

Capsaicin is a major pungent principle of chilli pepper and is a derivative of vanillic acid (Bennet and Kirby 1968). Capsaicin production in *Capsicum* fruits and callus cultures is well documented; it follows the phenylpropanoid pathway wherein ferulic acid, vanillin and vanillylamine are important precursors (Hall *et al.,* 1987, Johnson *et al.,* 1990, Sukrasno and Yeoman 1993). (Figure 1.7). The capsaicin biosynthetic pathway has two distinct branches, one of which utilizes phenylalanine and gives rise to the aromatic component vanillylamine, via the phenylpropanoid pathway. The second branch forms the branched-chain fatty acids by elongation of deaminated valine. Vanillylamine gets condensed with 8-methylnonanoic acid to produce capsaicin and the reaction is mediated by capsaicin synthase (Yeoman 1980). The phenylpropanoid pathway leading to capsaicin shares some common precursors with the lignin biosynthetic pathway (Sukrasno and Yeoman 1993).

Recent work on understanding of capsaicin synthesis using molecular tools has gained importance. Curry *et al* (1998) have isolated the cDNA forms of *Pal, Ca4h, Comt* from a library of cloned placental transcripts. These genes encode the first, second and fourth step of the phenylpropanoid branch of the capsaicinoid pathway. Based on the pattern of expression of these three genes during fruit development and across fruit of different pungency levels, Curry *et al*., (1998) have developed a hypothesis about the regulation of transcription for capsaicnoid biosynthetic enzymes. Transcripts of biosynthetic genes accumulate in the placenta early in fruit development and then decline in abundance; transcript levels of biosynthetic genes are proportional to the degree of pungency, the hottest Chilly has the greatest accumulation of transcripts. Curry *et al* (1998, 1999) have applied these transcript levels as a screening tool of a cDNA library of habanero placental tissue. Using this differential approach they have isolated a number of c-DNA clones and confirmed their differential patterns of expression. Two of the clones putatively encoded enzyme

activities predicted for capsaicinoid biosynthesis, beta-ketoacyl synthase and a transaminase.

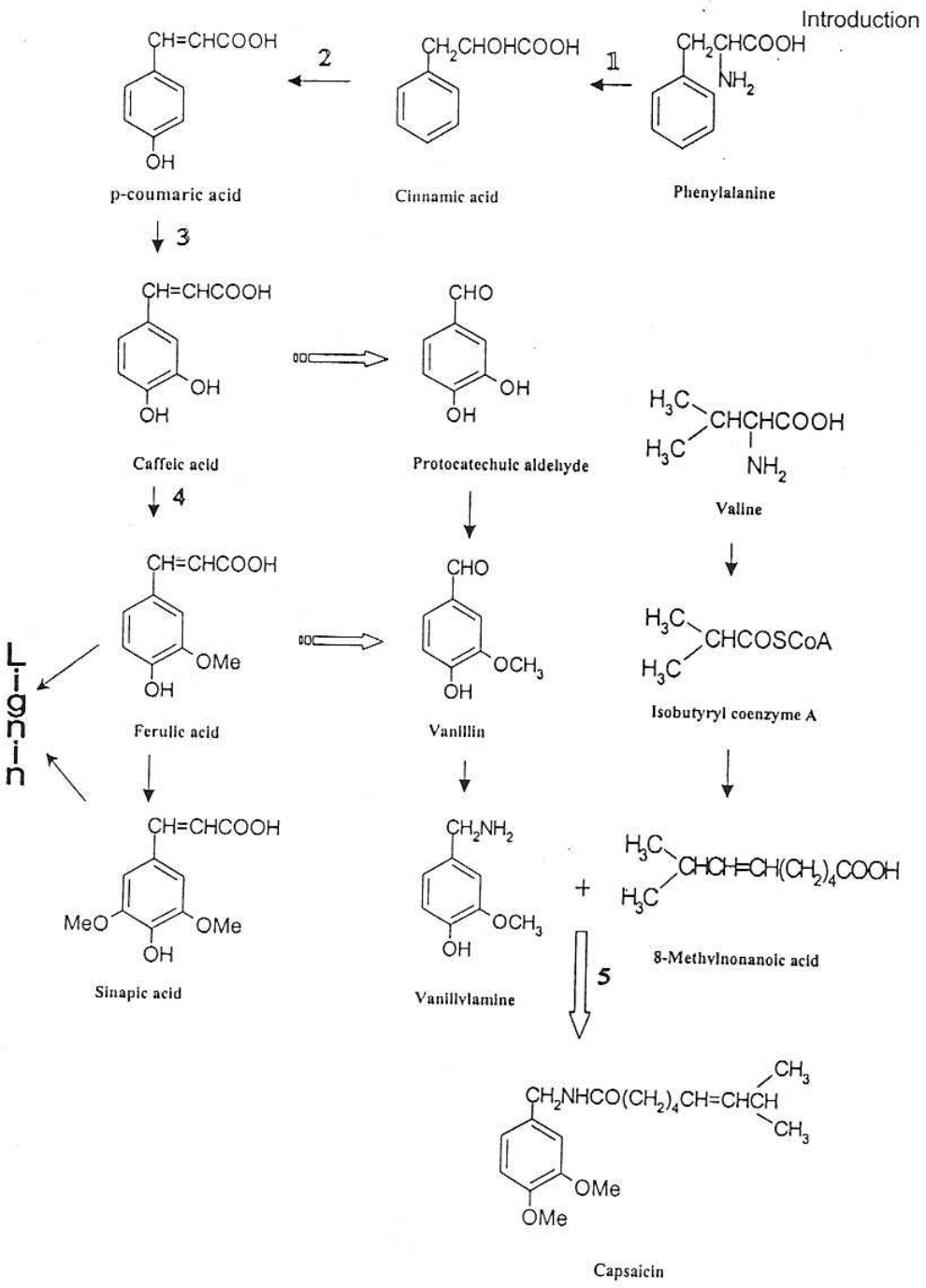


Fig. 1.7 Proposed biosynthetic pathway of capsaicin in *Capsicum frutescens* fruits and cell cultures (Source: Yeoman *et al.*, 1980)

Enzymes: **1**. Phenyl alanine ammonia lyase (PAL) **2**. *trans*-Cinnamic acid 4-hydroxylase **3**. *trans*-p-coumaric acid 3-hydroxylase **4**. Caffeic acid-O-methyl transferase (CAOMT) **5**. Capsaicin synthase

Lee *et al*., (1998 b) isolated and characterized O-diphenol-*O*-methyltransferase c-DNA clone in hot pepper. Matsui *et al*., (1997) have performed purification and molecular cloning of bell pepper fruit fatty acid hydroperoxide lyase. Aluru *et al* (1998a&b) and Curry *et al.,* (1999) have isolated a 3-ketoacyl-ACP synthase gene from habanero chilli, *Capsicum chinense* by screening cDNA libraries of transcripts from placental tissues.

The site of synthesis and accumulation of the capsaicinoids is the epidermal cells of the placenta (Suzuki *et al.,*1980). Within the cells, capsaicin-synthesizing activity has been demonstrated in the vacuolar fraction and capsaicinoids have been demonstrated to be accumulated in vacuoles (Suzuki *et al.,* 1980; Fujiwake *et al.,* 1982). Ultimately capsaicinoides are secreted extracellularly into receptacles between the cuticle layer and the epidermal layer of the placenta (Fujiwake *et al.,* 1982). These filled receptacles of capsaicinoids often appear as pale yellow to orange droplets on the placenta of the most pungent chilli fruits.

1.6.4.2 Plant cell culture mediated production of vanillin through biotransformation

The production of vanillin from ferulic acid, which is the principal intermediate in the general phenylpropanoid pathway was suggested by Zenk (1965) in case of cured vanilla pods. Funk and Brodelius (1990 a,b) investigated the phenylpropanoid metabolism in suspension cultures of *Vanilla planifolia* by feeding ferulic acid resulting in the formation of coniferyl aldehyde, along with traces of vanillylamine. Studies on feeding of phenylpropanoid precursors to cell suspension cultures of *C. frutescens*, for enhanced production of capsaicin (Lindsey and Yeoman 1984, Johnson *et al.,* 1996) and different vanilla flavour metabolites (Johnson *et al.,* 1996, Ramachandra Rao 1998), helped to elucidate the pattern of distribution of the precursors to the capsaicin and flavour pathways. It was also shown that not only phenylpropanoid intermediates but also other compounds, like cardiac glycosides (digitoxin), would be biotransformed to products of interest using cell suspension cultures of *C. frutescens* (Ramachandra Rao *et al.,* 2002).

1.6.5 Normal root cultures of Capsicum frutescens

Normal root cultures of *Capsicum* were very scarcely reported in the literature. The reason might be their inability to produce "capsaicin", which is the key product of interest. Thus most of the work has been concentrated on callus cultures, even though they produce less amounts of capsaicin. Normal root cultures were shown to be metabolically active in the biosynthesis of sesquiterpene phytoalexin - capsidol upon elicitation using cellulase (Chavez-Moctezuma and Lozoya-Gloria 1996). *C. frutescens* root cultures have been reported to accumulate various phenylpropanoids along with vanillylamine but not capsaicin (Ishikawa *et al.,* 1998).

1.6.6 Hairy roots of Capsicum frutescens

Hairy roots of chilli pepper (*Capsicum frutescens*) cv. Cayenne have been obtained using *Agrobacterium rhizogenes* mediated transformation by Sekiguchi *et al.,* (1996), where they have reported that capsaicin is not produced in hairy root cultures. Later Yamakawa *et al.,* 1998 successfully transformed *C. frutescens* with a phenylalanine ammonia lyase gene where the PAL cDNA from parsley (*Petroselium*) was linked to the CaMV 35 S promoter and subsequently transferred into hairy roots. They found that hairy roots harboring the PAL transgene showed different PAL activity with slow growth and altered morphology. Increased PAL activity was studied by Sekiguchi *et al.,* (1999) where the PAL activity was increased by the expression of parsly PAL 2 cDNA . The increase in PAL activity leads to increased lignin biosynthesis. They have also observed alteration in metabolism of aromatic compounds and the amino acid content.

1.7 Objectives of the present study

In the present study, the focus has been on utilization of root cultures for the production of secondary metabolites. As a model system, *Beta vulgaris* was chosen for pigment production and *Capsicum frutescens* for flavours. The detailed objectives are given below for each system.

1.7.1 **Beta vulgaris** *hairy root cultures for the production of betalaines*

1. To study the production of betalaines in hairy root cultures of *Beta vulgaris* especially on controlled production of betacyanin and betaxanthin.
2. Cultivation of hairy root cultures of *Beta vulgaris* in bioreactors for scale up of betalaine production process.
3. Studies on mass transfer to design a suitable bioreactor system for higher growth and metabolite production.
4. To develop effective down stream processing methodologies for release of pigments without affecting the culture to make the process a continuous one

1.7.2 Biotransformation studies using **Capsicum frutescens** *normal root cultures for the production of vanilla flavour metabolites*

1. To study the ability of *Capsicum frutescens* root cultures for biotransformation for production of vanilla flavour compounds from phenylpropanoids.
2. Optimisation of the parameters such as elicitors and β-cyclodextrin complexation for improved production of biotransformed compounds.
3. To examine the correlation of biotransformation capability with enzyme activities such as caffeic acid *O*-methyl transferase (CAOMT) for better understanding of the process.
4. Scale-up studies of developed biotransformation process in a bubble column bioreactor.

MATERIALS
&
METHODS

METHODOLOGY FOR *BETA VULGARIS* HAIRY ROOT SYSTEM

2.1.1 Plant material and establishment of aseptic seedlings
2.1.2 Glassware
2.1.3 Chemicals

2.1.4 Hairy root cultures of *B. vulgaris*
2.1.4.1 Establishment of axenic hairy root cultures
2.1.4.2 Confirmation of transformation
DNA isolation and PCR analysis
Southern blot analysis
2.1.4.3 Maintenance of hairy root cultures

2.1.5 Bioreactor studies
2.1.5.1 Acoustic mist bioreactor
2.1.5.2 Bubble column reactor
2.1.5.3 Nutrient sprinkle reactor

2.1.6 Biomass estimation
2.1.7 Conductivity and osmolarity measurements
2.1.8 Feeding of precursors, elicitors and effluxing agents
2.1.9 Betalaine analysis
2.1.10 Colour analysis of betalains
5. **Extraction and analysis of DOPA and dopamine**

2.1.1 Plant material and establishment of aseptic seedlings

Seeds of *Beta vulgaris* L. (variety- Ruby queen) were purchased from Indo-American Hybrid seeds Ltd, Bangalore, India. The seeds were washed in running tap water and were surface sterilized by rinsing them in 70% ethanol for 10 seconds followed by surface sterilization in aqueous solution of 0.1% (w/v) $HgCl_2$ for 3-5 min. in sterile distilled water. The MS basal media (Murashige and Skoog 1962) was supplemented with 3% sucrose (Hi Media, India). The pH of the media was adjusted to 5.8 ± 0.2 prior to gelling with 0.8 % agar-agar (Hi Media, India). The gelled media was autoclaved at 1.06 kg cm^{-2} pressure and 121^0C for 15 min. The seeds were inoculated on to MS basal media and incubated at 25 ± 2 ^{0}C under illumination (4.41 Jm^{-2} s^{-1} 18 hr day^{-1})

2.1.2 Glassware

The glassware such as conical flasks, culture tubes, pipettes, measuring cylinders etc. used for the entire studies were of Borosil or Vensil brand. The bioreactors used in the present study were made of corning glass.

2.1.3 Chemicals

Various chemicals used in the present studies were obtained from the Sigma chemical company, St. Louis, Missouri, USA. Sucrose and meso-inositol which were used in media preparation were obtained from Himedia Labs, Mumbai, India.

2.1.4 Hairy root cultures of *B. vulgaris*

2.1.4.1 Establishment of axenic hairy root cultures

Aseptic seedlings obtained were infected with *Agrobacterium rhizogenes* strain 15834 (wild strain obtained from Prof. P.I.J. Hooykaas Clusius Laboratorium, Rijks universitiet, Leiden, The Netherlands) on leaves and hypocotyl regions and again placed on fresh MS medium with 3% sucrose and 1% agar. The hairy roots started appearing from 10^{th} day of infection. In order to make the cultures free from the bacterium, after 20 days of infection the roots were cut and placed in MS liquid

medium containing 0.05% (w/v) cefotaxime (Alkem chemicals, India) for 2 days. The roots were then washed thrice with distilled water and transferred into fresh MS basal liquid medium with 3% sucrose and maintained in dark conditions on a rotary shaker at 90 rpm at a temperature on 25±1^{0}C.

2.1.4.2 Confirmation of transformation

The transgenic nature of hairy roots was confirmed by PCR analysis followed by southern blotting.

DNA isolation and PCR analysis

DNA from the hairy root clone (derived from the strain ATCC-15834) as well as normal roots of *B. vulgaris* was isolated according to Dellaporta *et al.,* (1983). Plasmid DNA from *Agrobacterium rhizogenes* strain ATCC-15834 was used as a positive control. The Polymerase chain reaction was used to detect the integration of *rol* A gene into the plant genome. The bacteria- free- roots of *Beta vulgaris* grown in MS liquid medium without any plant growth regulators were taken and washed in a solution of NaOH (200 mM) and SDS (1% w/v) for 5min and then rinsed in sterile deionised water (Damiano *et al*. 1995). The roots were dried on sterile filter paper and quickly frozen in liquid nitrogen. Genomic DNA from hairy roots and normal roots was extracted according to the method of Dellaporta *et al.* (1983). PCR was used to detect the *rol A* gene using primers which were designed according to Hamil *et al*. (1991) and obtained from Bangalore Genei. Pvt Ltd, Bangalore, India. For amplification of *rol* A gene, the primers used were, forward- 5'-AGA GAA TTA GCC GGA CTA-3' and, reverse- 5'-GTA TTA ATC CCG TAG GTT GTT T-3'. The PCR mixture (total volume 25 µl) contained 50 ng of DNA prepared from hairy roots and normal roots respectively as the template, 1X PCR buffer, 25 p moles of each primer, 2.5 mM of dNTPs and 1 unit of Taq DNA polymerase (Bangalore Genei Pvt Ltd.). PCR for *rol* A was carried out under the following conditions in Perkin-Elmer thermal cycler: initial denaturation at 94^o C for 5 min followed by 35 cycles of 1 min denaturation at 94^o C, 1 min annealing at 55^o C and 1 min extension at 72^o C with a final extension of 72^o C for 10 min. The amplicons were analyzed by running on 1.2%

agarose gel (Hi Media India Pvt. Ltd, India) along with 100-bp marker (MBI Fermentas). Plasmid DNA from *Agrobacterium rhizogenes* strain ATCC 15834 was used as control.

Southern blot analysis

Transformation of *Beta vulgaris* for hairy roots induced by *A. rhizogenes* was confirmed by Southern blot hybridization. The PCR amplified DNA was run on 1.2% agarose gel to separate the DNA fragments and transferred to nylon membrane (Pall Gellman) using standard protocols (Sambrook et al., 1989). PCR amplified fragment of *rol* A gene obtained from *Agrobacterium rhizogenes* strain ATCC 15834 was used as probe after labeling with digoxigenin (DIG). The labeling, hybridization and detection were carried out according to the manufacturer's instructions (Boehringer Mannheim, Germany) as given below.

The purified PCR fragment (15 µl) was denatured in boiling water for 10 min followed by snap cooling on ice. After centrifuging the denatured DNA, 2µl hexanucleotide mix, 2µl of dNTP mixture, and 1µl Klenow enzyme were added on ice. The contents were mixed by a brief spin and incubated at 37 ^{0}C for 20 h. The reaction was stopped by adding 2µl of 0.2M EDTA. Subsequently, 2.5µl of 4M LiCl and 75µl of prechilled ethanol were added and mixed well. The samples were kept at –20°C overnight for precipitation. After centrifugation at 4°C for 15 min, the pellet was washed with 100µl of 70% ethanol and allowed to dry. Later the pellet was dissolved in 50µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at –20°C.

Prehybridization was carried out at 68^{0}C with mild agitation for 6 hours followed by hybridization at 68^{0}C overnight (5X SSC, N-lauroylsarcosine, 0.1% (w/v) SDS, 0.02% (w/v)). Further, the membrane was washed in 2X SSC (3M NaCl, 0.3M sodium citrate pH 7.2, 0.1%SDS), for 2x10 min at room temperature and subsequently, with 50ml 0.1%SSC, 0.1%SDS, for 2x15 min at 68°C under mild agitation. The membrane was rinsed briefly at room temperature in maleic acid buffer (0.1M maleic

acid, 0.15 M NaCl, adjusted to pH 7.5) and incubated in 1X blocking solution (Boehringer Mannheim, Germany) (1X working solution is prepared by diluting the stock solution 1:10 in maleic acid buffer) for 30 min. 10 ml antibody solution (1:5000 Anti-DIG-AP conjugate in 1X blocking solution) was added to the membrane and incubated for 1 h at room temperature. The membrane was washed for 2x15 min in 50ml maleic acid buffer. The membrane was incubated in 20ml detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5) for 5 min. 10ml freshly prepared BCIP/NBT solution (substrate for alkaline phosphatase) was then added and the color was developed by incubating overnight in the dark without shaking.

2.1.4.3 Maintenance of hairy root cultures

The hairy roots of *Beta vulgaris* were maintained by sub culturing in Murashige and Skoog (1962) medium without any phytohormones and with 3% sucrose. The pH was adjusted to 5.8 before autoclaving. The flasks were kept on a shaker at 90 rpm and cultured in dark.

2.1.5 Bioreactor studies

2.1.5.1 Acoustic mist bioreactor

The schematic diagram of the acoustic mist bioreactor used in the present study is shown in Figure 2.1. It has 2 major components the bioreactor vessel made up of polycarbonate and the acoustic transducer (Holmes Co., USA). The vessel was placed on the transducer at a standardized distance and the vessel had a support inside with two-tier autoclavable nylon mesh (Muhlen Bou, Bangalore, India) support to place hairy roots. The mist cycle was controlled by a simple control box (Initial 30 min on set; with 10/20 min. on and off set up for the whole culture period). The acoustic field intensity was found to vary with the axial distance from the transducer. Hence the bioreactor was kept at a distance of 2.5 cm from the transducer for maximum mist generation. The volume of the vessel is 300 mL and the working volume was 100 mL. The reactor vessel also had openings for air inlet (via a PTFE filter of 0.22μm) which passes through the sparger, out let connected to $CaCl_2$ flask and one more port for medium recirculation from a reservoir. The high-energy ultra

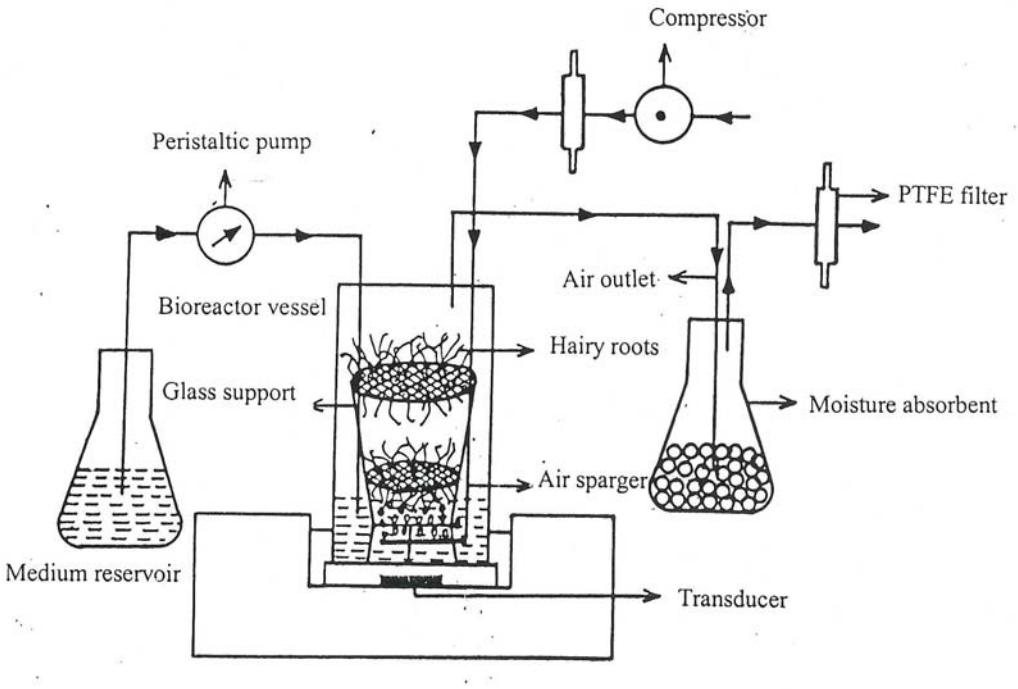


Fig 2.1: Schematic diagram of an acoustic mist bioreactor

sound waves generated by the transducer (1.7 MHz) passed through the liquid medium via the polycarbonate vessel creating a liquid mist, which coalesces and falls on the roots providing required nutrients to the hairy roots. It was observed that the mist generation rate was constant even though the volume of the medium was varied. It has an advantage of delinking the bioreactor vessel with the transducer facilitating the autoclaving of the bioreactor vessel separately preventing the damage to the transducer.

2.1.5.2 Bubble column reactor

The bubble column reactor (Figure 2.2) was made up of corning glass (Length 22 cm, diameter 14 cm) of 3L volume and the working volume was 1.75 L. The reactor lid was provided with openings for air inlet, outlet, inoculation port and for sample drawing. The compressed air through the PTFE filter (0.22μm) was sparged at a rate of 33.4 cm^2/sec, which results in bubbling supplying oxygen to the hairy roots. The hairy roots were inoculated in a plastic autoclavable basket (Height 10.5 cm, diameter 8.5 cm) supported by a stainless steel stand at a distance of 7 cm from the bottom of the reactor. It is opened on the top and has pores of 0.5 cm size on the sides and the bottom to facilitate oxygen transfer through the bubbles. Due to coalescence, the diameter of the bubbles varied from the sparger to the liquid surface. The reactor was operated in dark and the medium sample was drawn for osmolarity and conductivity measurements at 4-day intervals.

2.1.5.3 Nutrient sprinkle reactor

The schematic diagram of the nutrient sprinkle bioreactor used in the present study is shown in Figure 2.3. This reactor had 2 vessels, one used as cultivation vessel and other as a media reservoir. The working volumes were 1L and 1.5L respectively. The volumes of the vessels were 3L and made up of corning glass (Length 22 cm, diameter 14cm). The medium was recirculated between the 2 vessels through silicon tubes by peristaltic pumps. The vessels have openings for air inlet, outlet, inoculation port (In the functional reactor) and sampling port besides the openings for air circulation. The roots were placed on an autoclavable nylon mesh support (Muhlen

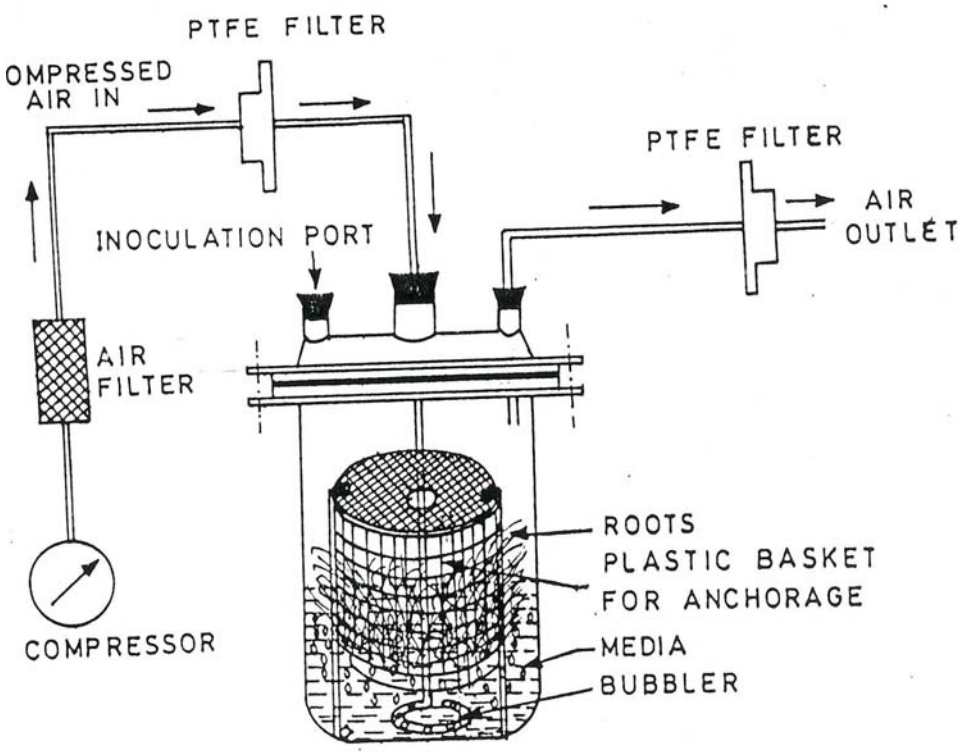


Fig 2.2: Schematic diagram of a bubble column bioreactor

Bou, Bangalore, India) which was further attached to a stainless steel stand. The medium from the reservoir was sprinkled on the root biomass by a cross-shaped opening on top of the support providing nutrients to the growing tissues.

2.1.6 Biomass estimation

After harvesting, the roots were dried on a filter paper and the fresh weight (FW) was noted. The roots then were dried in a hot air oven at 70^0C for 24 hours for dry weight (DW) estimation.

2.1.7 Conductivity and osmolarity measurements

Conductivity measurements were done using a conductivity meter (Wiss-teelm-werkstalten model LF-54, Wielhalm, Germany) at a constant temperature of 25^0C and expressed in milli Siemens. Osmolarity was measured by an automatic cryoscopic osmometer(Osmomat-3, Gontech, Germany) and expressed in milli Osmol/Kg. The sensitivities are less than ± 2%.

2.1.8 Feeding of precursors, elicitors and effluxing agents

Spermidine (spd) and putrescine (put) were dissolved in required amount of distilled water and fed to the cultures grown in bioreactor after filter-sterilization with 0.22 μ membrane filters (Sartorius India Ltd.) in a laminar air flow chamber. Methyljasmonate (MJ) was first dissolved in ethanol and made the volume with distilled water and fed to the cultures in bioreactor after filter-sterilization using 0.22 μ membrane filters (Sartorius India Ltd.).

CTAB (Cetyl Trimethyl Ammonium Bromide) a cationic surfactant was purchased from Merck chemicals, Darmstadt, Germany. It was dissolved in distilled water and fed to the cultures after filter-sterilization with 0.22 μ membrane filters (Sartorius India Ltd.) in a laminar air flow under aseptic conditions. CTAB has not affected the pH of the medium.

Tyrosine was first dissolved in a few drops of 0.1 N NaOH, made up the volume with distilled water, filtersterilized, and fed to the cultures at required concentrations. Similarly, catechin and caffeic acid were dissolved in water and fed to the cultures after filter-sterilization.

2.1.9 Betalaine analysis

Known weight of hairy roots were extracted in a mortar and pestle with a known volume of mixture of 0.05% (w/v) citric acid and 0.01% (w/v) ascorbic acid. The homogenate was centrifuged at 5000 rpm and then the absorbance of the supernatant was measured in a UV-Spectrophotometer at 540 and 480 nm for betacyanins and betaxanthins respectively. The betalaine content was calculated according to Abeysekere *et al.* (1990).

$$\text{Betacyanin content (mg/g fresh wt.)} = \frac{\text{Absorbance at 540 nm}}{1120} \times \frac{1000}{\text{Volume of sample}}$$

$$\text{Betaxanthin content (mg/g fresh wt.)} = \frac{\text{Absorbance at 480 nm}}{750} \times \frac{1000}{\text{Volume of sample}}$$

The sum of betacyanin and betaxanthin contents is considered as the total betalaine content in the sample (mg/g fresh wt.)

In case of studies with pigment release, the medium from the bioreactor was drawn aseptically, filtered through Whatman#1 filter paper, centrifuged at 2000 rpm for 10 min and then analysed for betalaines (mg L^{-1} medium) as mentioned above.

2.1.10 Colour analysis of betalaines

The hunter colour property of the pigments was estimated with a colour measuring system (Minolta Spectrophotometer, model: CM 3500D; Japan) and

expressed in terms of 'l' (lightness), 'a' (redness), and 'b' (yellowness), where the instrument calibration was done with a standard white tile.

2.1.11 Extraction and analysis of DOPA and dopamine.

The hairy roots (500 mg fresh weight) were extracted with 2% formic acid (2 ml) in a chilled mortar and pestle using neutralized glass powder under dark conditions. The extracts were centrifuged at 3000 rpm and filtered through 0.22 µ filters (Sartorius India) for HPLC analysis for quantification of DOPA and dopamine.

The HPLC analysis used in this present study was modified from Steiner *et al.,* (1999). The HPLC analysis was performed with a Shimadzu system (LC-10 A, programmable multi wave length detector and scanning fluorescence detector). The chromatograph was equipped with a 5 µm Nucleosil C 18 column (25 cm long, 4 mm i.d.; Shimpack, USA). The solvent system used was phosphoric acid (H_3PO_4, 1.5 % v/v) in water. Tyrosine, DOPA and dopamine were detected with a UV detector (280 nm) and a fluorescence detector (Xenon arc lamp; excitation at 280 nm and emission at 314 nm). DOPA and dopamine were quantified by comparing with authentic standard curves.

2.1. METHODOLOGIES FOR *CAPSICUM FRUTESCENS* ROOT CULTURES

2.2.1 Plant material, establishment of aseptic seedlings and root cultures
2.2.2 Growth measurement
2.2.3 Feeding precursors
2.2.4 Complexation of precursors with β-cyclodextrin (BCD)
2.2.6 Biotransformation studies in bioreactor
2.2.7 Extraction and estimation of capsaicin
2.2.8 Extraction and estimation of vanillin and related metabolites
2.2.9 Caffeic acid *O*-methyl transferase (CAOMT) activity
2.2.10 Protein estimation

2.2.1 Plant material, establishment of aseptic seedlings and root cultures

Capsicum frutescens Mill. (IHR 1203) seeds were obtained from Indian Institute of Horticultural Research (IIHR, Bangalore, India). The methodology of obtaining aseptic seedlings is described in section 2.1.1. Aseptically grown seedlings of *C. frutescens* after 30 days of germination had developed a good root system. The roots were subcultured into fresh half-strength MS liquid medium with 2.23 μM naphthalene acetic acid (NAA), and kept at 25 ± 1 ^{0}C in the dark, on a rotary shaker at 90 rpm.

2.2.2 Growth measurement

As described in section 2.1.6

2.2.3 Feeding precursors

Phenylpropanoid precursors such as- ferulic acid, protocatechuic aldehyde, caffeic acid and veratraldehyde were dissolved in 80% (v/v) ethanol and fed externally, after filter-sterilization, to the root cultures of *Capsicum.* The final alcohol concentration was kept below 0.1% (v/v) to avoid the inhibitory effect of ethanol on the growth of culture as reported by Ramachandra Rao (1998). The addition of precursors to the shake flasks was done on day 10 of the culture period.

Methyl jasmonate (MJ) was first dissolved in ethanol and appropriate volumes were made up with distilled water and fed to the cultures on 10th day after filter sterilization using 0.22 μ membrane filters (Sartorius India Ltd.)

2.2.4 Complexation of precursors with β-cyclodextrin (BCD)

Ferulic acid was complexed with β-cyclodextrin (BCD) in aqueous solutions at 1:1 molar concentration, and the complex was fed to the *C. frutescens* root cultures after filter sterilization using 0.22 μ membrane filters (Sartorius India Ltd.).

2.2.5 Biotransformation studies in bioreactor

The bioreactor used for this study was a 3-L bubble column type as described in the section 2.1.5.2.

The bioreactor was inoculated with 5 g (fresh weight) of 1-week old root cultures of *C. frutescens*. Ferulic acid (1 mM) and β-cyclodextrin (1 mM) complex was added to the bioreactor (dissolved in methanol and filtersterilized) after 12 days. 10 ml aliquots of medium from the reactor was drawn aseptically every 3 days, then extracted with ethyl acetate, concentrated to dryness and used for HPLC analysis for detection and analysis of vanilla flavour compounds. After the reactor run was over, the roots were taken out and ground with ethyl acetate in a mortar and pestle, using glass powder; the extract was concentrated, and finally dissolved in methanol for HPLC analysis of resultant biotransformed products.

2.2.6 Extraction and estimation of capsaicin

The normal roots of *C. frutescens* were ground with ethyl acetate in a chilled mortar and pestle using glass powder. The extract was centrifuged at 2000 rpm for 15 min. and the supernatant was evaporated. The residue was then dissolved in known aliquots of ethyl acetate and used for capsaicin analysis. The media samples were also extracted with ethyl acetate in a separating funnel. The pooled organic layers were dried and finally dissolved in a known volume of ethyl acetate for HPLC analysis.

The HPLC analysis was carried out according to Johnson *et al*., (1992). The capsaicin analysis was done on a C 18 Bondapak column using authentic capsaicin standard (Sigma chemicals, St. Louis, USA) with detection at 280 nm. The isocratic mobile phase was acetonitrile : water (1 % acetic acid) (40 : 60 v/v) and a flow rate of 1 ml/min, was used.

2.2.7 Extraction and estimation of vanillin and related metabolites

The roots were ground with ethyl acetate in a chilled mortar and pestle using glass powder. Samples of the medium were extracted with ethyl acetate in a

separating funnel (3 times); thereafter, the organic layers were pooled and concentrated to dryness under reduced pressure (Rotovac, Heidelph, Germany). The extracts were finally dissolved in methanol for HPLC analysis. HPLC analysis was carried out using a Shimadzu LC-10A system on a Shim-pack column (4.6 mm x150 mm, 5µm pore size). The mobile phase with isocratic solvent system consisted of methanol: acetic acid: water (20:5:75) at a flow rate of 1 ml/min and sample was detected at 280 nm. Chromatographic peaks were determined by comparison with authentic standards.

2.2.8 Caffeic acid *O*-methyl transferase (CAOMT) activity

The activity of CAOMT was determined by the method reported by Shimada *et al.,* (1970). Fresh root tissue of 500 mg fresh weight was homogenized in chilled mortar with 6 ml of 100mM Tris-HCl buffer, pH 7.5. The extract was filtered through guaze cloth and centrifuged at 6000 rpm for 20min at 4 ^{0}C. The supernatant was used as the enzyme extract. The reaction mixture contained 0.2 ml of 1M Tris-HCl, pH 8.0, 0.1 ml each of 10 mM caffeic acid, 40 mM $MgCl_2$, 5mM S-adenosyl-L-Methionine (SAM) and 1ml of the enzyme solution. The reaction was carried out at 30^0 C for 30min. The enzyme reaction was terminated by the addition of 0.1 ml of 1.0 M HCl. The mixture was extracted with diethyl ether (2.5 ml x 2). The solvent was evaporated at 22^0 C under reduced pressure, and the residue was dissolved in 0.5 ml of absolute ethanol. Samples were chromatographed on silica gel GF_{254} TLC plates developed with benzene: acetic acid (4:1). Ferulic acid was detected under UV light and the fluorescent spot on the plate was recovered and eluted with 4 ml of absolute methanol. The amount of ferulic acid was determined by measurement of absorbance at 323 nm using Spectrophotometer. The specific activity of CAOMT was expressed in unit/mg protein. One unit of the enzyme was defined as formation as µg ferulic acid formed per 30 min at 37^0 C under the assay conditions.

2.2.9 Protein estimation

The protein content in enzyme preparations was determined by Lowry's method (Lowry *et al.,* 1951) using bovine serum albumin (BSA) as reference standard.

RESULTS

3. 1 RESULTS OF THE STUDIES WITH *BETA VULGARIS* HAIRY ROOT SYSTEM

3. 1 Results of the studies with *Beta vulgaris* hairy root system

The objectives of the present study include the production of betalaines using various bioreactor designs by hairy roots of *B. vulgaris* to evaluate the mass transfer

effects and optimization of a suitable bioreactor design for scale-up studies. Attempts were also made for increased pigment production and on release of pigments without inhibiting the root growth.

3.1.1 Initiation of hairy root cultures

Hairy roots obtained from different strains of *Agrobacterium rhizogenes* have shown differences in branching patterns as well as growth. Among the strains used for initiation of hairy roots, such as ATCC 15834, LMG 150 and A_4, the frequency of hairy root initiation and their subsequent growth was higher in case of hairy roots obtained from the strain ATCC 15834 (Fig 3.1). Moreover, among all the clones obtained, hairy roots obtained from strain ATCC 15834 have shown faster growth as well as high metabolite production. Hence, for all further experiments the hairy roots obtained from the strain ATCC 15834 were used. The hairy roots at different culture times are shown in Fig. 3.2.

3.1.2 Confirmation of transgenic nature of *B. vulgaris* hairy roots

The hairy root clone (ATCC-15834) showed the presence of *rol* A gene on PCR amplification (Fig. 3.3a), where as the untransformed roots showed the absence of the same. The primers used were, forward- 5'-AGA GAA TTA GCC GGA CTA-3' and, reverse- 5'-GTA TTA ATC CCG TAG GTT GTT T-3'. *rol* A primers produced a fragment of 360 bp as compared with the λ Pst I molecular marker. Southern hybridization also revealed the transgenic nature of hairy roots obtained upon infection with *Agrobacterium rhizogenes* (Fig. 3.3b).

3.1.3 Growth and betalaine production studies of hairy roots in shake flasks.

The hairy roots in the initial stages of growth were pale coloured, and with progression of culture time, they turned red in colour indicating the accumulation of betalaines. They exhibited fast growth rate, wherein the maximum biomass of 14.5 g

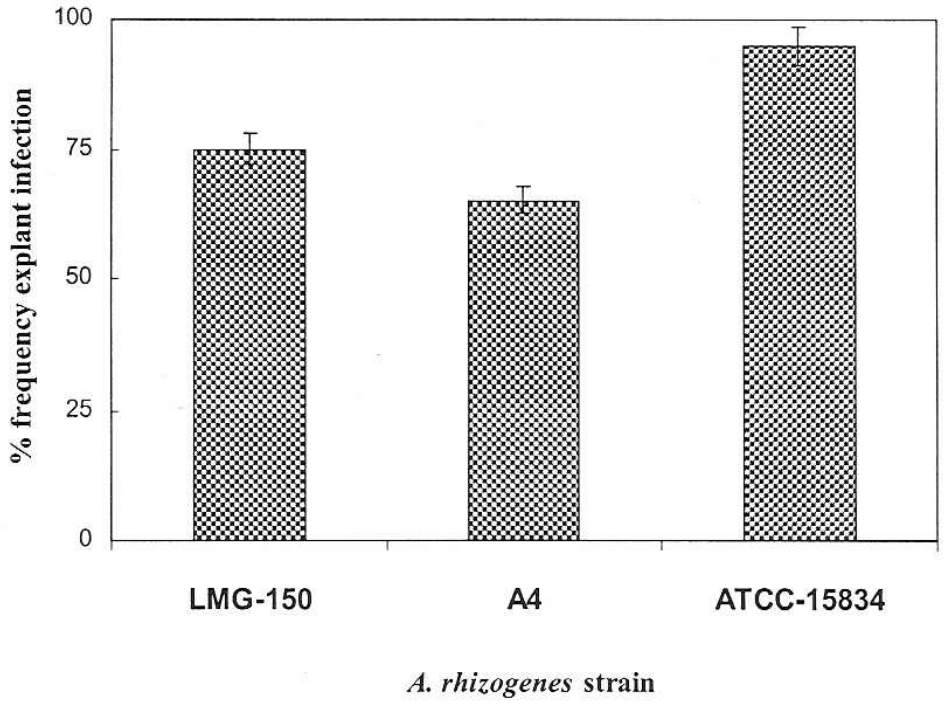
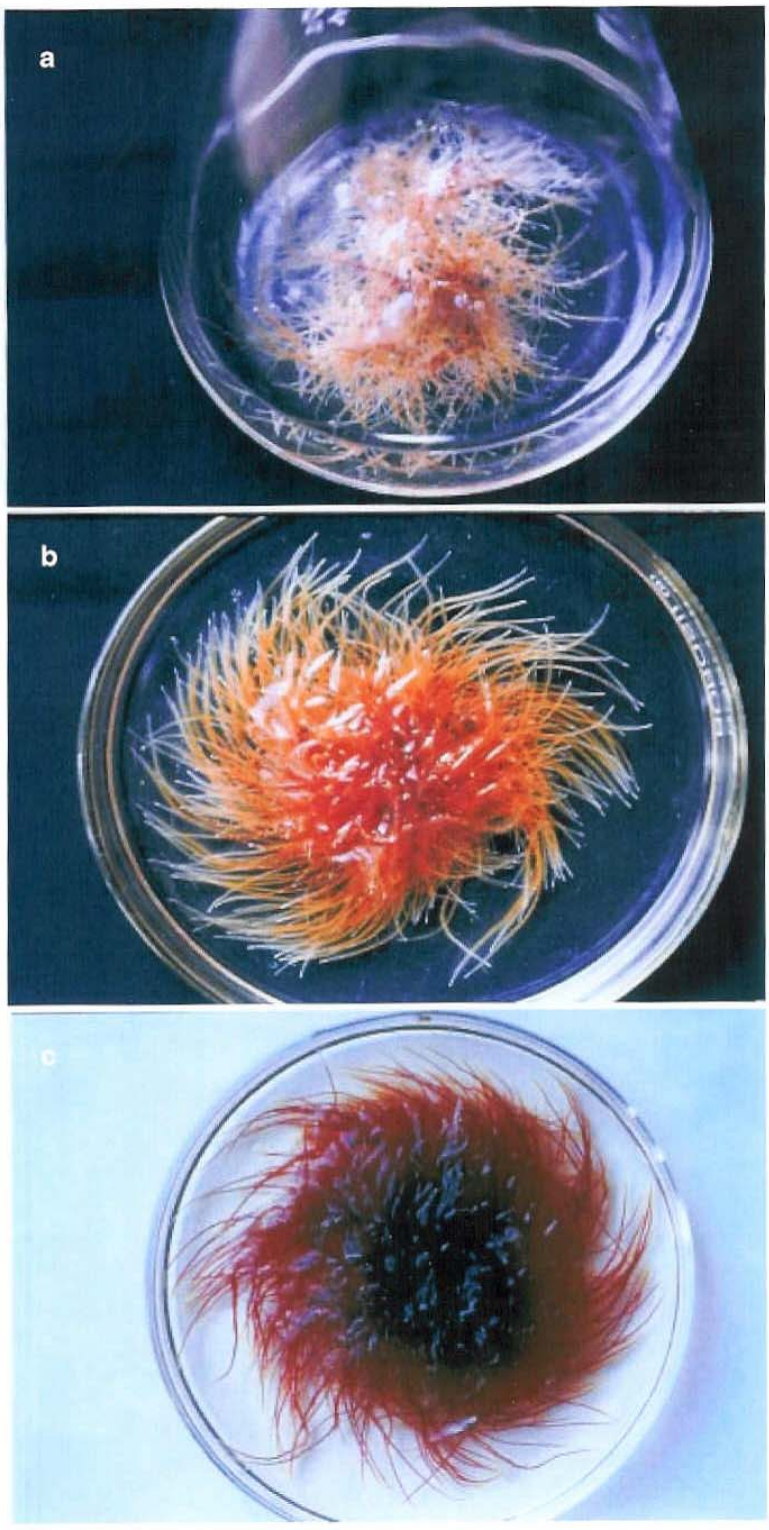
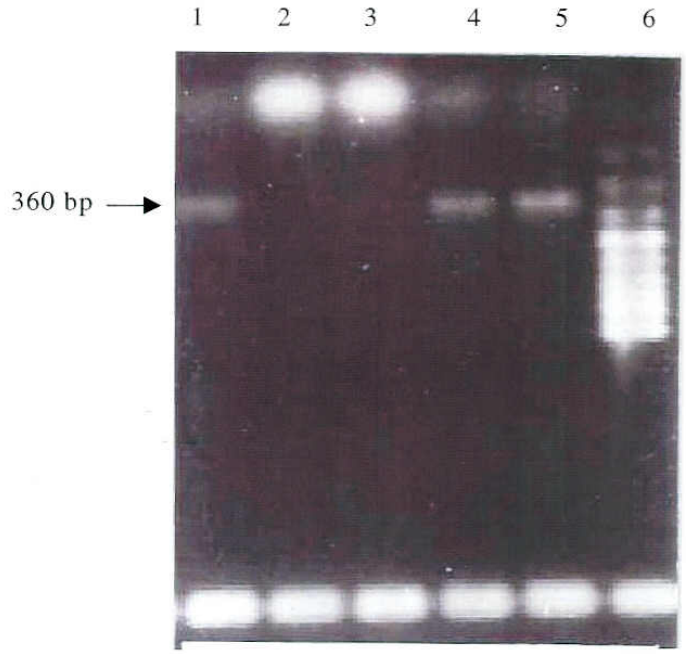


Fig 3.1: Effect of various strains of *Agrobacterium rhizogenes* on % frequency of explant infection in *B. vulgaris*


a
b
c



ig 3.3: (a) Agarose gel electrophoresis of PCR amplified *rol* A genes (360 bp)
Lane 1. Genomic DNA from *Agrobacterium rhizogenes*
Lane 2&3. Control/ untransformed
Lane 3&4. Transformed roots of *B. vulgaris*
Lane 6. Molecular marker (λ Pst I)

(b) Southern blotting
Hybridization of the PCR product using DIG labeled probe for 360 bp fragment from the *rol* A sequence -
Forward- 5'-AGA GAA TTA GCC GGA CTA-3' &
Reverse- 5'-GTA TTA ATC CCG TAG GTT GTT T-3'

DW L^{-1} was obtained on the day 24 of culture from an initial inoculum of 0.3 g DW L^{-1}. There was a 48.3 fold increase in biomass recorded as on day 24. With further increase in culture time, the biomass decreased (Fig 3.4). Similarly, betalaine content also increased with culture time, reaching a maximum value of 160 mg L^{-1} on the day 24 of culture. However, betalaine levels reduced subsequently.

3.1.4 Estimation of biomass using medium conductivity and osmolarity

To know the effect of other environmental changes on conductivity and osmolarity of the medium, controlled experiments without any biomass were carried out. Change in pH from 4-6 has not affected the conductivity and osmolarity. Increase in temperature from 5^0 to 50 ^{0}C resulted in an increase of conductivity from 3.1 to 7.5 mS but has not affected medium osmolarity (Table 3.1 a).

Table 3.1a: Variation of conductivity and osmolarity with temperature in controls (MS medium without inoculum).

Temperature (^{0}C)	Conductivity (mS)	Osmolarity (m Osmol Kg^{-1})
5	3.1	94
7	3.9	"
10	4.4	"
20	5	"
25	5.4	"
30	6.1	"
35	6.5	"

Increase in sucrose and glucose concentrations from 1-6% showed a decrease in conductivity from an initial value of 5.2 mS to 4.8 and 4.7 mS, respectively. Increase in glucose and sucrose concentration from 1-6 % increased the osmolarity significantly to reach a maximum of 430 and 432 m Osmol Kg^{-1}, respectively (Table 3.1 b,c).

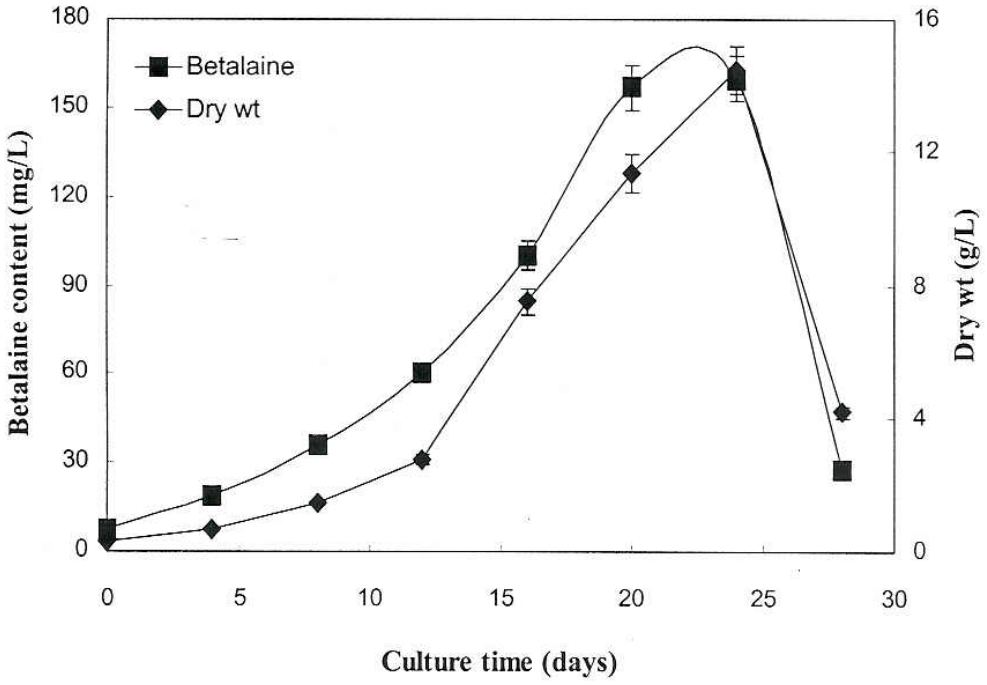


Fig 3.4: Growth and betalaine production in hairy roots of *B. vulgaris* grown in shake flasks

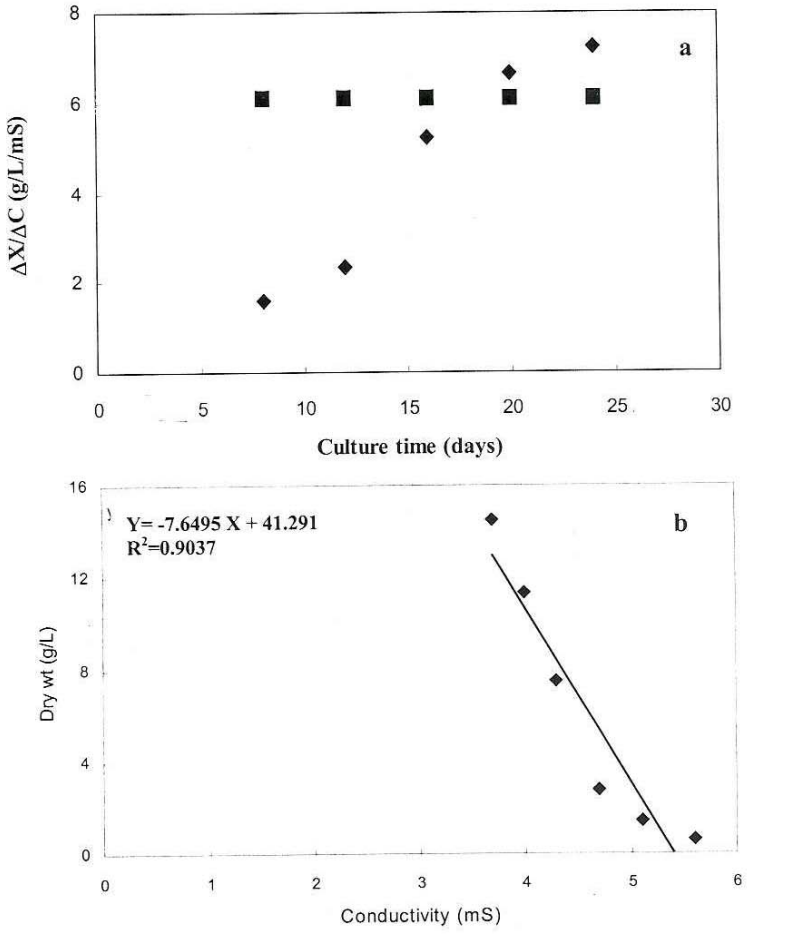


Fig 3.6: (a) Variation of the ratio between dry weight and conductivity (ΔX/ΔC) for *B. vulgaris* hairy root cultures as a function of culture time (■). ♦ Indicates the value obtained from a linear correlation of the dry wt. data forced through the origin of Fig. 3.5b (6.109g (dry wt) L^{-1} mS^{-1}).

(b) Relationship between conductivity and biomass concentration during batch culture of *B. vulgaris* hairy roots.

Table 3.1 b: Variation of conductivity and osmolarity with sucrose concentration in controls (MS medium without inoculum)

Sucrose (% W/V)	Conductivity (mS)	Osmolarity (m Osmol Kg^{-1})
1	5.2	119
2	5	149
3	5	180
4	5	208
5	4.8	261
6	4.8	432

Table 3.1 c: Variation of conductivity and osmolarity with glucose concentration in controls (MS medium without inoculum)

Glucose (% W/V)	Conductivity (mS)	Osmolarity (m Osmol Kg^{-1})
1	5.2	171
2	5.1	218
3	5.05	263
4	5	325
5	4.8	374
6	4.7	430

3.1.4.1 Conductivity as a method of indirect estimation of biomass

The variation of hairy root biomass concentration and conductivity during batch culture of *B. vulgaris* L. is shown in Fig. 3.5 a. The medium conductivity gradually decreased with culture time from an initial value of 5.6 mS to 3.7 mS on the day 24 of culture. Taya *et al.,* (1989) and Ryu *et al.,* (1990) have shown that increase in cell concentration (ΔX) can be correlated directly with decrease in the medium conductivity (ΔC) in case of plant cell and tissue cultures in shake flasks. A linear relationship between hairy root mass grown (ΔX) and decrease in conductivity (ΔC)

was observed during batch culture of *B. vulgaris* as shown in Fig. 3.5 b. Here ΔX is $X-X_0$ where X is dry biomass at any given time 't' (g L^{-1}); and ΔC is C_0-C where C_0 is initial conductivity of the medium (mS) and C is the conductivity at any given time 't'. The proportionality constant, 'k' obtained by linear regression forcing through origin is 6.109 g (dry wt) L^{-1} mS^{-1} in the present case. ΔX/ΔC against culture time was plotted to check whether the involvement of initial values leads to errors during correlation between conductivity and biomass (Fig. 3.6 a). Ballica et al. (1993) have indicated a much simpler and more useful relationship in y=mX+C form. The plot according to this relationship for *B. vulgaris* hairy root culture is shown in Fig. 3.6 b. The m and C values were found to be –7.64 and 41.29, respectively. These values were used to calculate the biomass as predicted from conductivity values.

3.1.4.2 Osmolarity as a method of indirect estimation of biomass

The variation of hairy root biomass growth with osmolarity during batch culture of *B. vulgaris* is shown in Fig. 3.7 a. The medium osmolarity increased initially to a maximum of 232 mOsmol kg^{-1}, as recorded on 8^{th} day from an initial value of 220 mOsmol kg^{-1} and then decreased gradually to reach a value of 168 mOsmol kg^{-1} on 24^{th} day. The correlation between hairy root biomass growth and decrease in osmolarity during batch culture of *B. vulgaris* is shown in Fig. 3.7 b. A linear relationship was observed between change in biomass (ΔX) and change in osmolarity (ΔO) from 8^{th} day of culture period. Here ΔX is $X-X_0$ where X_0 is the initial biomass concentration and X is the dry biomass concentration at any given time 't' (g L^{-1}) and ΔO is O_0-O where O_0 is the initial osmolarity of the medium (mOsmol kg^{-1}) and O is the osmolarity at any given time 't'. The proportionality constant 'k' obtained by linear regression forcing through origin was found to be 0.228 g (dry wt) L^{-1} mOsmol kg^{-1}. As mentioned earlier for conductivity, in this case also ΔX/ΔO was plotted against culture time to check whether the involvement of initial values in the calculation leads to any errors during correlation of biomass with osmolarity (Fig. 3.8 a). An alternative method which does not involve differential modes (ΔX or ΔO) was used to correlate biomass with osmolarity in the form of y=mX+C. The plot according to this relationship is shown in Fig. 3.8 b. The m and C

values of *B. vulgaris* hairy root cultures have found to be -0.215 and 49.234, respectively. These values were used to calculate the biomass as predicted from osmolarity values.

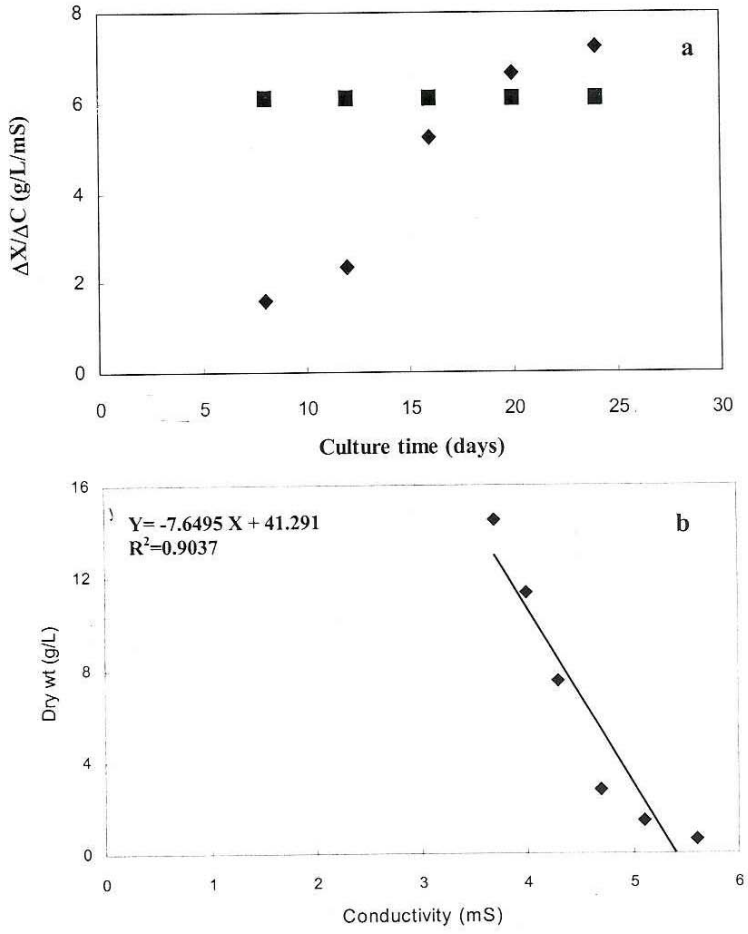


Fig 3.6: (a) Variation of the ratio between dry weight and conductivity (ΔX/ΔC) for *B. vulgaris* hairy root cultures as a function of culture time (■). ♦ Indicates the value obtained from a linear correlation of the dry wt. data forced through the origin of Fig. 3.5b (6.109g (dry wt) L^{-1} mS^{-1}).

(b) Relationship between conductivity and biomass concentration during batch culture of *B. vulgaris* hairy roots.

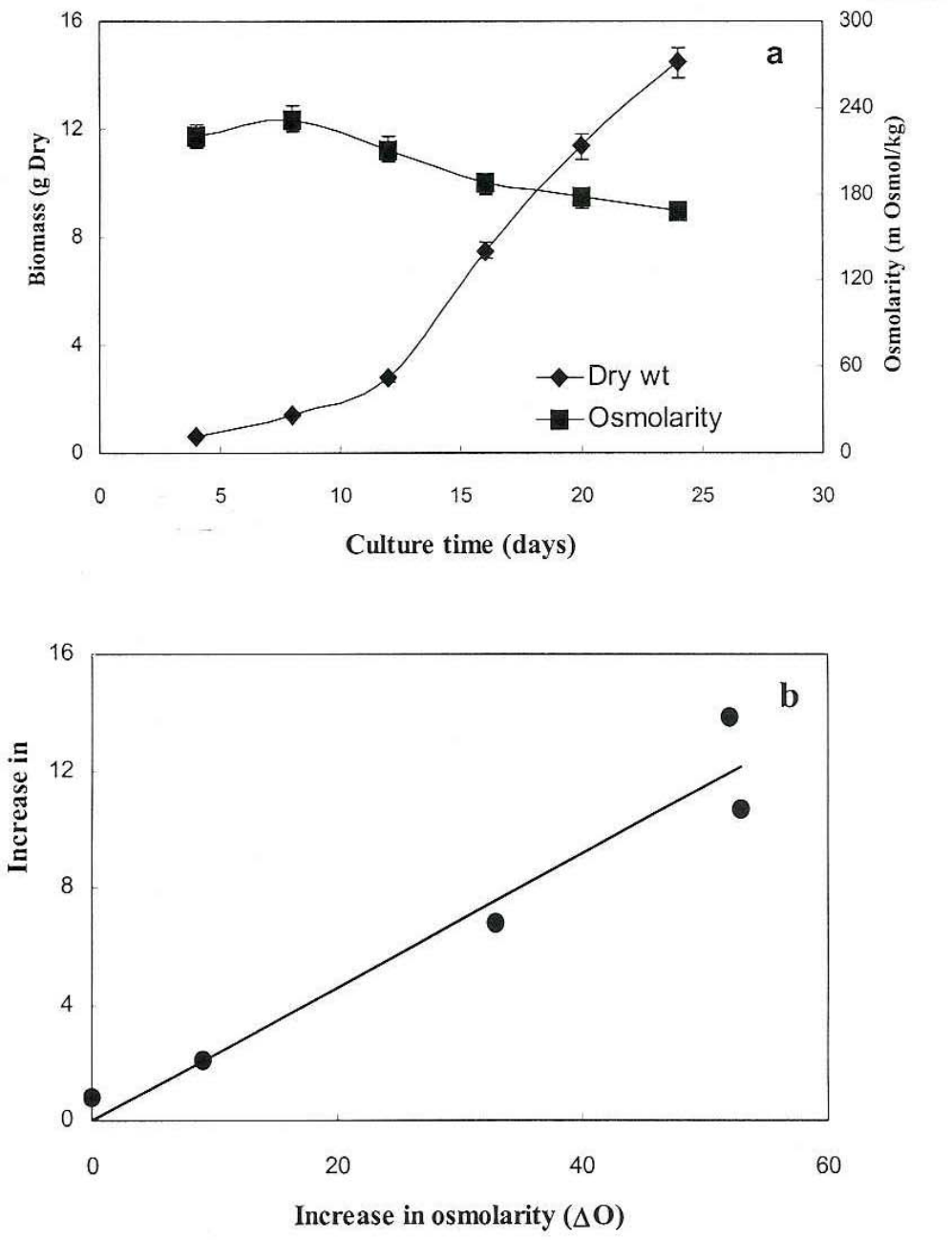


Fig 3.7: (a) Relationship between osmolarity and biomass concentration during batch culture of *B. vulgaris* hairy roots.
(b) Increase in biomass concentration (ΔX) as a function of decrease in osmolarity (ΔO) during batch culture of *B. vulgaris* hairy roots.

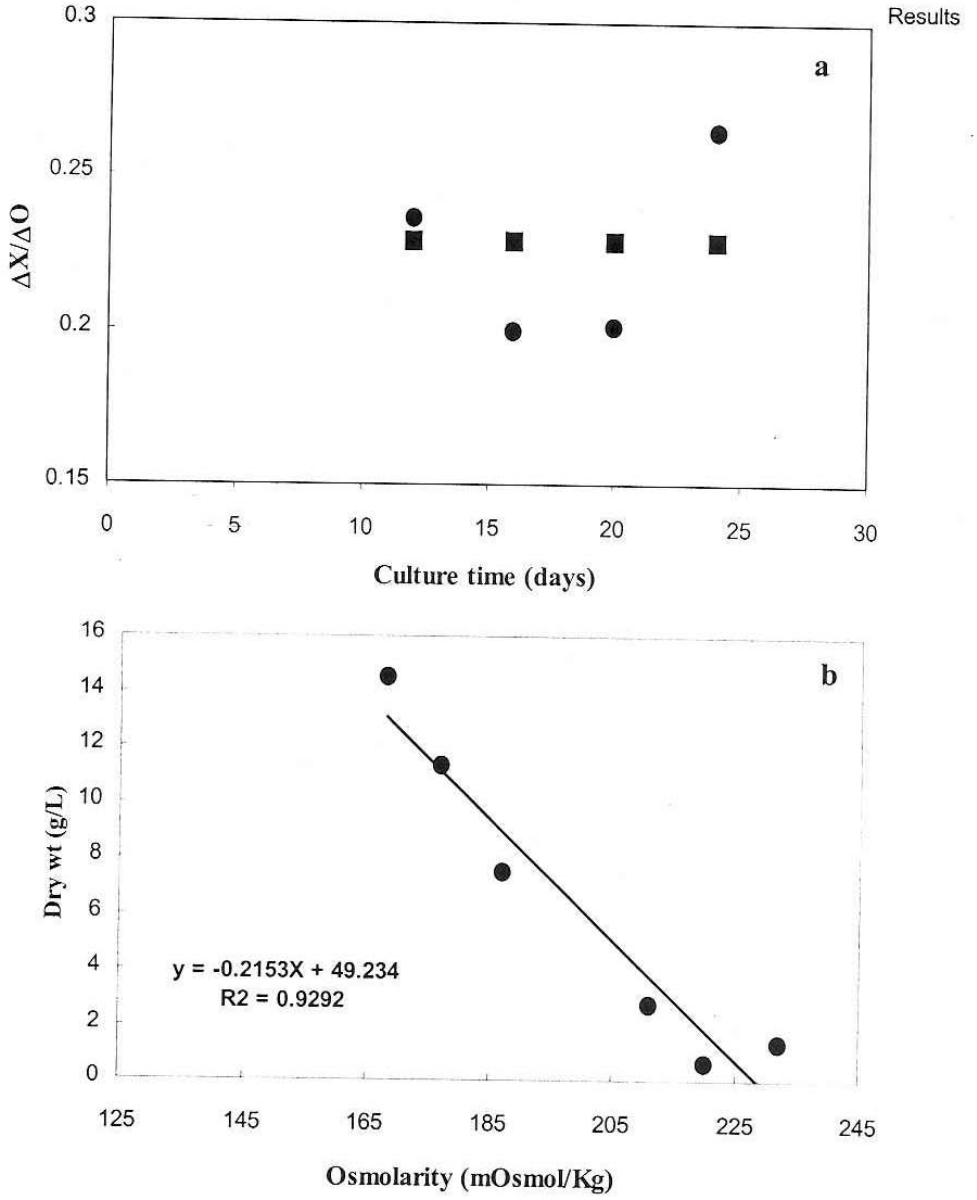


Fig 3.8: (a) Variation of the ratio between dry weight and osmolarity (ΔX/ΔO) for *B. vulgaris* hairy root cultures as a function of culture time. (■) Indicates the value obtained from a linear correlation of the dry wt. data forced through the origin of Fig. 3.7 (0.228 g (dry wt) L^{-1} mOsmol kg^{-1}).

(b) Relationship between osmolarity and biomass concentration during batch culture of *B. vulgaris* hairy roots.

Bioreactor systems for *Beta vulgaris* hairy roots

3.1.5.1 Performance of bubble column bioreactor

The 3 L- bubble column bioreactor (as described in section 2.1.5.2) had a working volume of 1.75 L. MS basal medium was used in the bioreactor as a nutrient medium for hairy roots. The reactor after autoclaving at 121 ^{0}C for 15 min at 1.06 kg cm^{-2} pressure, was inoculated with 8.57 g FW L^{-1} of beet hairy root inoculum (4-day old). Samples from the media were drawn every 4-days aseptically in a laminar air flow for conductivity and osmolarity measurements. The biomass in the bioreactor was harvested after 24 days of culture and the roots were analysed for growth and betalaine contents. *B. vulgaris* hairy roots grown in bubble column bioreactor are shown in Fig. 3.9. The final biomass obtained was recorded as 3.37 g DW L^{-1} after 24 days of culture (Fig 3.10a). The growth rate of hairy roots is recorded as 0.118 g dry wt L^{-1} day^{-1} (Fig. 3.10b). There was a gradual decrease in the conductivity with culture time from an initial value of 5.4 mS to 3.8 mS as recorded on the day 24 of culture (Fig 3.11 a). However, the osmolarity increased initially to 275 mOsmol Kg^{-1} (on the day 8 of culture) from an initial value of 220 mOsmol Kg^{-1}, which decreased further with culture to reach a final value of 200 mOsmol Kg^{-1}, as recorded on the day 24 of culture (Fig. 3.11 b). The harvested roots were analysed for betalaine content which was recorded as 31.36 mg g $^{-1}$DW (Fig 3.12)

3.1.5.2 Performance of nutrient sprinkle bioreactor

The nutrient sprinkle bioreactor (as described in section 2.1.5.3) had 2 vessels, one working as a functional reactor and other as a media reservoir. The working volume of the reactor was 2.5 L. The reactor vessels with MS basal medium were autoclaved and the functional reactor was inoculated with hairy root biomass of (4-day old) 6 g FW L^{-1}. The growth of hairy roots in the reactor was monitored for 24 days, wherein media samples drawn at 4-day intervals were analysed for conductivity and osmolarity measurements. The roots grown in this bioreactor were shown in Fig. 3.13. The final biomass obtained was recorded on the day 24 as 1.55 g DW L^{-1} (Fig 3.10a). The growth rate was recorded as 0.044 g dry wt L^{-1} day^{-1} (Fig. 3.10b). The conductivity values

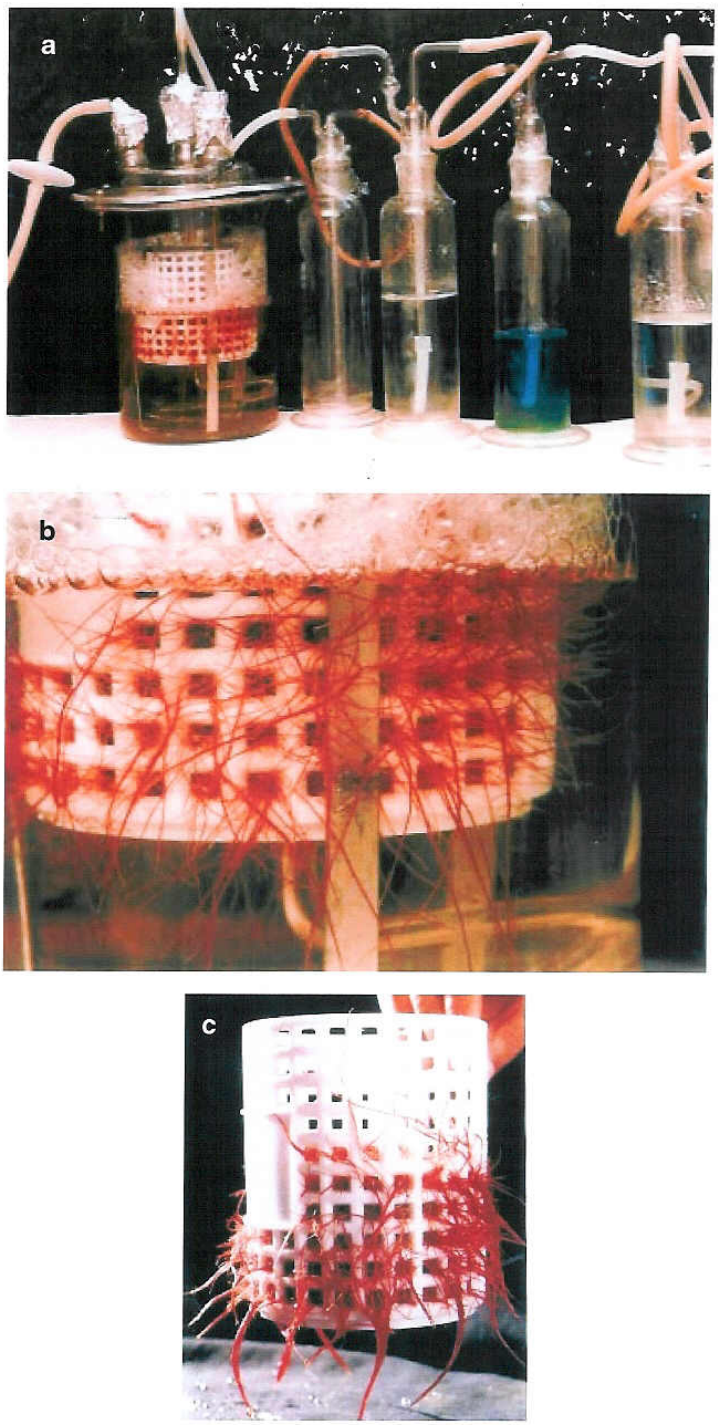


Fig 3.9: *B. vulgaris* hairy root cultures grown in a 3L bubble column bioreactor

(a) The bubble column reactor is made up of corning glass (3L) with a working volume of 1.75 L (MS basal medium, with 3% sucrose and pH 5.8).
(b) Plastic basket acting as a support for growing hairy roots
(c) Harvested hairy root cultures with accumulated betalaine pigments.

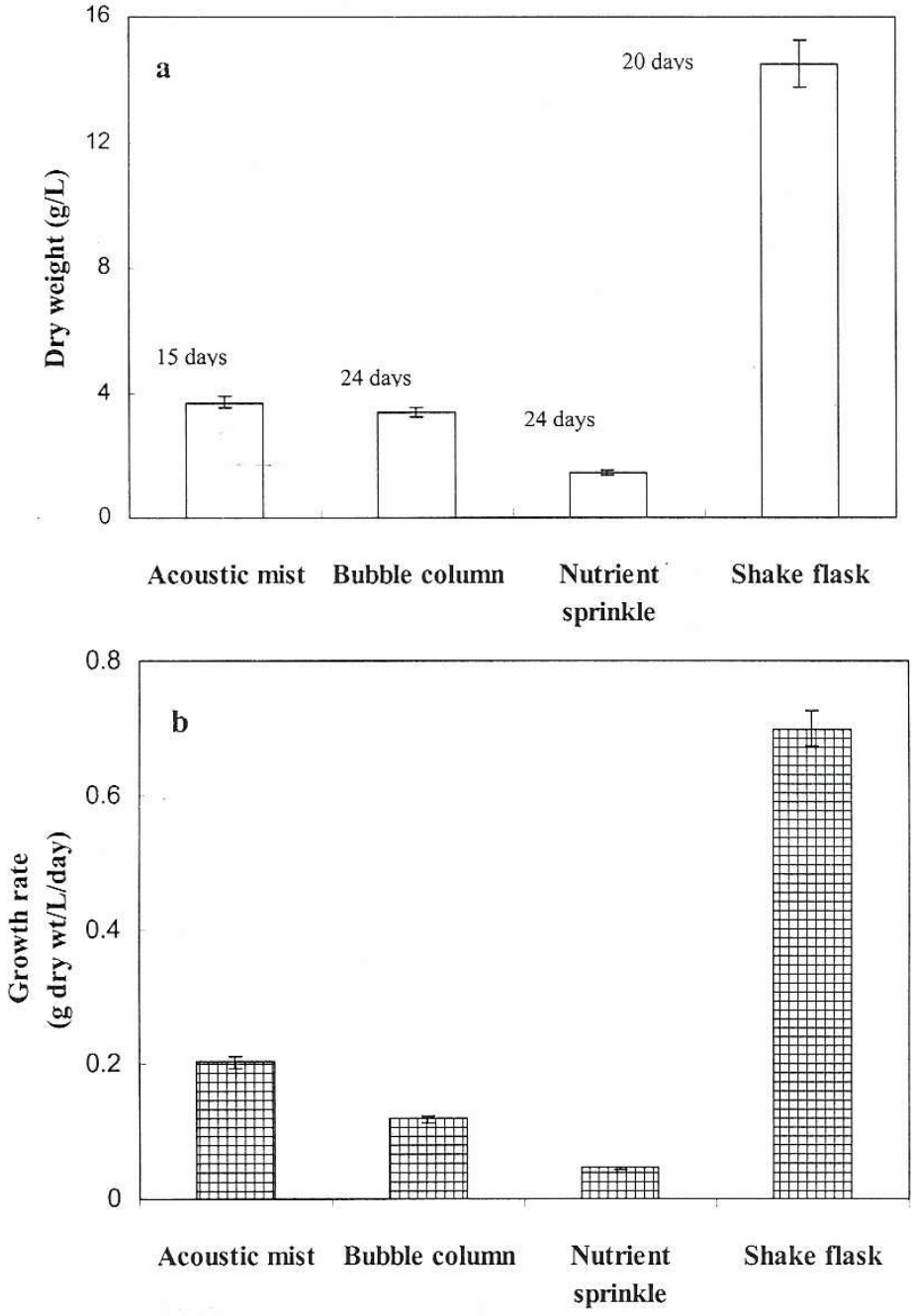


Fig 3.10: Variation of (a) final biomass and (b) growth rate of *B. vulgaris* hairy roots in different bioreactors in comparison with shake flask values.

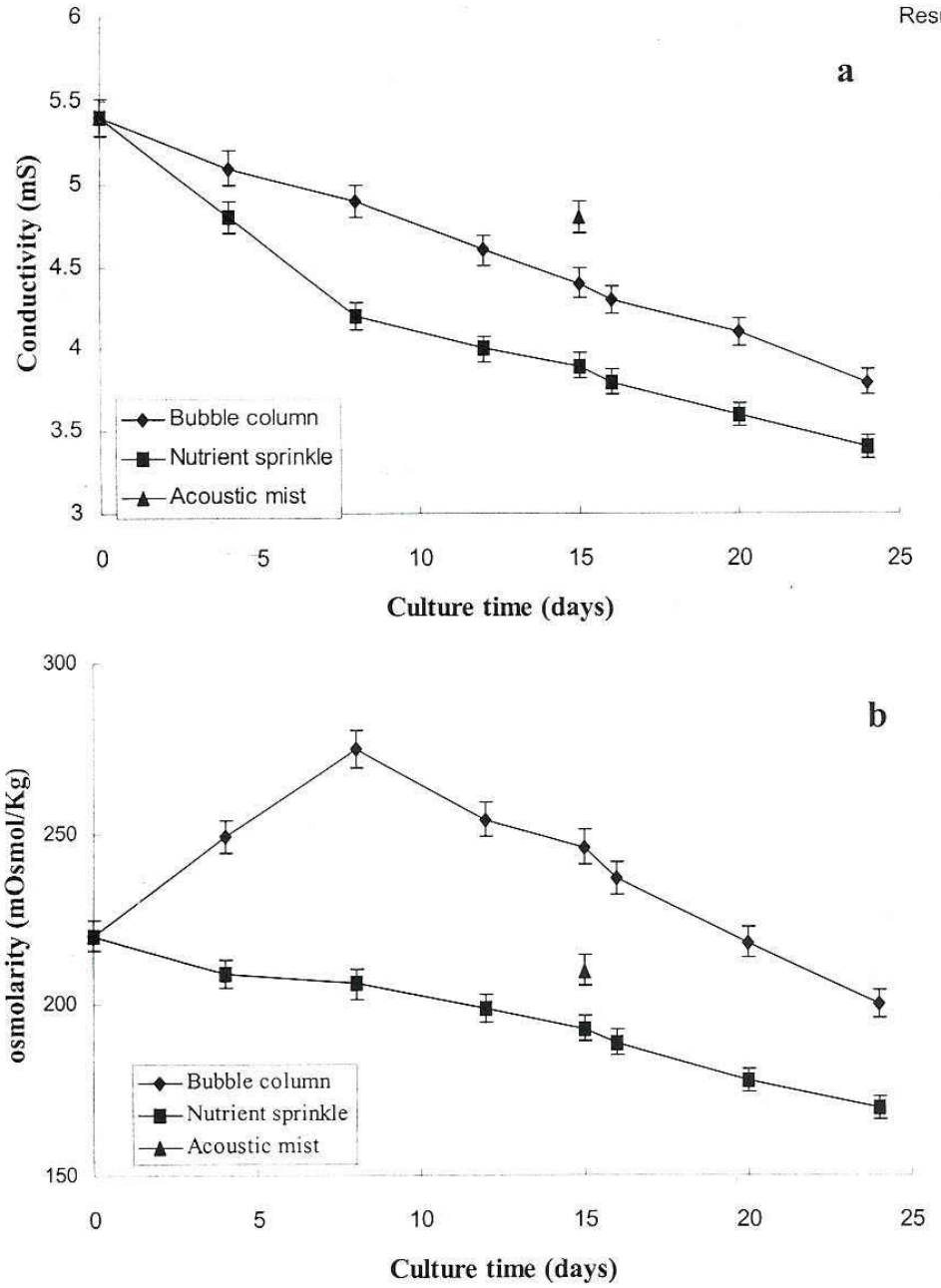


Fig 3. 11: (a) Variation of conductivity of culture medium in different bioreactors for *B. vulgaris* hairy roots

(b) Variation of osmolarity of culture medium in different bioreactors for *B. vulgaris* hairy roots

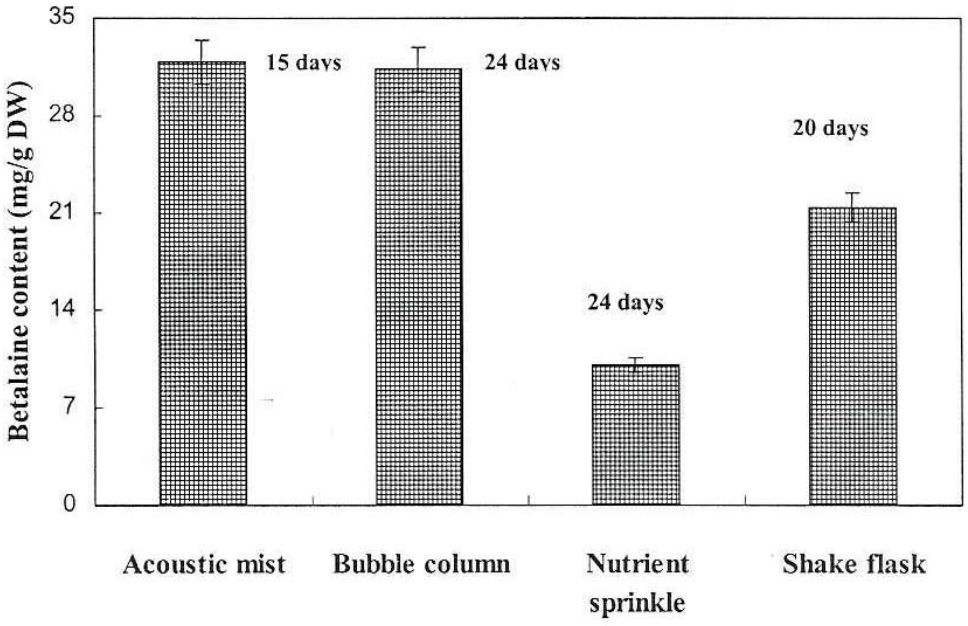


Fig 3.12: Final betalaine content obtained in different bioreactors at varied culture times inoculated with *B. vulgaris* hairy root cultures

decreased gradually with culture time from an initial value of 5.4 mS to a minimum of 3.4 mS recorded on the day 24 of culture (3.11 a). The osmolarity values were also decreased gradually with culture time from an initial value of 220 m Osmol Kg^{-1} to a minimum of 170 m Osmol Kg^{-1} as on the day 24 (Fig. 3.11 b). The betalaine content was recorded as 10.03 mg g^{-1} DW on the day 24 (Fig. 3.12).

3.1.5.3 Performance of acoustic mist bioreactor

The design of the acoustic mist bioreactor used in the present study was described in section 2.1.5.3.1, which is a prototype of bioreactor with a total volume of 300 ml, and a working volume of 100 ml. The bioreactor was autoclaved and later was inoculated with an inoculum of 8.5 g FW L^{-1}. The hairy roots grown in this bioreactor are shown in Fig 3.14. The reactor was run for 15 days and the biomass was harvested and analysed for betalaine content. The final biomass obtained was 3.67 g DW L^{-1} recorded on the day 15 of the culture (Fig 3.10a). The growth rate in this case was recorded as 0.202 g dry wt L^{-1} day^{-1} (Fig. 3.10b). The conductivity and osmolarity values decreased to reach a minimum of 4.8 mS and 210 m Osmol Kg^{-1}, from initial values of 5.4 mS and 220 m Osmol Kg^{-1}, respectively (Fig.3.11a, 3.11 b). The betalaine content was recorded as 31.9 mg g^{-1}DW on day the 15 (Fig. 3.12). In all the reactors studied, there was no sucrose limitation observed as the spent medium still had notable sucrose content (~ 0.5 %, i.e $1/6^{th}$ of initial sucrose content). Therefore there was no requirement of external addition of sucrose. The pH of the medium reduced from an initial value of 5.8 to 4.8 ± 0.2 during growth in bioreactors. However, this change in pH has not affected the root growth or betalain content. No leaching of pigment was observed in any of the bioreactor run.

Among all the reactors studied, Acoustic mist bioreactor showed a maximum specific growth rate, followed by bubble column and nutrient sprinkle (Table 3.1) Even though the biomass accumulation was more in shake flasks (14.5 g DW/L after 20 days), acoustic mist bioreactor showed maximum specific growth rate of 0.055 day^{-1} which was higher than shake flask cultures (0.048 day^{-1}). However, bubble column and nutrient sprinkle bioreactors exhibited lesser specific growth rates.

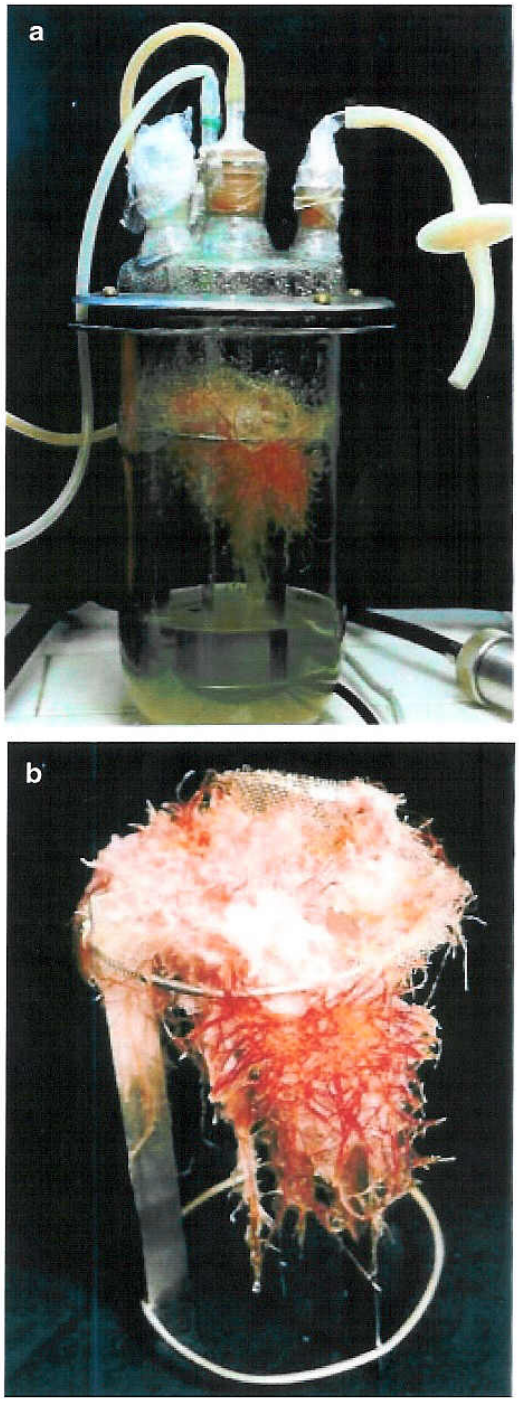


Fig 3.13: *B. vulgaris* hairy root cultures grown in a nutrient sprinkle bioreactor
The reactor vessel (a) with nylon mesh support (b) anchoring the hairy roots. The bioreactor has 2 vessels, one acting as a reservoir of medium and the other functioning as a bioreactor where the roots were grown. The medium was circulated using peristaltic pumps

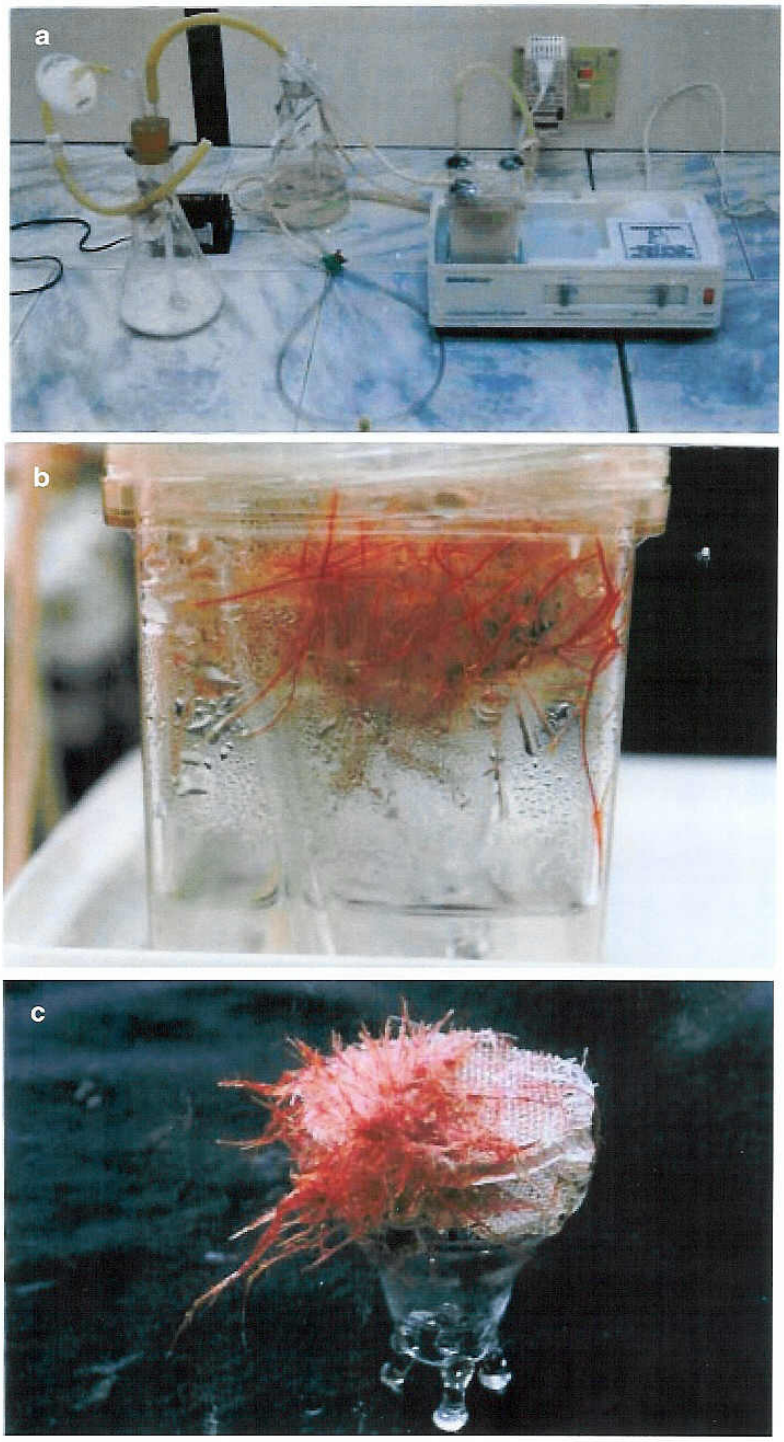


Fig 3.14: *B. vulgaris* hairy root cultures grown in an acoustic mist bioreactor

The acoustic mist bioreactor (a) is made up of a polypropylene vessel (b) wherein the support (c) for hairy root anchorage is embedded. The roots were placed on the autoclavable nylon mesh. The ultrasonic waves generated by transducer creates mist inside the reactor vessel, which coalesce and drip on the growing hairy roots providing nutrients

Table 3.2: Specific growth rates of* B. vulgaris *hairy roots in different reactors

Type of bioreactor	Sp. Growth rate (day^{-1})
1. Acoustic mist	0.055
2. Bubble column	0.035
3. Nutrient sprinkle	0.031
4. Shake flask	0.048

3.1.6 Validity of osmolarity and conductivity measurements in indirect estimation of biomass in bioreactors

In the present study, the biomass values of different bioreactors employed are predicted using an emperical equation obtained based on the shake flask data. The experimental values for biomass were as reported in the present study with different designs of bioreactors. The predicted values of biomass were obtained by substituting the values obtained from shake flask data in the equation y=mX+C. The m and c values of biomass predicted from conductivity studies were –7.64 and 41.29, respectively (Section 3.1.3.1). In case of biomass values predicted from osmolairity, the m and c values were –0.215 and 49.234, respectively (Section 3.1.3.2). The experimental and predicted values of biomass calculated by using conductivity and osmolarity measurements for all three bioreactors *viz*. acoustic mist, bubble column and nutrient sprinkle were given in Fig 3.15. From the figure 3.15, it is understood that prediction of biomass yield by osmolarity measurements was more effective than those predicted by conductivity measurements.

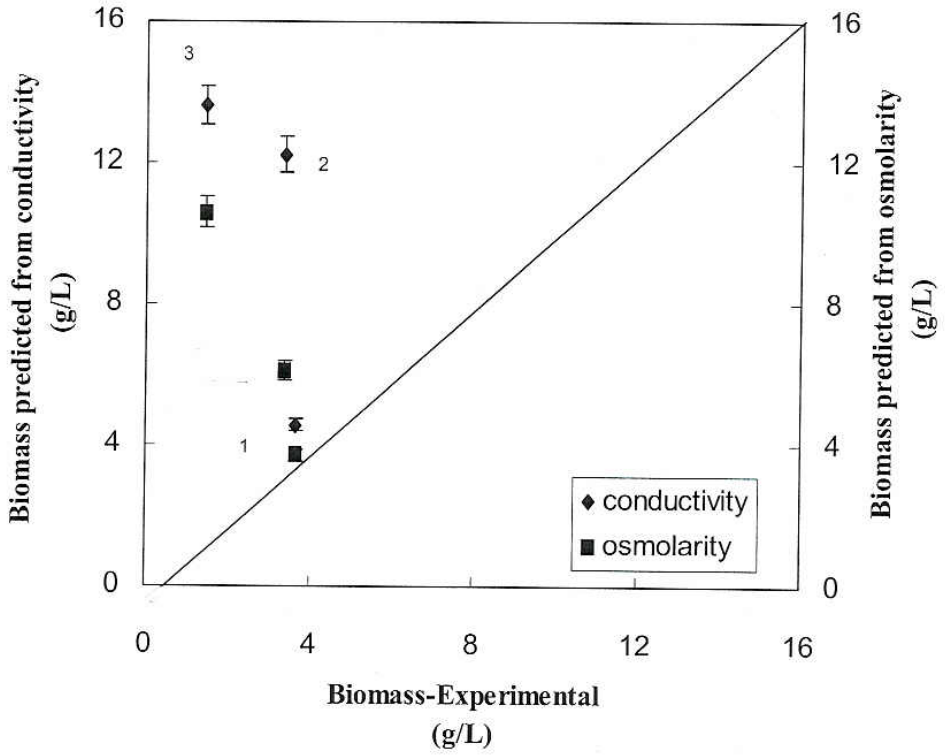


Fig 3.15: Variation of biomass values obtained from osmolarity and conductivity values in three different bioreactors as compared with experimental.
1. Acoustic mist 2. Bubble column 3. Nutrient sprinkle

3.1.7 Studies on improvement of growth and metabolite production in bioreactors

Based on the results mentioned above, it was proposed to study the possible methodologies for improved growth and betalaine contents in the bioreactor. Even though, acoustic mist bioreactor performed better with higher biomass and production, as it is a prototype reactor, further experiments were carried out in a 3-L bubble column bioreactor (working volume of 1.75 L).

3.1.7.1 Effect of polyamines on hairy root growth and metabolite production in a 3L-bubble column bioreactor

Earlier work in our lab (Bais et al 2000) using *Beta vulgaris* hairy roots in shake flasks suggested that combined feeding of spermidine and putrescine (0.75 mM each) resulted in a higher yield of biomass as well as betalaine production. Thus the same level was adapted in the present experiment. The addition of polyamines on 5th day resulted in a very narrow increase in pH to 5.87/5.92 which is still in the range of optimum pH for hairy root growth. The reactor run was terminated after 24 days and the hairy roots were harvested for growth and metabolite analysis. The final biomass was recorded as 39.2 g FW L^{-1} (i. e 2.7 g DW L-1) which was 1.23 fold higher than that of control (without any elicitor). The betalaine content was also recorded to be higher, 32.9 mg g^{-1}DW, which was 1.27 fold higher than in control (Fig 3.16).

3.1.7.2 Influence of methyl jasmonate (MJ) on growth and metabolite production in a 3L-bubble column bioreactor

Pilot studies were carried out using shake flasks to determine the optimum concentration that could induce higher metabolite production. The hairy roots grown in shake flasks were fed with MJ at concentrations of 10, 20, 40, 70 and 100 µM after 5 days of culture period and hairy root growth and betalaine content were monitored periodically. MJ at higher concentrations i.e. 70 and 100 µM showed a slight decrease in pH to 5.7, whereas and at lower concentrations there was no significant change. The results shown in Figure 3.17 a indicate that the biomass accumulation was

inhibited with an increase in the concentration of MJ. Maximum biomass (6.3 g

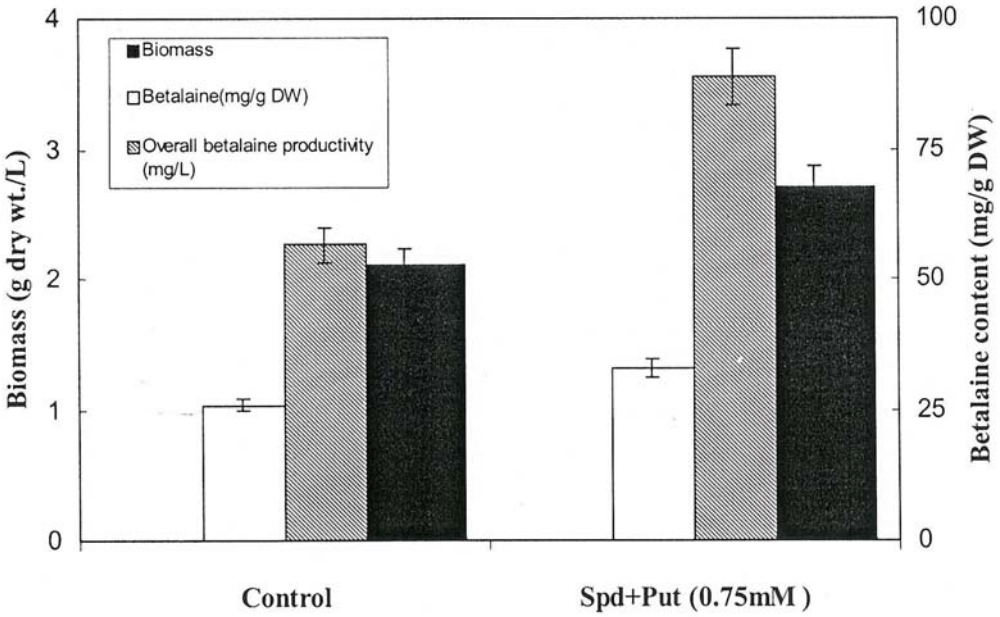


Fig 3.16: Influence of polyamines on growth and betalaine content of *B. vulgaris* hairy roots in a 3L-bubble column bioreactor (working volume of 1.75L)

FW/40 ml culture medium after 20 days) was recorded in control cultures without MJ which was 1.05, 1.1, 1.17, 1.54, and 1.61 fold higher than 10, 20, 40, 70 and 100 µM methyl jasmonate fed cultures respectively. At levels beyond 100 µM the growth was severely affected. Contrarily, the MJ treatments enhanced betalaine formation to extents significantly higher than control cultures (Figure 3.17 b). High levels of MJ (70-100 µM) treatment resulted in higher betalaine content on 15th day of culture (i.e 10th day after its addition) whereas lower levels of MJ showed maximum pigment accumulation on 20th day of culture i.e after 15 days of addition (Fig 3.17 b). Thus cultures fed with 100 µM MJ accumulated 18 mg g $^{-1}$ DW betalaines after 10 days of culture, which was 1.28 fold higher when compared with that of control. Among

various concentrations studied, 40 µM MJ resulted in a maximum betalaine content of 32.36 mg g^{-1} DW on 20^{th} day of culture which was 1.35 fold higher than control cultures on the same day. The overall productivity of betalaines (mg L^{-1}) was higher in 40 µM MJ fed cultures.

Based on the above observations, the bioreactor was fed with 40 µM MJ after 5 days culture period. On 18^{th} day the hairy roots grown in reactor were harvested and the final biomass obtained was 49.3 g FW (28.17 g FW L^{-1}). The betalaine content was recorded as 36.13 mg g^{-1} DW which was 1.4 fold higher than the control (Fig 3.18) . After the addition of MJ, within 2-3 days, roots started accumulating more pigment as observed visually compared to control. After 18 days of bioreactor run the roots started turning brown, hence cultivation was terminated.

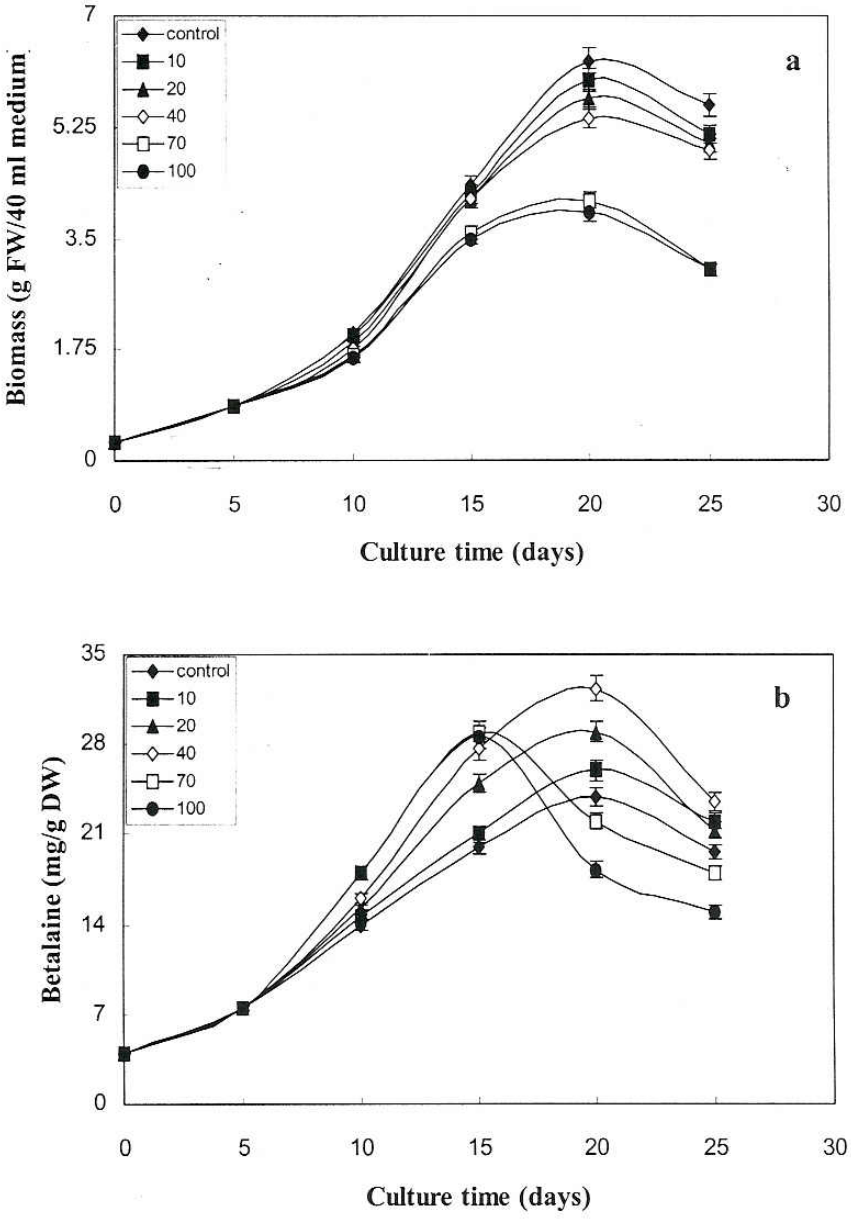


Fig 3.17: Influence of methyljasmonate (μM) on (a) growth and (b) betalaine content of hairy roots of *B. vulgaris* in shake flasks.

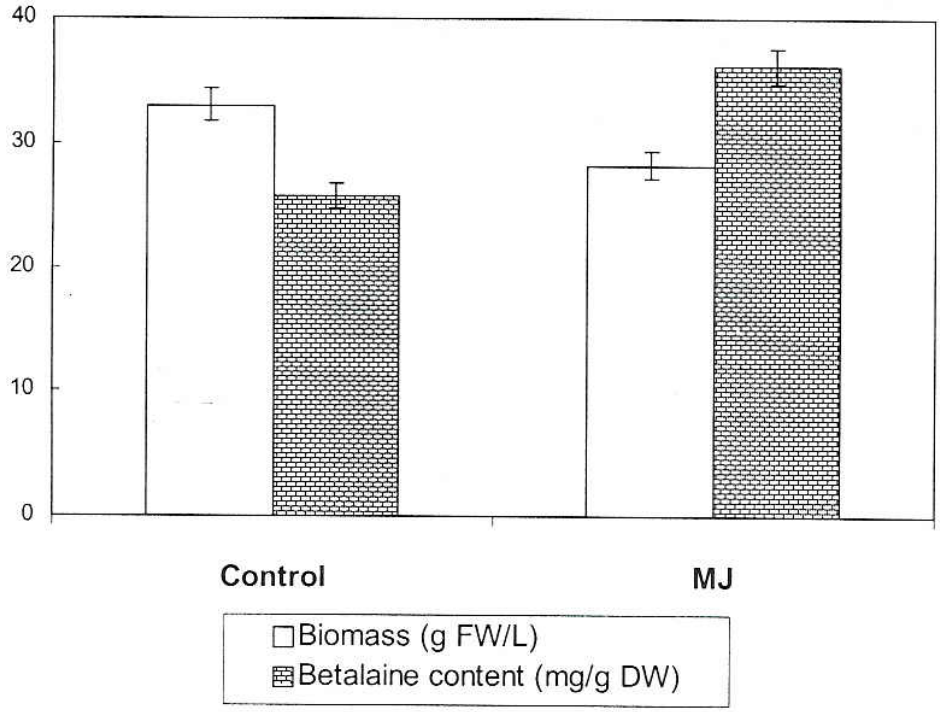


Fig 3.18: Influence of MJ on biomass and betalaine contents in a 3L-bubble column bioreactor (working volume 1.75 L) with *B. vulgaris* hairy roots. (data recorded on 18th day of culture).

3.1.8 Pigment release under the influence of Cetyl Trimethyl Ammonium Bromide (CTAB).

Cetyl Trimethyl Ammonium Bromide (CTAB), which is a known cationic surfactant and membrane permeabilizing agent was administered to the culture to obtain final concentrations of 0.001%, 0.002%, 0.005%, 0.01% and 0.05% (w/v). The

addition of CTAB at these concentrations did not show any change in the pH of the medium. After 17 days of hairy root growth in bioreactor, CTAB was added aseptically after filter sterilization in a laminar air flow chamber. Betalaine pigment release under the influence of CTAB in a 3L- bubble column bioreactor is shown in Fig. 3.19. The results presented in Fig 3.20a indicate that at high concentrations of CTAB (0.01 & 0.05%) the efflux was faster and betalaines started releasing into the medium a few hours after the addition of CTAB (Fig 3.20a). At low CTAB concentrations, it was observed that the release of pigments was slow and the viability of roots grown in bioreactor was not affected. In the case with 0.01-0.05% of CTAB, the roots started turning brown indicating necrosis, and therefore could not be used for further growth. At a concentration of 0.002 % the betalaine release was steady with an increase of incubation time. Maximum betalaine content efflux was observed at the 48^{th} hour, which was 17.3 mg L^{-1}. Further, the hue values of released pigments were also checked, the values varied with treatment time as shown in Fig 3.20 b. It was found that the lightness "l" values reduced from 91 to 70 recorded at 12 and 48 hours respectively, after CTAB (0.002%) addition. However, the redness and yellowness 'a' and 'b' values increased with incubation time in CTAB effluxed pigments. The final biomass obtained was 25.8 g FW L^{-1}. The harvested roots were analysed for betalaine content and found that they still have a betalaine content of 20 mg g^{-1} DW of roots.

There was no browning of tissue or necrosis at 0.002% of CTAB suggesting that roots can be used further in a continuous mode of operation for betalaine production.

3.1.8.1 Checking viability after CTAB treatments

In order to check the viability, the roots were taken out aseptically in a laminar air flow from the bioreactor and were subcultured into fresh MS basal medium. The comparative data of growth and betalaines production of control and permeabilized roots (subcultured after CTAB addition) is shown in Table 3.2. It is evident that the viability as well as metabolite production in roots after CTAB addition was unaltered indicating that they were physiologically active and viable.

Fig 3.19: Release of betalaine pigments upon CTAB treatment in 3L-bubble column bioreactor (1.75L working volume) with *B. vulgaris* hairy roots

Table 3.3: Growth and betalaines accumulation in hairy root cultures of *Beta vulgaris* subsequent to treatment with CTAB (0.002% w/v) as compared to untreated control cultures.

Culture time (days)	Control		Permeabilized with CTAB (0.002%)	
	Biomass (g FW/40 mL)	Betalaine (mg g^{-1}DW)	Biomass (g FW/40 mL)	Betalaine (mg g^{-1}DW)
0	0.12 ± 0.06	3.22 ± 0.72	0.13 ± 0.06	4 ± 0.9
7	0.62 ± 0.1	6.9 ± 0.86	0.615 ± 0.12	7.2 ± 0.9
14	3.6 ± 0.28	18.65 ± 1.13	3.8 ± 0.26	18.32 ± 1.2
21	5.9 ± 0.2	23.65 ± 1.9	5.86 ± 0.19	24 ± 1.67
28	5.3 ± 0.16	19.89 ± 1.85	5 ± 0.18	18.3 ± 1.53

3.1.9 Studies on accumulation of L-dihydroxyphenylalanine (DOPA) and dopamine in hairy root cultures of *B. vulgaris*

Growth and betalaine production in *B. vulgaris* hairy roots in the present study is shown in Fig 3.21 wherein maximum biomass and pigment production were recorded on 20th day of culture as 6.3 g FW/ 40 ml and 24 mg/ g DW, respectively. The variation of DOPA and dopamine contents with culture period of *Beta vulgaris* hairy roots is shown in Fig 3.22 a. Hairy root cultures in the initial stages of growth accumulated more DOPA than dopamine (3.22 b). Maximum DOPA accumulation (30 μg/g DW) was recorded on 5th day of culture, which decreased further with culture to reach a minimum of 7.4 μg/g DW on 20th day of culture. Maximum dopamine accumulation was recorded on 15th day of culture as 0.975 mg/g, which decreased further with culture time (3.22 b). The HPLC profile of the metabolites is shown in Fig 3.23.

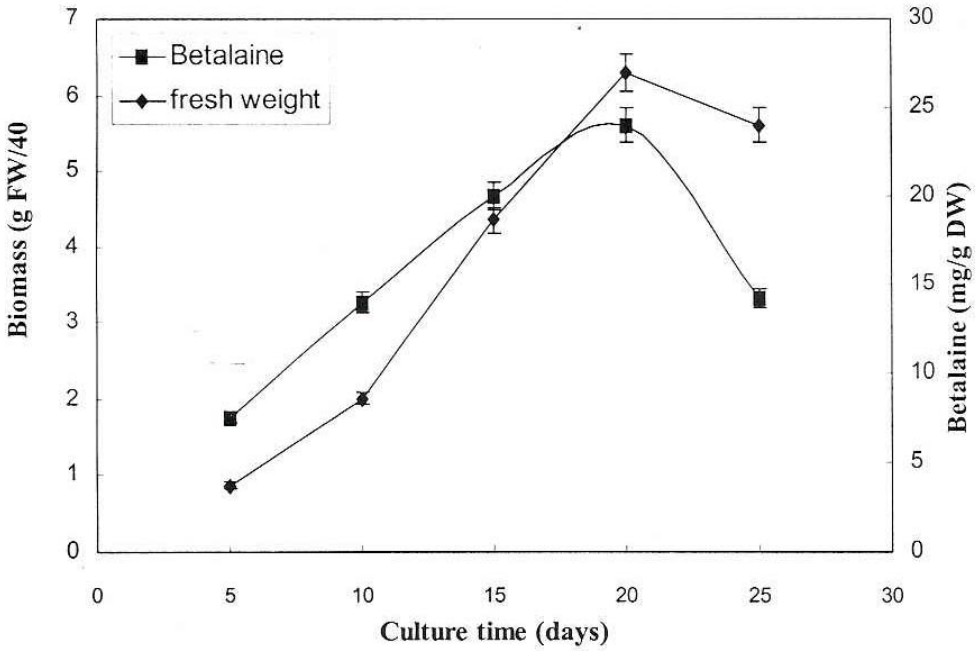


Fig 3.21. Growth and betalaine production profile of *B. vulgaris* hairy roots

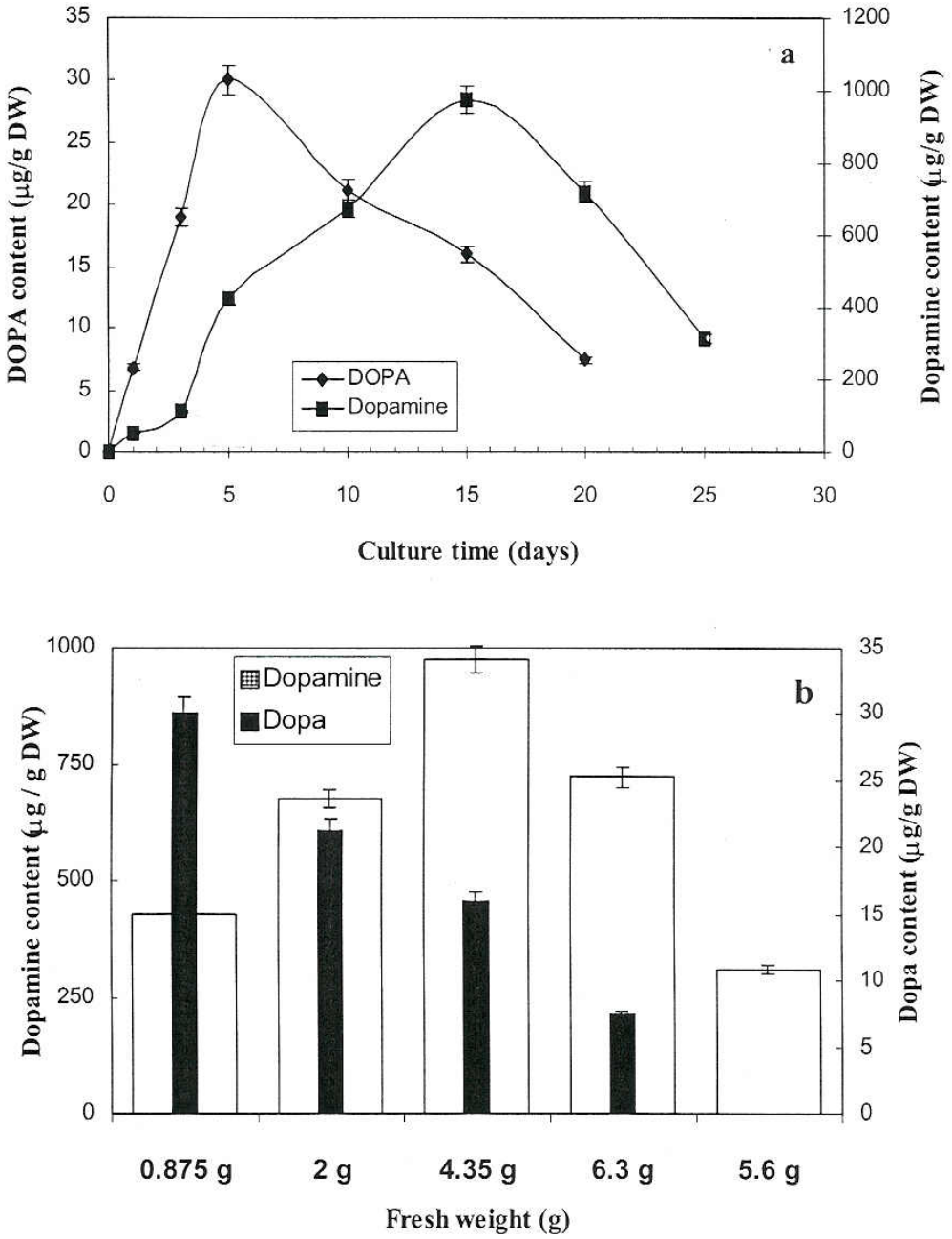


Fig 3.22: Variation of DOPA and dopamine contents with (a) culture time and (b) fresh weight in hairy roots of *B. vulgaris*

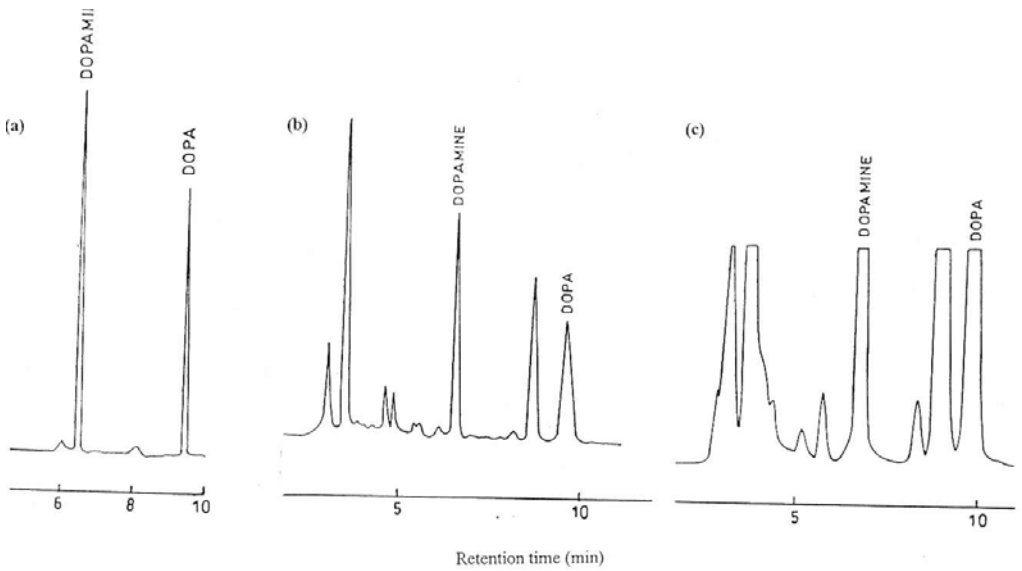


Fig 3.23: HPLC profiles of DOPA and dopamine from hairy root cultures of *B. vulgaris*
(a) Authentic standards (b) UV detector-280 nm (c) Fluorescence detector (Xenon arc lamp; excitation at 280 nm and emission at 314 nm)
Conditions: Column: C 18 column (25 cm long, 4 mm i.d.); Mobile phase: H_3PO_4 (1.5 % v/v) in water.

3.1.9.1 Influence of Tyrosine feeding on DOPA & dopamine accumulation in hairy root cultures of B. vulgaris.

Tyrosine which is a major precursor of DOPA was fed at a concentration of 1 mM to the *B. vulgaris* hairy root cultures on 5th day of culture where maximum DOPA was recorded. Addition of tyrosine inhibited the growth (Fig 3.24a) with 1.82 fold lesser biomass (3.13 g FW after 17th day of culture) when compared to the control. The pH of the medium increased from 5.8 to 6.2 ± 0.2 in tyrosine fed cultures. Further increase in the concentration of tyrosine led to browning and necrosis of the roots. Similarly betalaines content was also affected by the tyrosine feeding (Fig 3.24 b). The roots started turning brown in the later stages of culture growth. However, at a concentration of 1 mM the roots were not dead even though, they showed a decrease in the biomass accumulation. Betalaine content in tyrosine fed cultures was 1.49 times lesser (16 mg g^{-1} DW) when compared to control on 17th day

of culture. After 17 days of culture, the betalaines content decreased further. The hairy roots changed gradually to black indicating the probable accumulation of phenolics. The accumulation of DOPA and dopamine in relation to tyrosine feeding is shown in Fig 3.25 a and 3.25 b, respectively. Maximum DOPA accumulation was recorded on the 8^{th} day of culture as 48.8 µg/g DW which was 1.75 fold higher in comparison with control cultures on the same day. With further increase in culture time, DOPA content in both control as well as tyrosine fed cultures decreased and reached a minimum of 9 and 17.36 µg/g DW on 17^{th} day of culture. More than DOPA, tyrosine feeding enhanced the levels of dopamine wherein a maximum of 1.62 mg/g DW on 11^{th} day of culture was obtained which was 2.31 fold higher compared to control. From the above data it is evident that addition of tyrosine facilitated more DOPA and dopamine accumulation with concomitant reduction in betalaine levels.

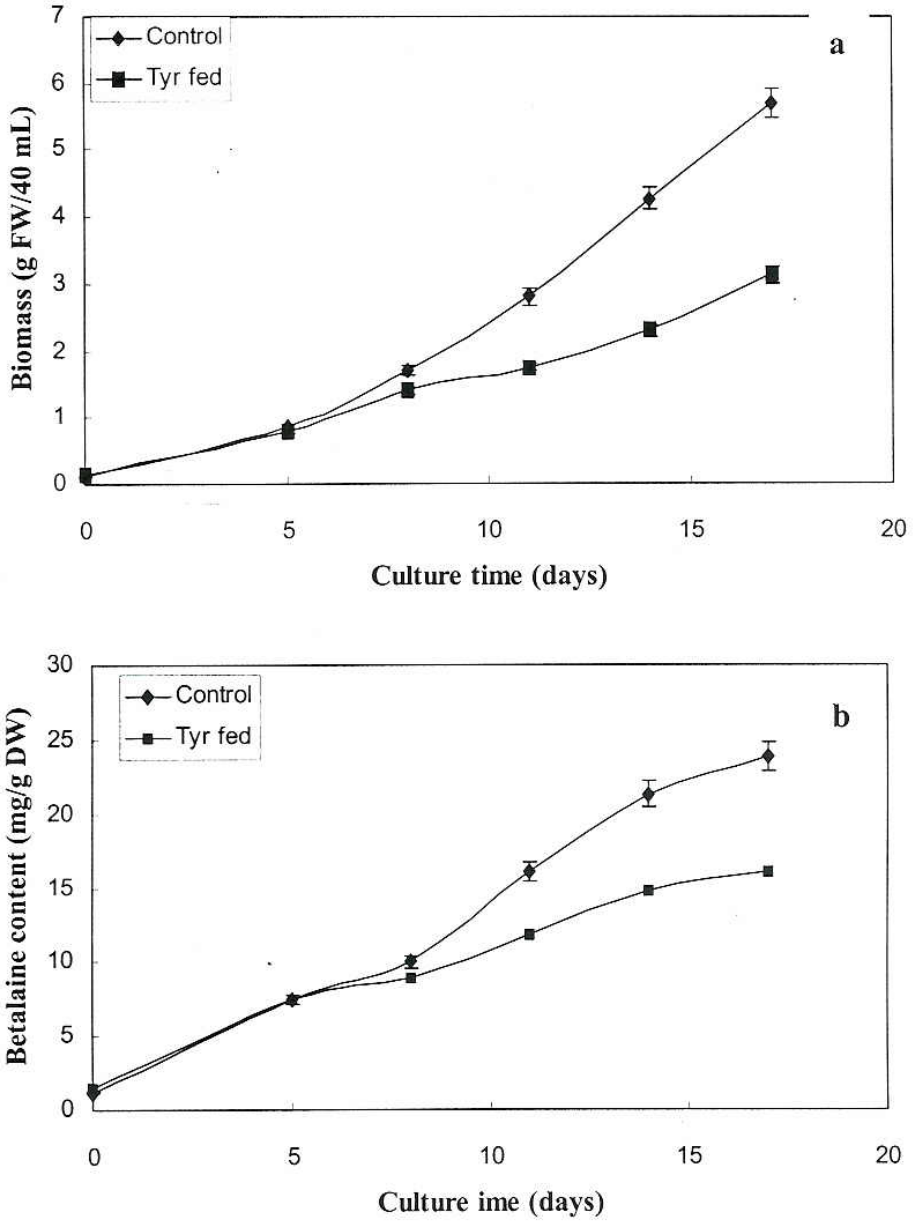


Fig 3.24: Influence of tyrosine feeding on (a) biomass and (b) betalaine contents in hairy roots of *B. vulgaris*.

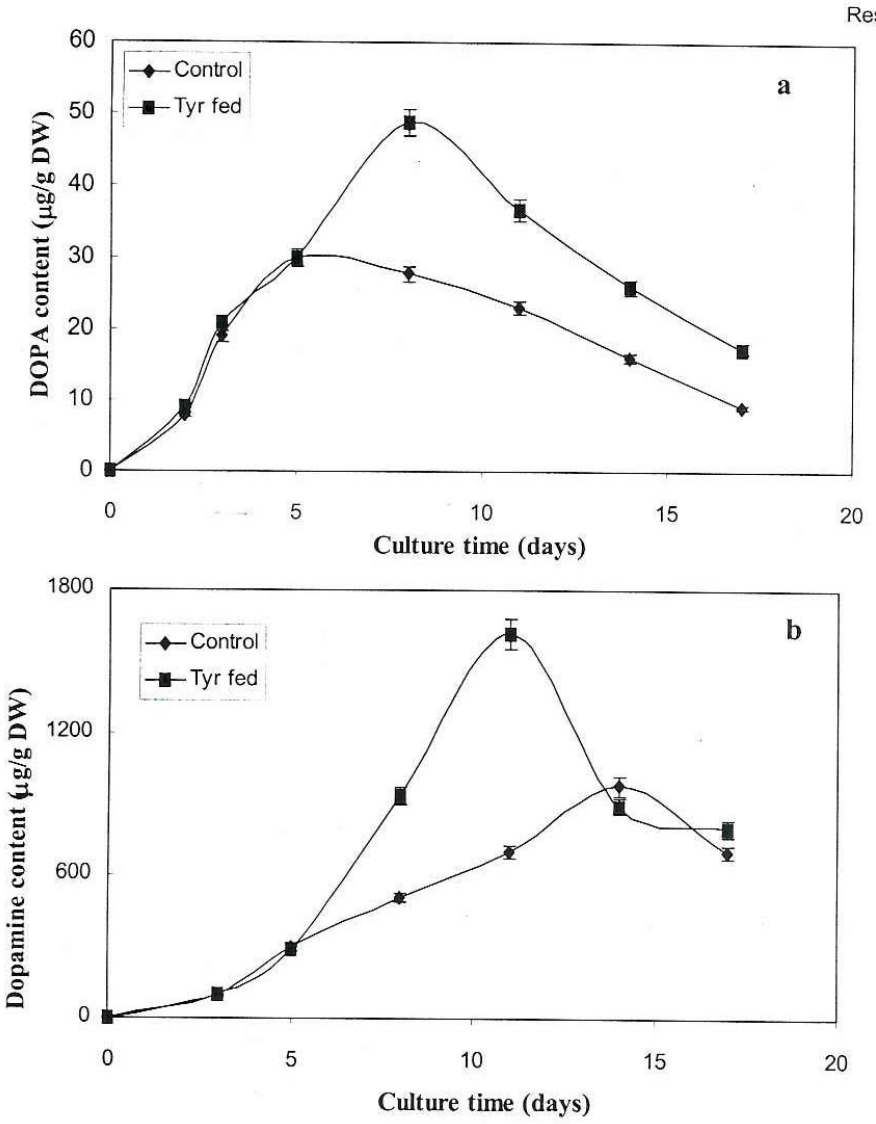


Fig. 3.25. Influence of tyrosine feeding on (a) DOPA and (b) dopamine contents in hairy roots of *B. vulgaris*

3.1.9.2 Influence of caffeic acid and catechin on DOPA and dopamine accumulation in hairy root cultures of B. vulgaris

Caffeic acid and catechin which are known as oxygenase inhibitors were added at a concentration of 50 µM on the 12th day of culture. The pH of the medium decreased to 5.2 ± 0.3 in treated cultures. Both these compounds have led to a decrease in the biomass as well as betalaines content (Fig 3.26 a, 3.26 b). There was a

1.67 and 1.7 fold lesser biomass obtained in cultures fed with caffeic acid and catechin respectively compared with control on 20th day of culture. Similarly betalaine accumulation was significantly affected which was 3.75 and 4 fold less in caffeic acid and catechin fed cultures, respectively when compared to control cultures. However, caffeic acid and catechin influenced enhanced accumulation DOPA and dopamine with the latter one being major (Fig. 3.27 a, 3.27 b). Maximum DOPA content was recorded on the 18th day of culture as 50 and 41.7 µg/g DW in cultures fed with caffeic acid and catechin respectively, which were 3.49 and 2.91 fold higher than in untreated control cultures. Dopamine content reached a maximum on the 16th day of culture as 1.9 and 1.67 mg/g DW in caffeic acid and catechin fed cultures, respectively, which was 2 and 1.76 fold higher when compared to control cultures.

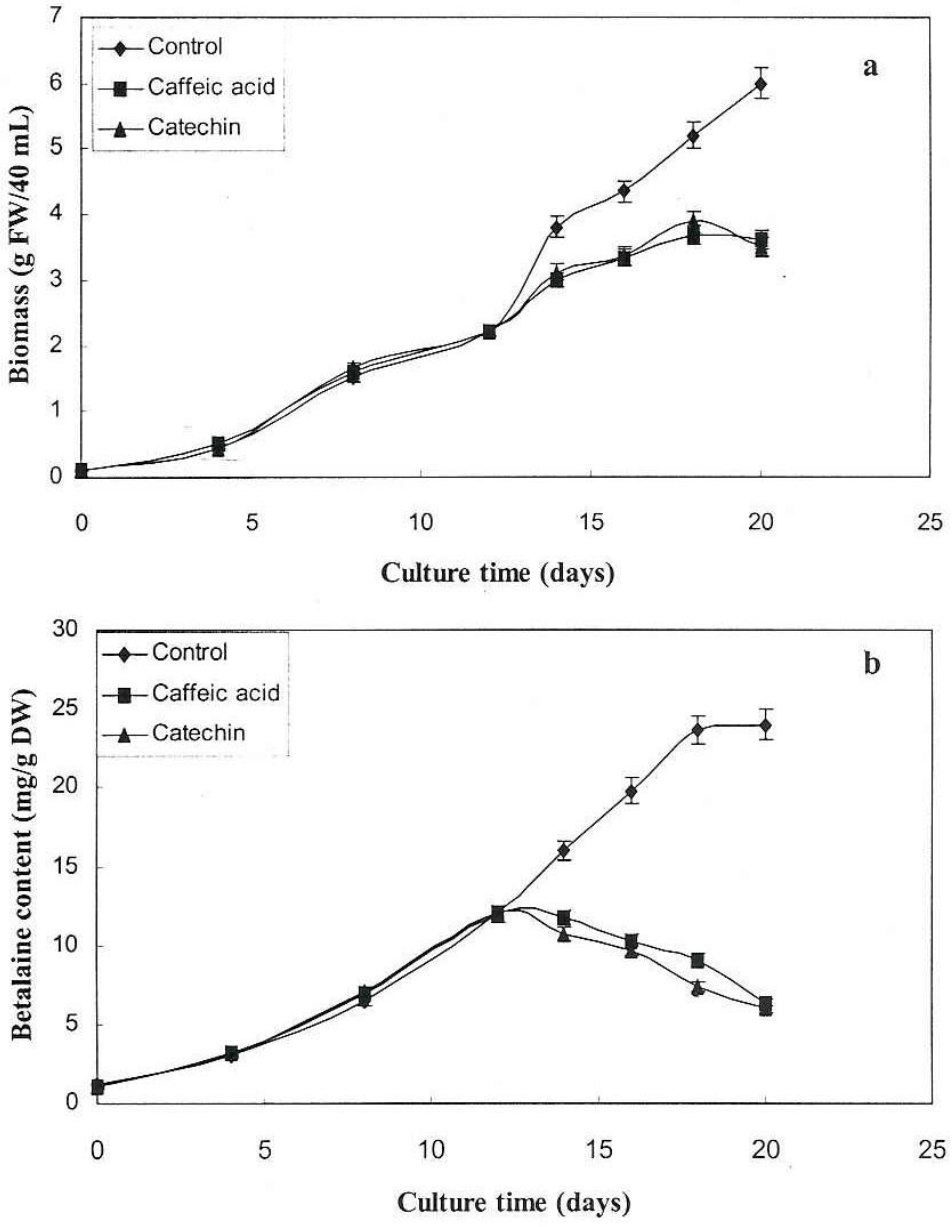


Fig 3.26: Variation of (a) biomass and (b) betalaine contents in hairy root cultures of *B. vulgaris* treated with oxygenase inhibitors

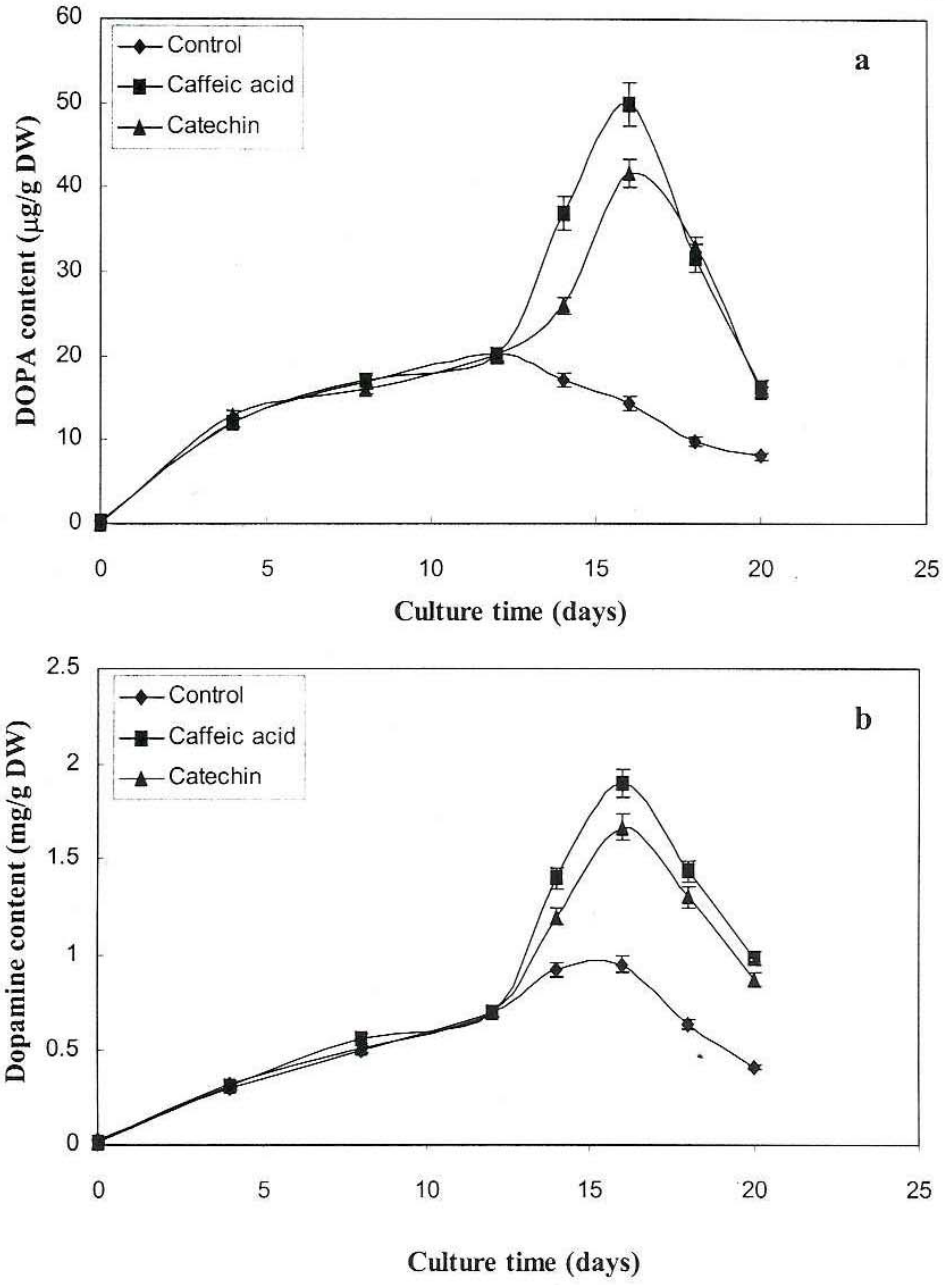


Fig 3.27: Influence of oxygenase inhibitors on (a) DOPA and (b) dopamine contents in hairy root cultures of *B. vulgaris*

3.2. RESULTS OF THE STUDIES ON *CAPSICUM FRUTESCENS* NORMAL ROOT CULTURES FOR BIOTRANSFORMATION

3.2.1 Growth characteristics of root cultures

3.2.2 Phenylpropanoid metabolite accumulation in normal roots

3.2.3 Biotransformation studies using normal roots of *C. frutescens*

3.2.3.1 Biotransformation of ferulic acid to vanillin related metabolites

Biotransformation of 1mM ferulic acid

Biotransformation of 2mM ferulic acid

Biotransformation of ferulic acid complexed with β-cyclodextrin (2mM each)

3.2.3.2 Bioreactor studies on biotransformation of ferulic acid

3.2.3.3 Biotransformation of protocatechuic aldehyde to vanillin related compounds

3.2.3.4 Biotransformation of caffeic acid to vanillin related metabolites

Biotransformation of caffeic acid

Biotransformation of caffeic acid along with methyl jasmonate

Effect of MJ on 3-O-Caffeic acid O-methyl transferase (3-O-CAOMT) activity

3.2.3.5 Biotransformation of veratraldehyde to vanillin related metabolites

3.2.1 Growth characteristics of root cultures

The normal root cultures of *Capsicum frutescens* were thick and succulent (Figure 3.28a). The pale white colour of the roots in the initial growth period was gradually transformed to light brown colour at the end of the culture time. After 20 days of growth, the maximum biomass was recorded as 6.5 g FW 40 mL^{-1} of culture medium. The roots started forming callus like structures after 20 days of culture, which increased with culture time (Fig 3.28b). The growth pattern is shown in Fig 3.29, wherein a gradual increase in biomass was attained. This was formed from an initial inoculum of 0.3 g. However, there was a 21.6-fold increase in the biomass over the initial value.

3.2.2 Phenylpropanoid metabolite accumulation in normal roots

HPLC analysis of normal root extracts revealed the presence of phenylpropanoid intermediates such as cinnamic acid, caffeic acid, coumaric acid and ferulic acid along with vanillylamine (Fig 3.30). Among all the intermediates, caffeic acid accounted to a maximum of 197 μM g^{-1} FW. However, capsaicin was not detected in root cultures.

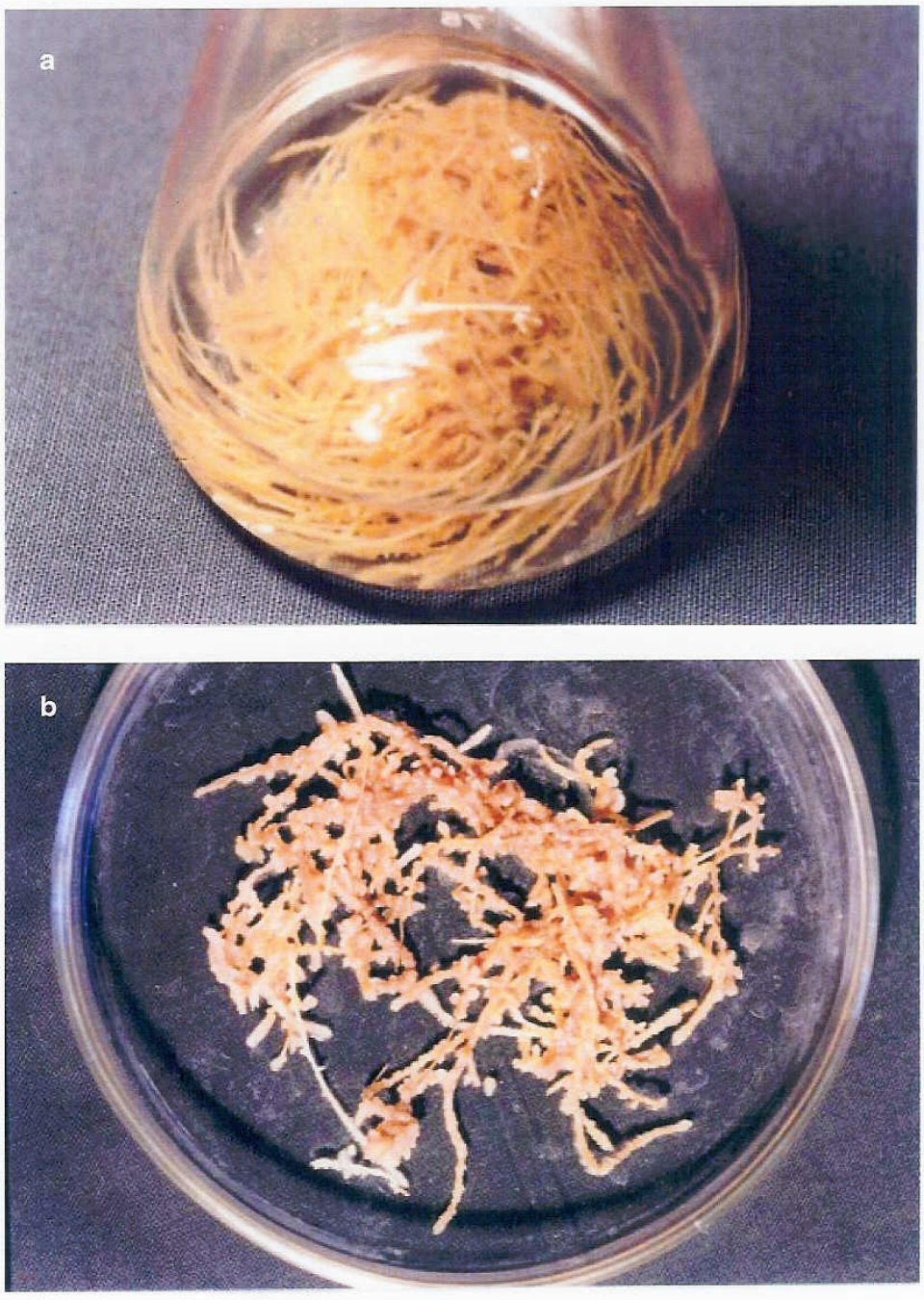


Fig 3.28: (a) Normal roots of *C. frutescens* grown in MS medium with 0.5 mg L^{-1} NAA (b) Callusing observed after 20 days of culture

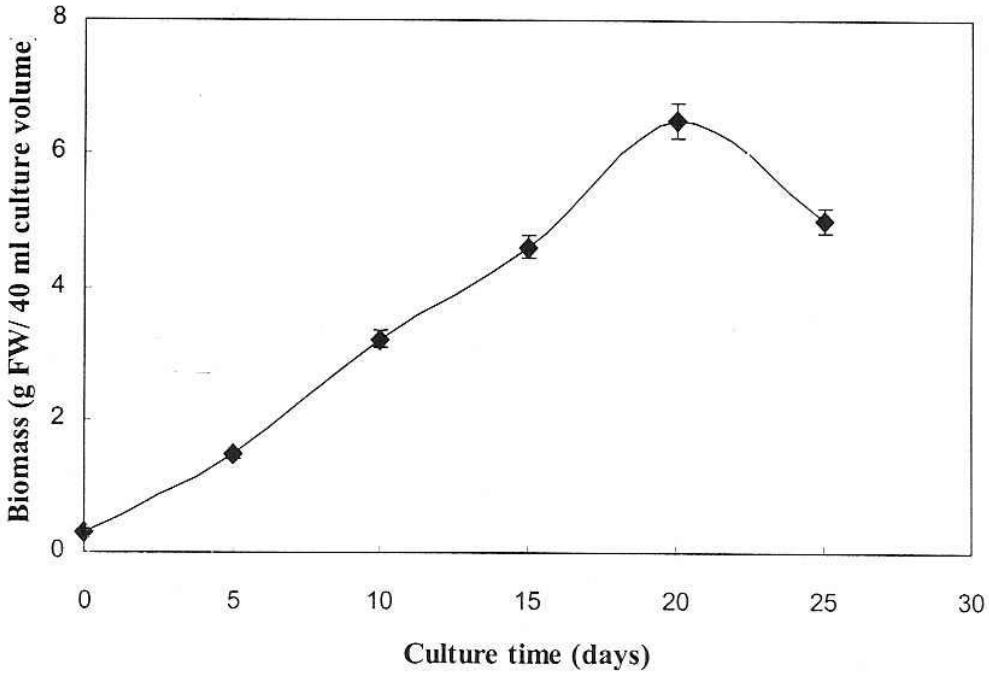


Fig 3.29: Growth pattern of *C. frutescens* normal roots in MS medium with 0.5 mg L^{-1} NAA

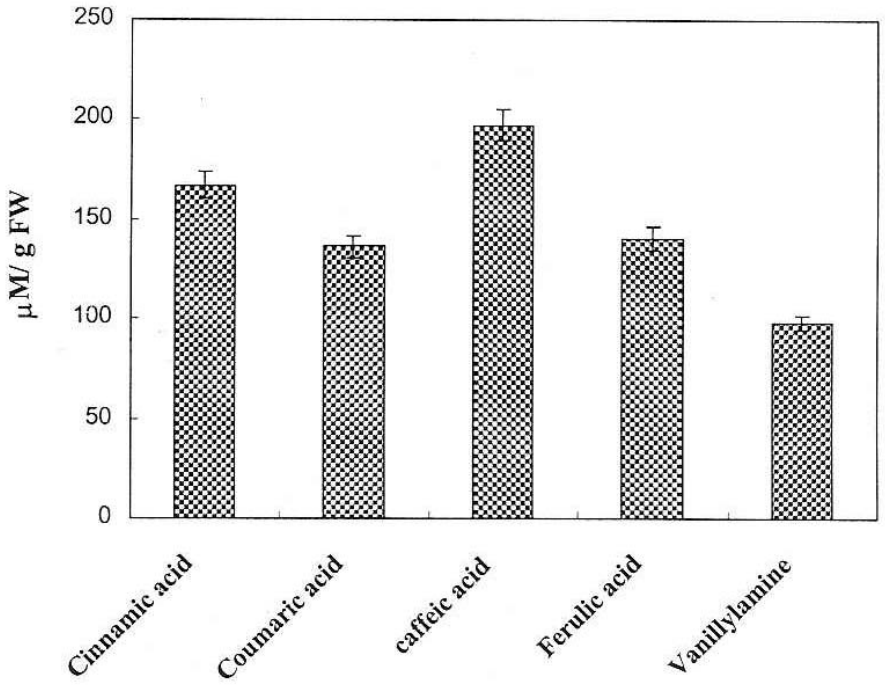


Fig 3.30: Accumulation of phenylpropanoid intermediates in normal roots of *C. frutescens* (data recorded on 20th day of culture)

3.2.3 Biotransformation studies using normal roots of *C. frutescens*

3.2.3.1 Biotransformation of ferulic acid to vanillin related metabolites

Biotransformation of 1 mM ferulic acid

Ferulic acid at 1mM did not exhibit any significant inhibitory effect on the growth of normal roots (Fig. 3.31). Maximum biomass of 6.2 g FW 40 mL^{-1} was recorded on the day 8 after precursor addition, where as the biomass in control cultures was 6.5 g FW 40 mL^{-1}. Root cultures not fed with ferulic acid as well as autoclaved root cultures fed with ferulic acid have not shown formation of vanillin

related metabolites. This suggests that the root cultures convert the precursors to various products by enzymatic action, while other factors such as temperature, light, or pH are of no significance. Feeding of ferulic acid at a concentration of 1 mM led to accumulation of various vanilla flavour metabolites which were detected and quantified by HPLC analysis after comparison of peaks with authentic standards (Figure 3.32). The concentration of these vanilla flavour compounds was measured during root cultivation as shown in Figure 3.33 a. The maximum vanillin production of 1.88 mg L^{-1} was recorded on the day 6 after precursor addition. Similarly, vanillylamine, vanillic acid and protocatechuic acid levels were maximum on day 6, amounting to 1, 1.4, and 0.85 mg L^{-1}, respectively. Other metabolites, such as vanillyl alcohol and parahydroxy benzoic acid, reached their maximum accumulation on day 8 after precursor addition (*viz.* 1.05 and 1.75 mg L^{-1}, respectively). Interestingly, a greater accumulation of vanillin was recorded in the culture medium than in the roots (Figure 3.33 b). Vanillin content in the medium was recorded as 1.37 mg L^{-1} on the day 6 after precursor addition, which is 2.8-fold higher than that accumulated in roots.

Biotransformation of 2 mM ferulic acid

Ferulic acid fed at a level of 2 mM led to higher production of biotransformed products than when fed at a level of 1mM (Figure 3.34a). However, at 2 mM concentration of ferulic acid, the root biomass was affected leading to lower biomass production. Maximum biomass of 5.2 g FW 40 mL^{-1} was recorded after 6 days of ferulic acid addition, which was 1.15 times lesser when compared with control cultures on the same day (5.96 g FW 40 mL^{-1}). Addition of ferulic acid (2 mM) however, did not stop the biomass but progressively inhibited the growth. This was evident from the figure 3.31 that the roots have exhibited growth even after the addition of ferulic acid but with further increase in culture time (after 6 days of ferulic acid addition) the root growth was inhibited. Maximum accumulation of vanillin (2.5 mg), vanillylamine (1.67 mg), vanillic acid (1.75 mg), and protocatechuic acid (1 mg) were recorded on day 6 after ferulic acid addition; these values were 1.33, 1.67, 1.21 and 1.17 times higher respectively, than in the cultures fed with 1 mM of ferulic acid. Vanillyl alcohol, p-hydroxy benzoic acid reached their maximum concentration (i.e.

2.12 and 2.15 mg L^{-1}, respectively) on day 8 after ferulic acid addition. Along with these, p-coumaric acid was also detected; its maximum production (0.3 mg L^{-1}) was recorded on day 8 after ferulic acid addition. In this case also, the amount of biotransformed products were more in media than in roots (Fig. 3.34b).

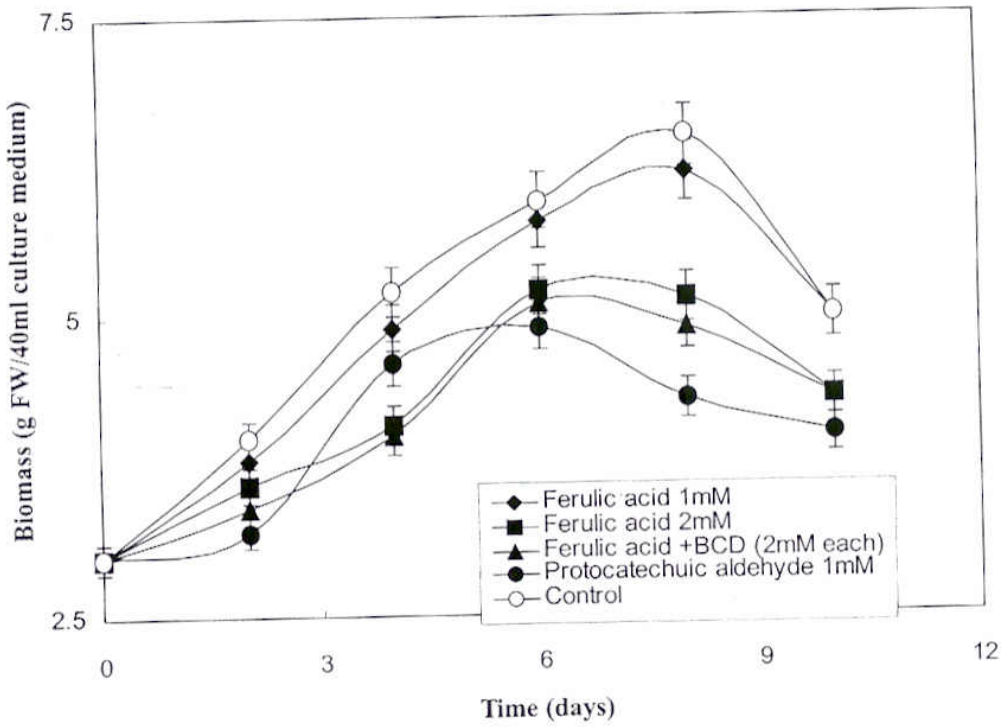


Fig. 3.31: Influence of ferulic acid and protocatechuic aldehyde on biomass accumulation of normal roots of *C. frutescens*.

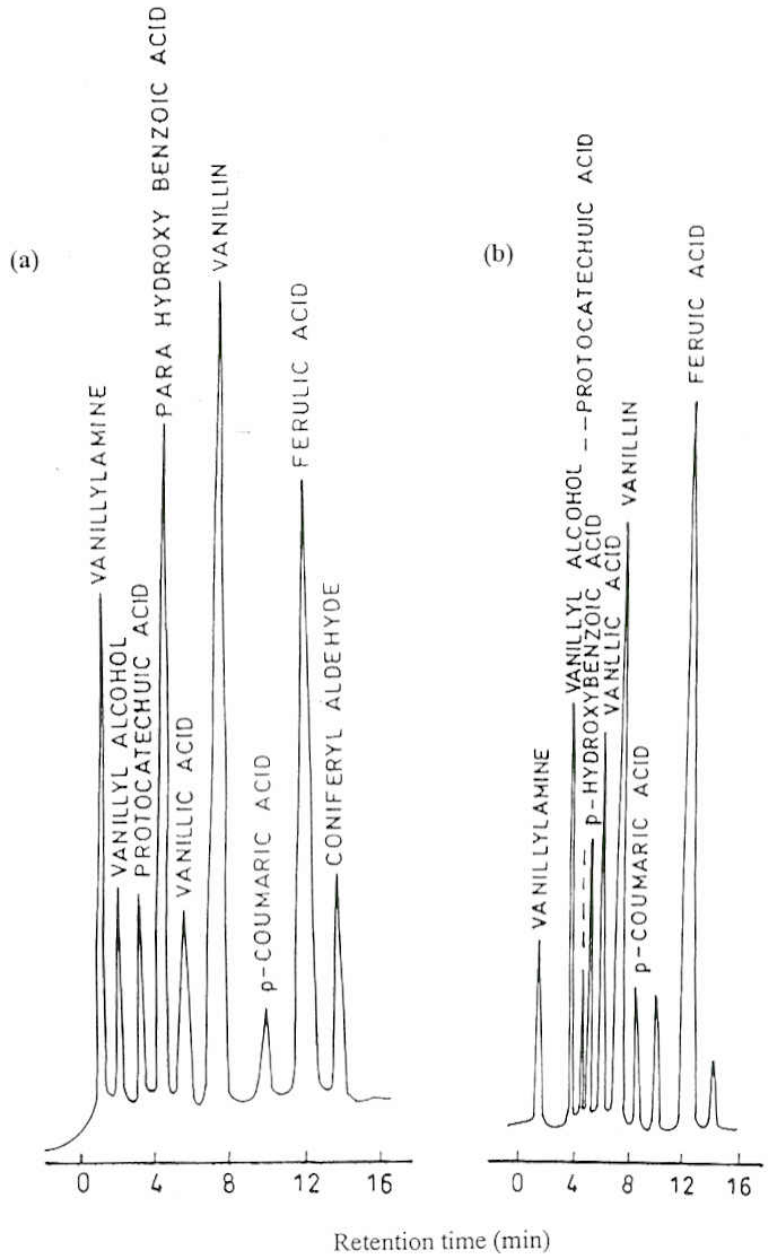


Fig 3.32: HPLC profile of biotransformed products in ferulic acid fed normal root cultures of *C. frutescens*
(a) authentic vanillin related standards; (b) Ferulic acid fed cultures
Column: C- 18 column (25 cm long, 4 mm i.d.)
Mobile phase: CH_3OH:CH_3COOH:H_20 (25:5:75)
Detector: UV-280 nm

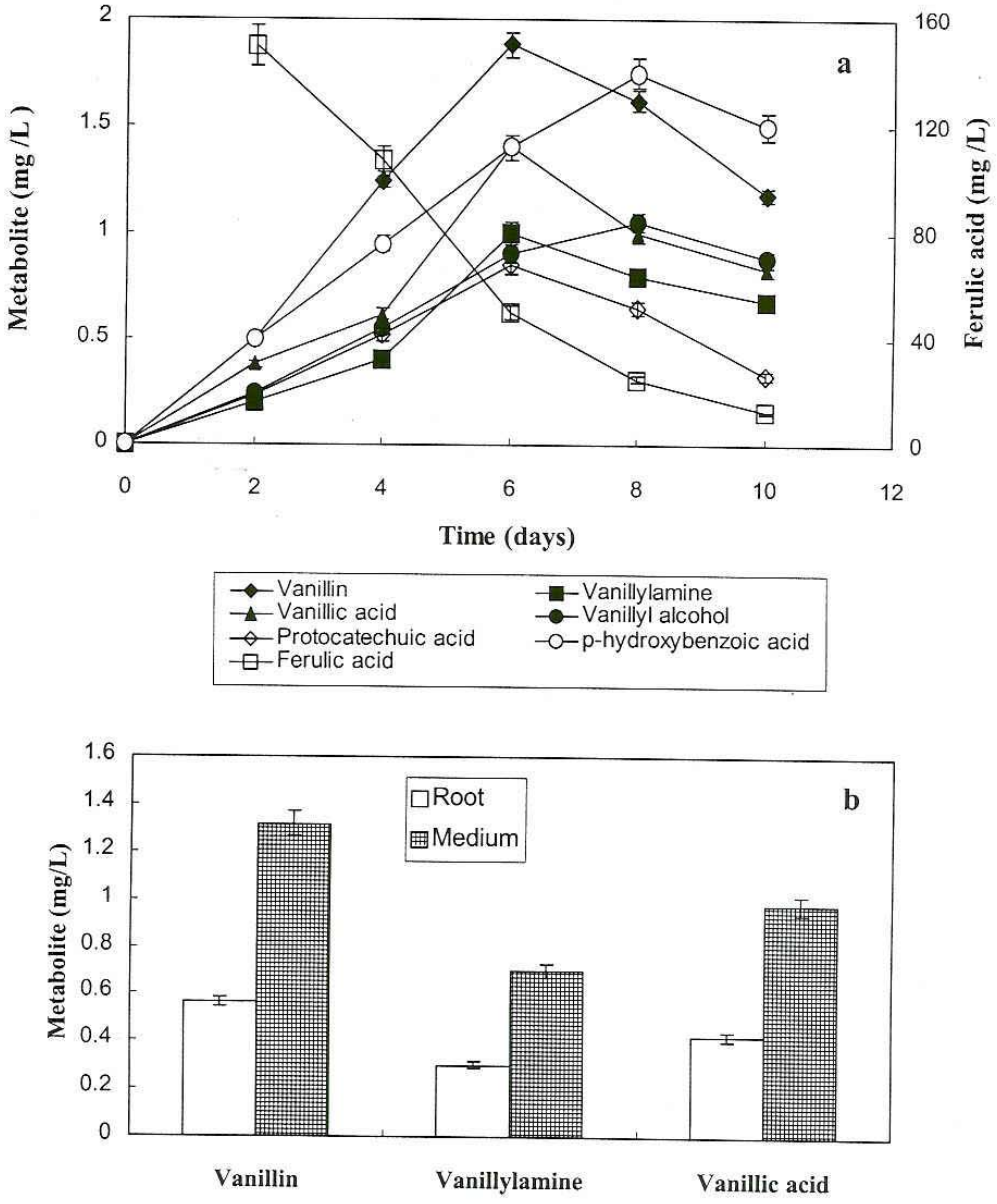
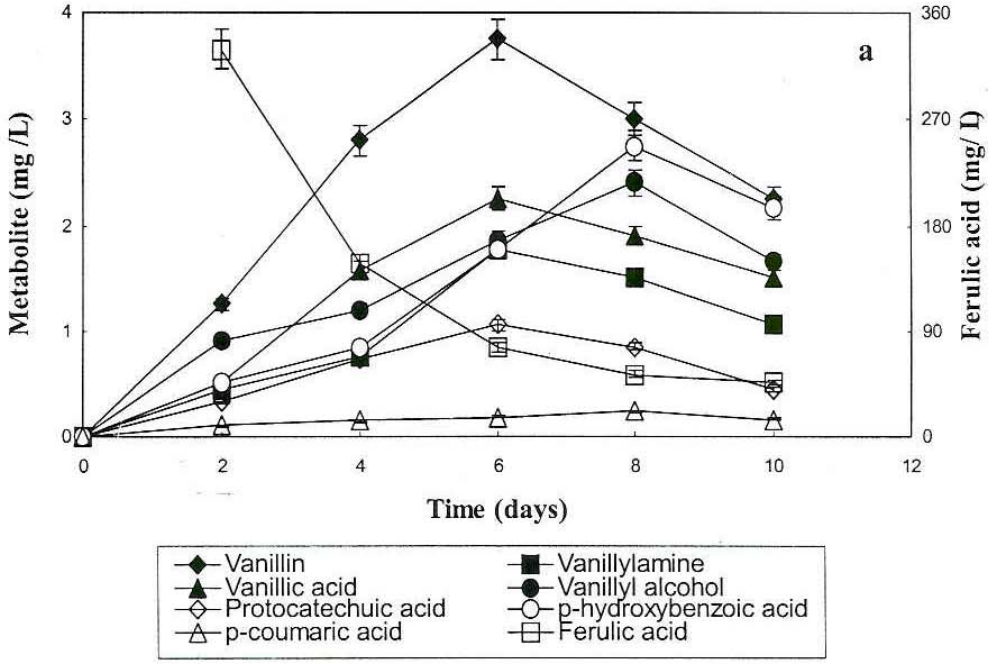


Fig 3.33: (a) Biotransformation of ferulic acid (1 mM) to vanillin related metabolites in *C. frutescens* normal roots
(b) Distribution of vanillin related metabolites in media and roots during biotransformation of ferulic acid
(data recorded on 6th day after ferulic acid addition)



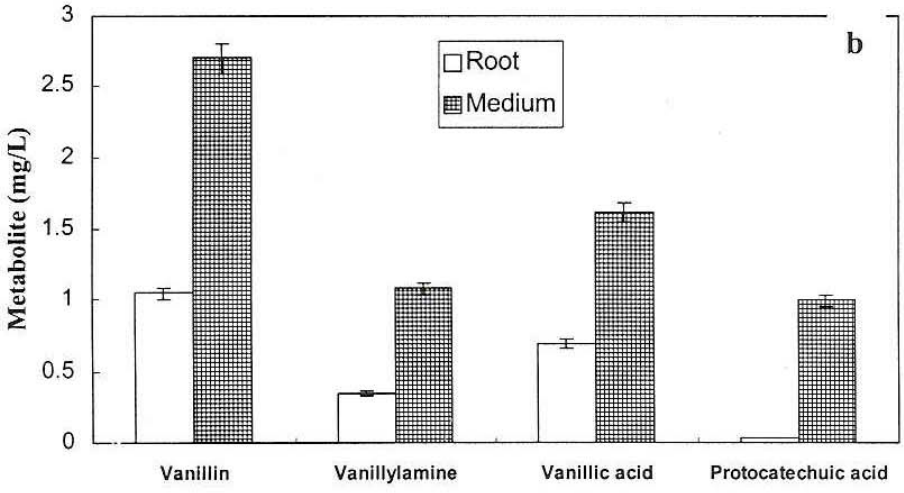


Fig 3.35: (a) Biotransformation of ferulic acid in presence of BCD (2 mM each) to vanillin related metabolites in normal roots of *C. frutescens*
(b) Distribution of vanillin related metabolites in roots and medium during biotransformation
(data recorded on 6th day after precursor feeding)

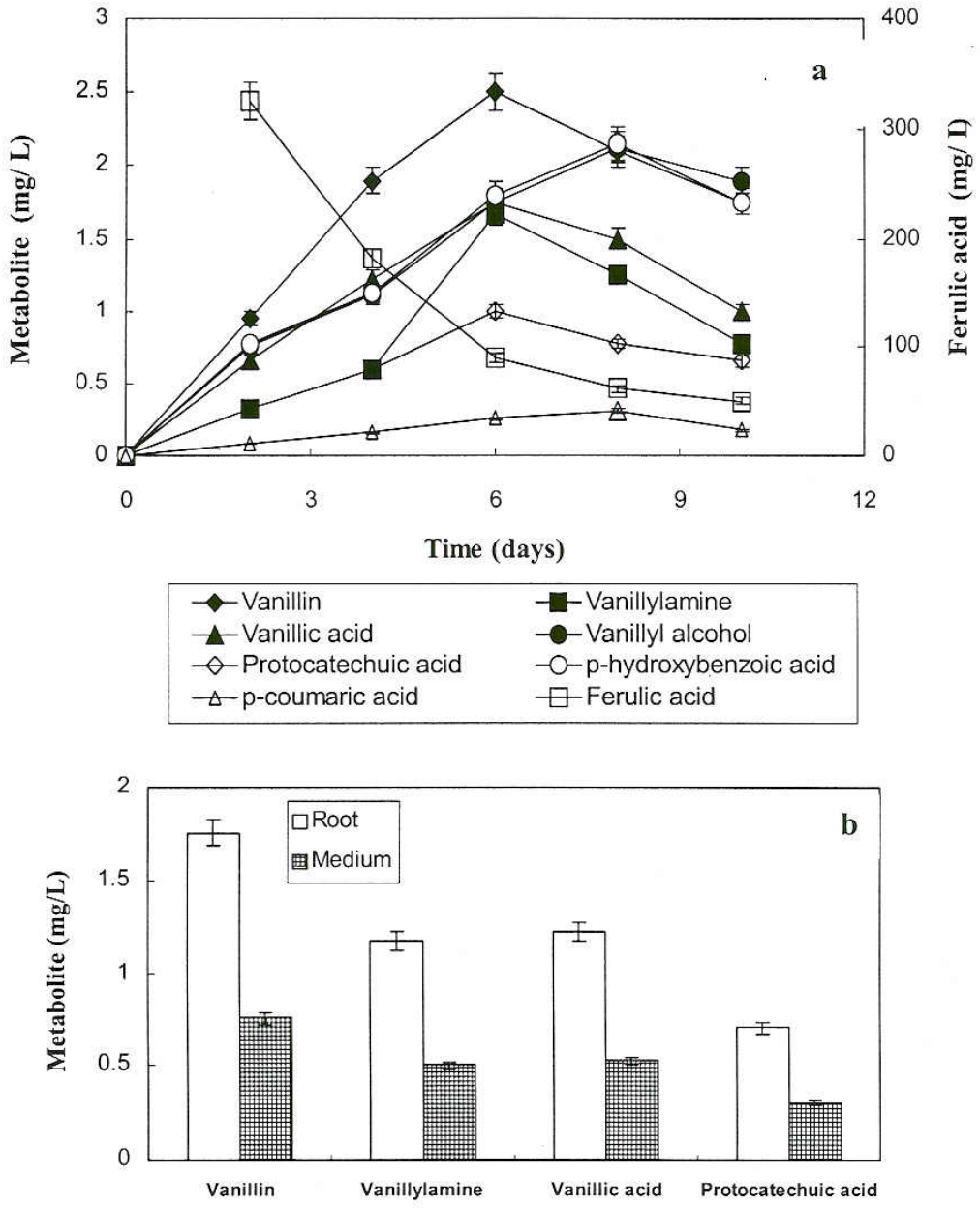


Fig 3.34: (a) Biotransformation of ferulic acid (2 mM) to vanillin related metabolites in normal roots of *C. frutescens*
(b) Distribution of vanillin related metabolites in roots and medium during biotransformation of ferulic acid (2 mM)
(data recorded on 6th day after ferulic acid feeding)

Biotransformation of ferulic acid complexed with β-cyclodextrin (2mM each)

Since 2mM ferulic acid produced more biotransformed product than at 1mM level, and is inhibitory to tissue growth at the 2 mM level, β-cyclodextrin complexation was attempted to enhance the efficiency of biotransformation. Cultures fed with ferulic acid complexed with β-cyclodextrin (2mM each) did not show significant improvement of growth of roots (Fig. 3.31). Root cultures have shown a maximum biomass of 5.1 g FW 40 ml^{-1} after 6 days of addition of ferulic acid-BCD complex, which is almost equal to that of cultures fed only with ferulic acid (2 mM). But when compared to cultures fed with ferulic acid alone, a higher production of vanilla flavour metabolites was observed (Figure 3.35a). Maximum vanillin accumulation recorded on day 6 after addition of ferulic acid –BCD complex was 3.754 mg L^{-1} which is 1.5 fold higher when compared to cultures fed on ferulic acid alone (2 mM). Similarly, the accumulation of other vanillin related metabolites was high when compared with the cultures fed with ferulic acid alone. Maximum vanillylamine (1.43 mg), vanillic acid (2.3 mg), and protocatechuic acid (1.04 mg) were also accumulated on the day 6 after ferulic acid addition. However, vanillyl alcohol (2.4 mg), parahydroxybenzoic acid (2.7 mg), and para coumaric acid (0.24 mg L^{-1}) were found to be maximum on day 8 after ferulic acid addition. Media samples have shown higher contents of biotransformed products than roots (Fig. 3.35b).

3.2.3.2 Bioreactor studies on biotransformation of ferulic acid

Based on the results obtained from the shake flask data it was proposed to carryout experiments with lesser concentration of ferulic acid i.e at 1 mM than 2 mM- as at the latter concentration, the roots were affected even though the biotransformation efficiency was more (Fig 3.31). The bioreactor was fed on the day 12 of culture with ferulic acid complexed with BCD (1 mM each). The accumulation of vanillin related metabolites in the medium over culture time is shown in Figure 3.36. Maximum accumulation of metabolites was recorded on day 9 after precursor addition. Maximum vanillin accumulation was 1.63 mg L^{-1} in the culture medium; the contents of other metabolites are as follows:- vanillylamine-1.1 mg L^{-1}, vanillic acid-1.3 mg L^{-1}, vanillyl alcohol- 1 mg L^{-1}, protocatechuic acid- 0.57 mg L^{-1} and

parahydroxy benzoic acid- 1.38 mg L^{-1}. Initially vanillylamine, vanillic acid and protocatechic acid were not detected, and started appearing only on day 6 after precursor addition.

However, after 24 days of reactor run, the roots were harvested and extracted and analysed by HPLC. The final weight of the roots was recorded as 21.76 g. Except protocatechuic acid and p- coumaric acid, all other metabolites were found in the roots with vanillin having the maximum value of 0.181 mg L^{-1}. The total vanillin production (medium and roots) was recorded as 1.28 mg L^{-1} (on day 12 after precursor addition). The total vanillin related metabolite production on day 12 after precursor addition, is shown in Table 3.4. In this case also only 5 % of the precursor was converted to further products.

Table 3.4: Distribution of vanillin related metabolites in medium and root samples of *Capsicum* grown in 3L bubble column bioreactor.

Vanillin & related metabolites	Amount in roots (µM)	Total amount((µM)
Vanillin	1.19	8.43
Vanillylamine	0.87	5.7
Vanillylalcohol	0.49	5.03
Vanillic acid	0.54	5.3
Protocatechuic acid	ND*	2.43
Parahydroxybenzoic acid	0.56	8.4

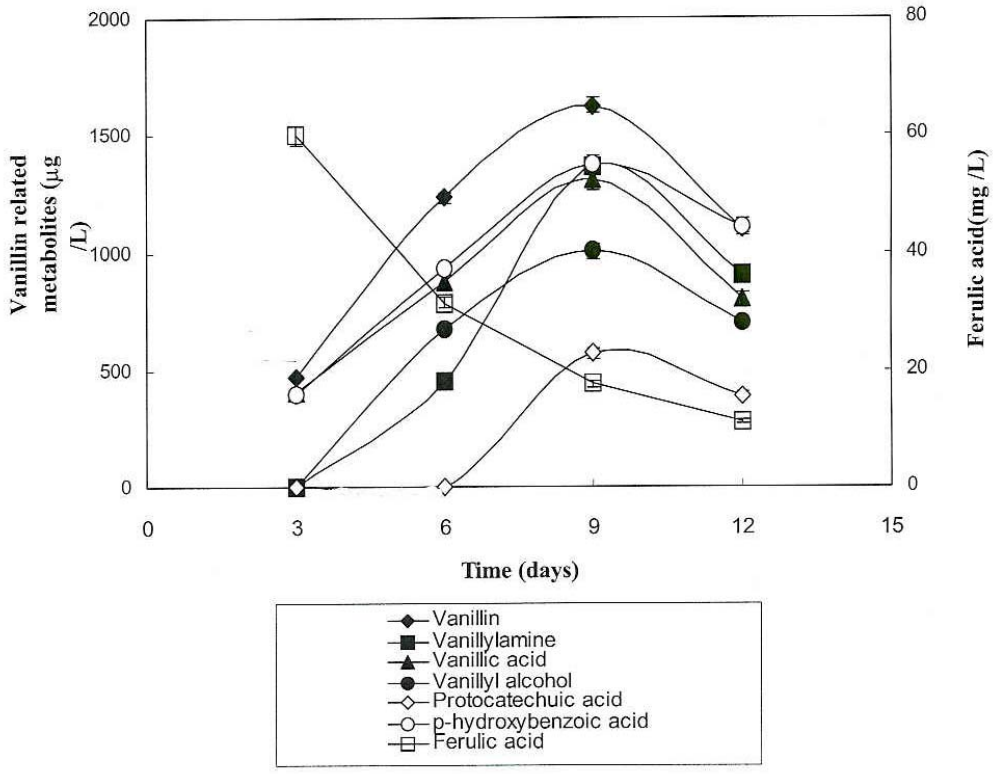


Fig 3.36: Biotransformation of ferulic acid-BCD complex (1 mM each) to vanillin related metabolites using normal roots of *C. frutescens* in a 3L-bubble column bioreactor (1.75 L working volume)

3.2.3.3 Biotransformation of protocatechuic aldehyde (1 mM) to vanillin related compounds

The growth of normal roots of capsicum was affected by the addition of protocatecuic aldehyde at 1mM concentration (Fig. 3.31). Maximum biomass accumulation was recorded as 4.9 g FW $40ml^{-1}$ culture medium, on day 6 after precursor addition, which is 1.32 times lesser than that of un-fed control. The biotransformed products were detected and quantified by HPLC analysis, and compared with authentic standards of vanilla flavour metabolites (Fig 3.37). Control

cultures with out protocatechuic aldehyde and autoclaved roots with protocatechuic aldehyde did not show accumulation of vanilla flavour metabolites.

Maximum vanillin (1.2 mg L^{-1}), vanillylamine (0.5 mg L^{-1}), and protocatechuic acid (0.57 mg L^{-1}) accumulation was recorded on the day 6 after precursor addition (Figure 3.38a). There is a considerable accumulation of vanillin related compounds in the medium. But in this case, the cultured roots also have shown significant contents of vanillin related metabolites ranging 30-50% of the total production (Fig 3.38b).

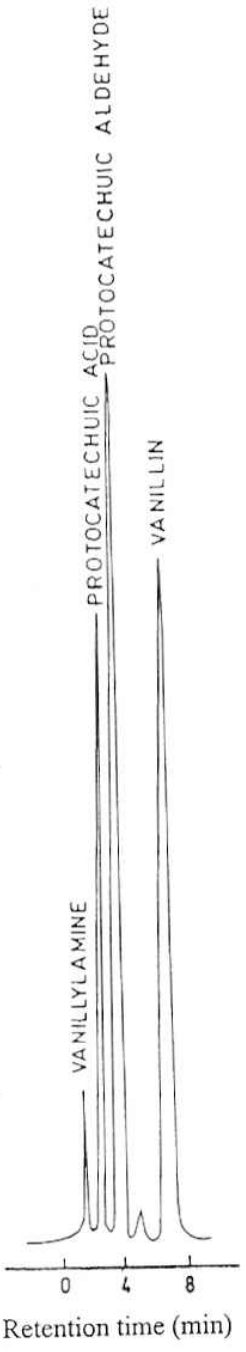


Fig 3.37: HPLC profile of biotransformed products in protocatechuic aldehy
fed normal root cultures of *C. frutescens*
Column: C- 18 column (25 cm long, 4 mm i.d.)
Mobile phase: $CH_3OH:CH_3COOH:H_20$ (25:5:75)

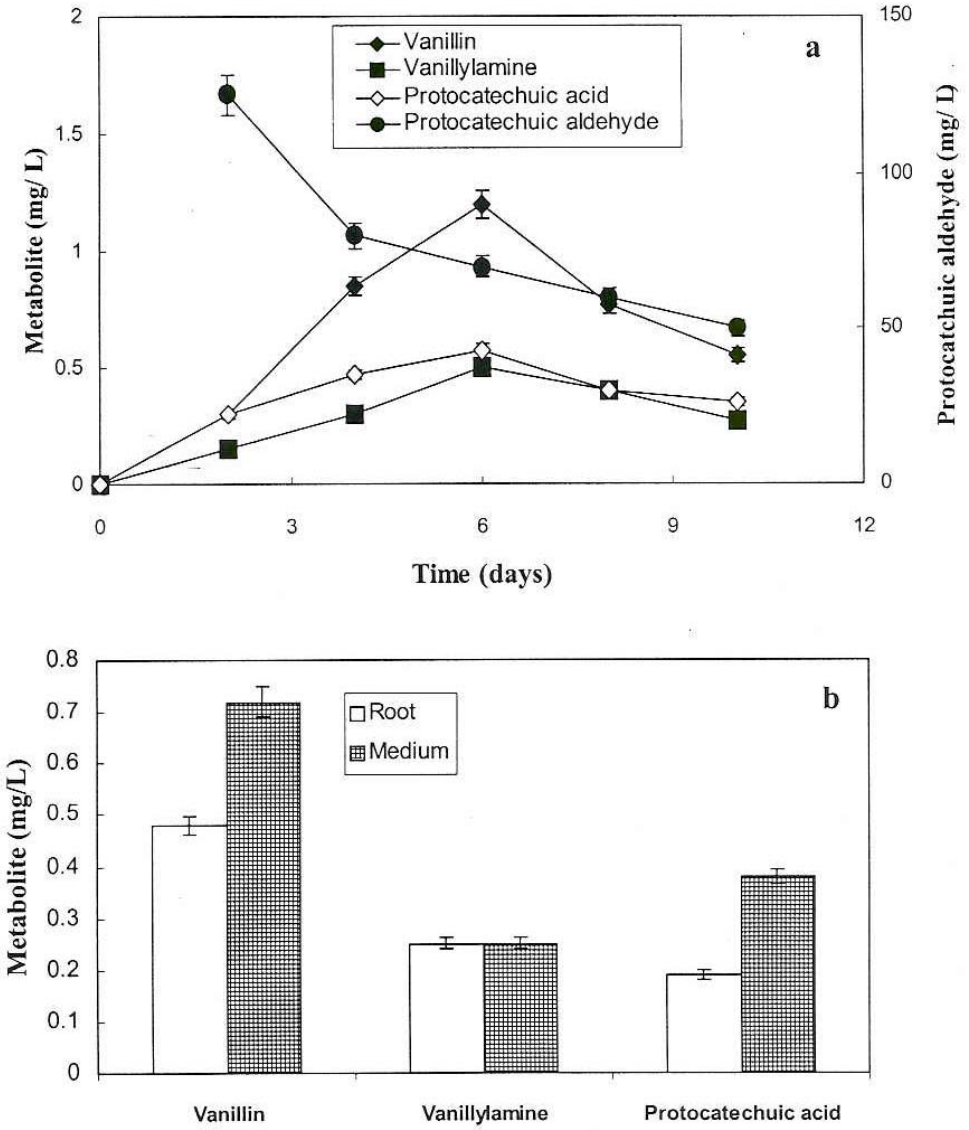


Fig 3.38: (a) Biotransformation of protocatechuic aldehyde (1 mM) to vanillin related metabolites in normal roots of *C. frutescens*
(b) Distribution of vanillin related metabolites in roots and medium during biotransformation of protocatechuic aldehyde (data recorded on 6th day after precursor feeding)

3.2.3.4 Biotransformation of caffeic acid to vanillin and related metabolites

Biotransformation of Caffeic acid

Caffeic acid was fed at a concentration of 1.5 mM to the normal root cultures on 10^{th} day of culture where the biomass was 3.8 ± 0.2 g FW 40 ml^{-1} culture medium. At this concentration caffeic acid showed inhibitory effect on biomass growth (Fig 3.39). After 16 days of culture, the biomass growth in caffeic acid fed cultures was recorded as 3.1 g fresh wt 40 mL^{-1} which was 1.67 times less when compared with unfed control on the same day. Control cultures without caffeic acid and autoclaved roots with caffeic acid have not shown vanillin related metabolite accumulation whereas caffeic acid fed roots could biotransform externally added caffeic acid to various vanillin related metabolites. The biotransformed products were identified by HPLC analysis and comparison with authentic vanillin related standards (Fig 3.40). The accumulation of biotransformed products over a period of time has been studied (Fig 3.41a). Ferulic acid and vanillin and started accumulating after 24 hrs of caffeic acid addition which was recorded as 685 and 467.5 µg L^{-1}, respectively. Ferulic acid levels showed a decrease with further culture time. Maximum total vanillin accumulation (roots and medium) was recorded as 2.097 mg L^{-1}on 4^{th} day of addition. Other vanilla flavour metabolites *viz*. vanillic acid and vanillylamine also were accumulated. Maximum vanillic acid (840 µg L^{-1}) and vanillylamine (532.5 µg L^{-1}) contents were recorded on 4^{th} and 5^{th} days after caffeic acid addition, respectively. Vanillin and vanillic acid contents were higher in the medium than roots contributing to 70% of total content whereas vanillylamine content was recorded more in roots (68.8% of the total) (Fig. 3.41b).

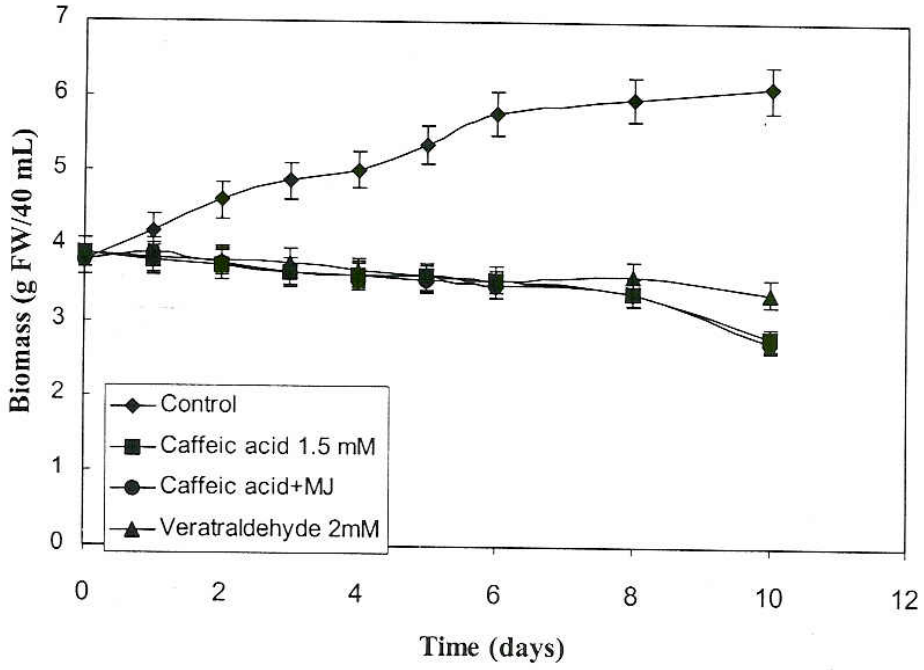


Fig. 3.39: Influence of caffeic acid and veratraldehyde on biomass accumulation in *C. frutescens* normal roots

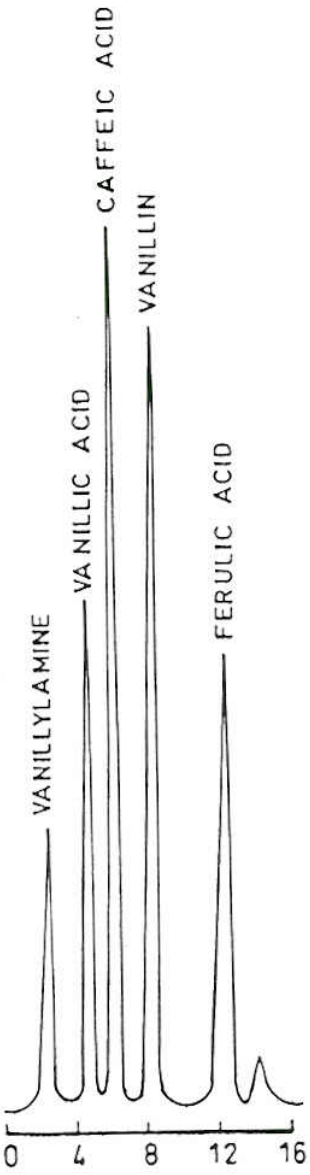


Fig 3.40: HPLC profile of biotransformed products in caffeic acid fed normal root cultures of *C. frutescens*
Column: C- 18 column (25 cm long, 4 mm i.d.)
Mobile phase: CH_3OH:CH_3COOH:H_20 (25:5:75)
Detector: UV-280 nm

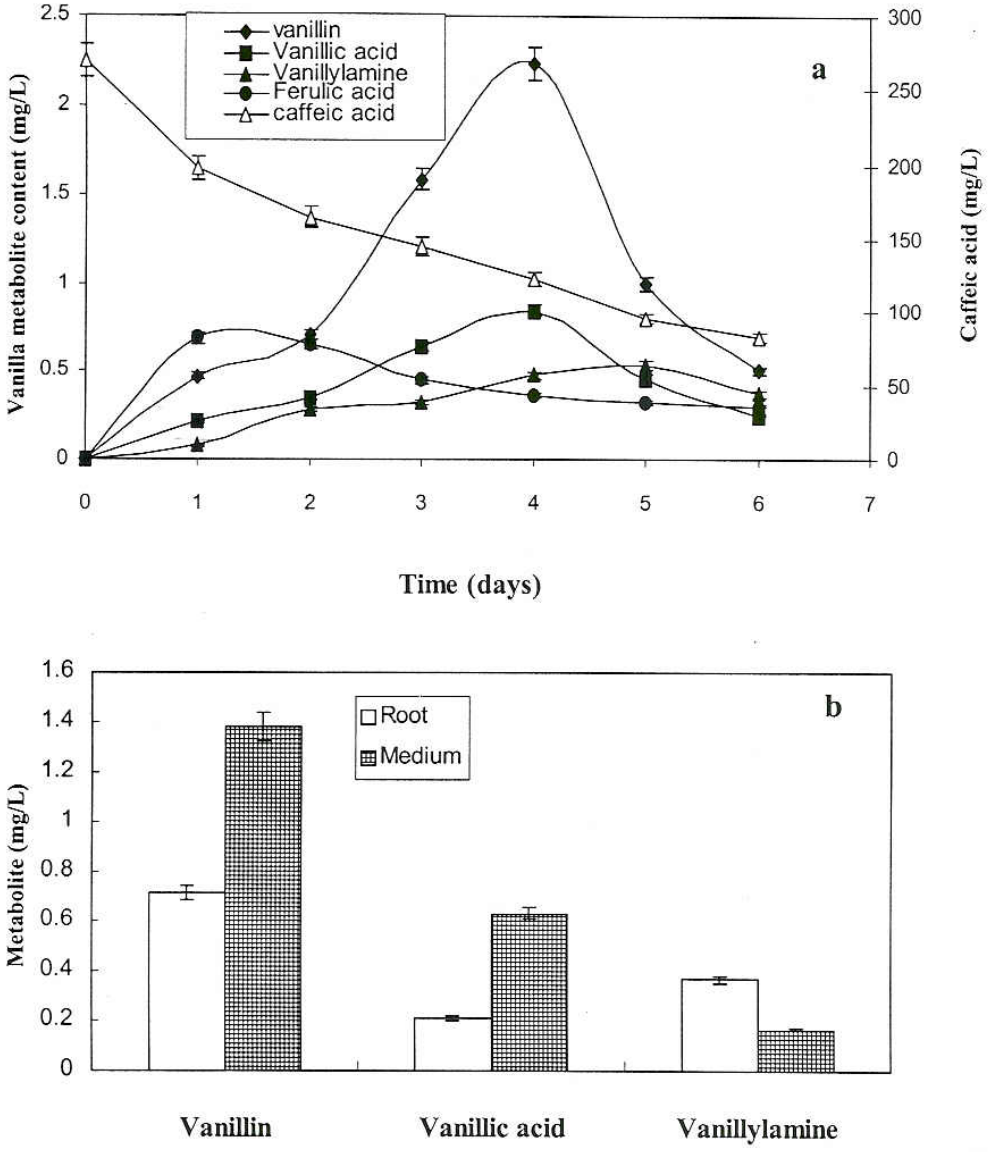


Fig 3.41: (a) Biotransformation of caffeic acid (1.5 mM) to vanillin related metabolites in normal roots of *C. frutescens*
(b) Distribution of vanillin related metabolites in roots and medium during biotransformation of caffeic acid (1.5 mM)
(data recorded on 4th day after precursor feeding)

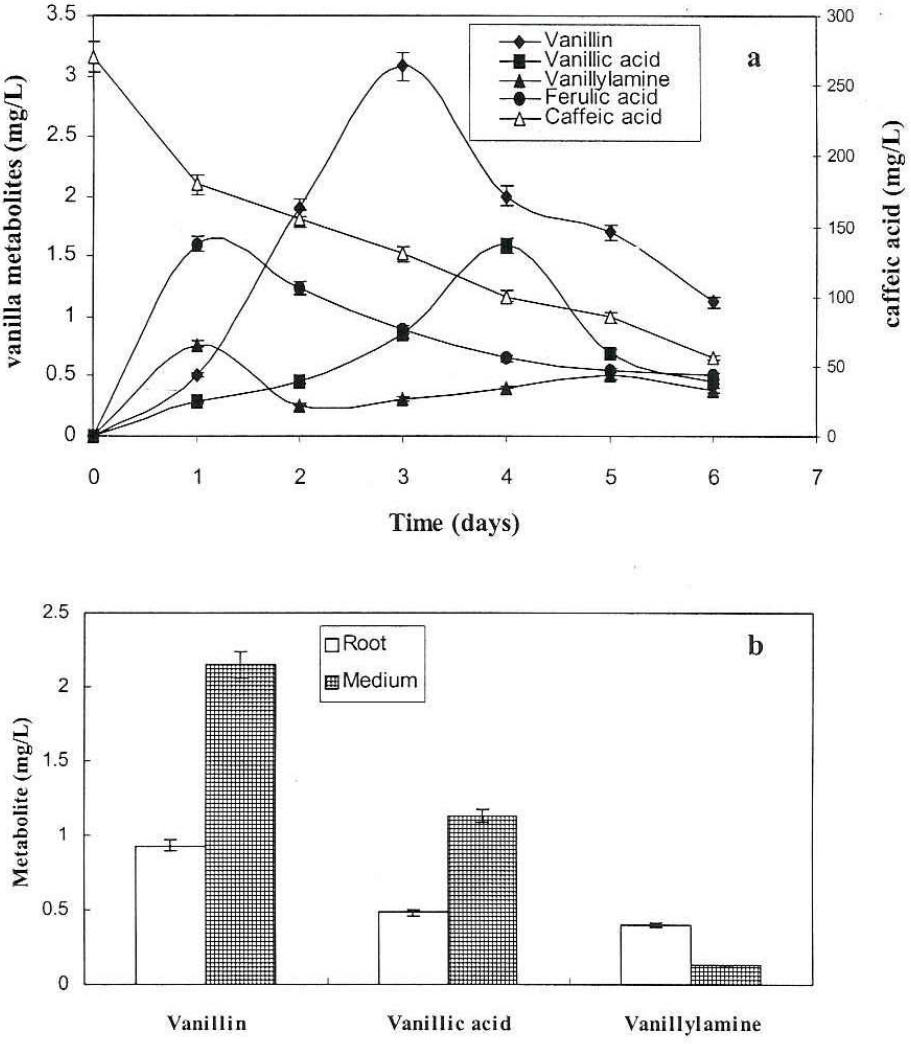


Fig 3.42: (a) Biotransformation of caffeic acid and MJ to vanillin related metabolites in normal roots of *C. frutescens*
(b) Distribution of vanillin related metabolites in roots and medium during biotransformation of caffeic acid and MJ (data recorded on 4th day after precursor feeding)

Biotransformation of caffeic acid along with methyl jasmonate

To check the influence of methyl jasmonate (MJ) on rate of biotransformation, it was added at a concentration of 10μM along with caffeic acid on 10^{th} day of culture. At this concentration, MJ has not shown any inhibitory effect on normal root growth (Fig 3.39). Maximum ferulic acid accumulation was recorded as 1.6 mg L^{-1} after 24 hours which was 2.33 fold higher when compared to cultures fed with caffeic acid only. Maximum vanillin accumulation was recorded on 3^{rd} day after precursor addition as 3.075 mg L^{-1} which was 1.37 fold higher when compared to control (2.24 mg L^{-1} on 4^{th} day) (Fig. 3.42a). Thus MJ not only elicited vanillin production, but also reduced the time taken for its maximum accumulation. It also resulted in increased vanillic acid formation, which was recorded to be the maximum on the 4^{th} day after the precursor addition as 1.59 mg L^{-1} (1.9 fold higher compared to control on the same day). There was no change in vanillylamine content observed in MJ fed cultures. The distribution of biotransformed products in roots and media is shown in Fig 3.42b. Thus MJ was found to be suitable as an elicitor for effecting biotransformation and also to produce higher levels of product.

Effect of MJ on 3-O-Caffeic acid O-methyl transferase (3-O-CAOMT) activity

3-*O*-CAOMT catalyzes the transfer of a methyl group from S-adenosyl-L-methionine to the 3-hydroxyl group of caffeic acid forming ferulic acid. CAOMT activity was assayed for caffeic acid fed cultures and MJ (caffeic acid +MJ) treated root cultures (Fig 3.43). MJ treatment led to an increase in the CAOMT activity in root cultures of *C. frutescens*. Maximum activity was recorded after 24 hours as 8.23 Units mg^{-1} protein which was 13.7 fold higher compared to control cultures (0.6 Units mg^{-1} protein). But in cultures fed with caffeic acid, maximum specific activity was recorded on 3^{rd} day of incubation which was 1.2 Units mg^{-1} protein.

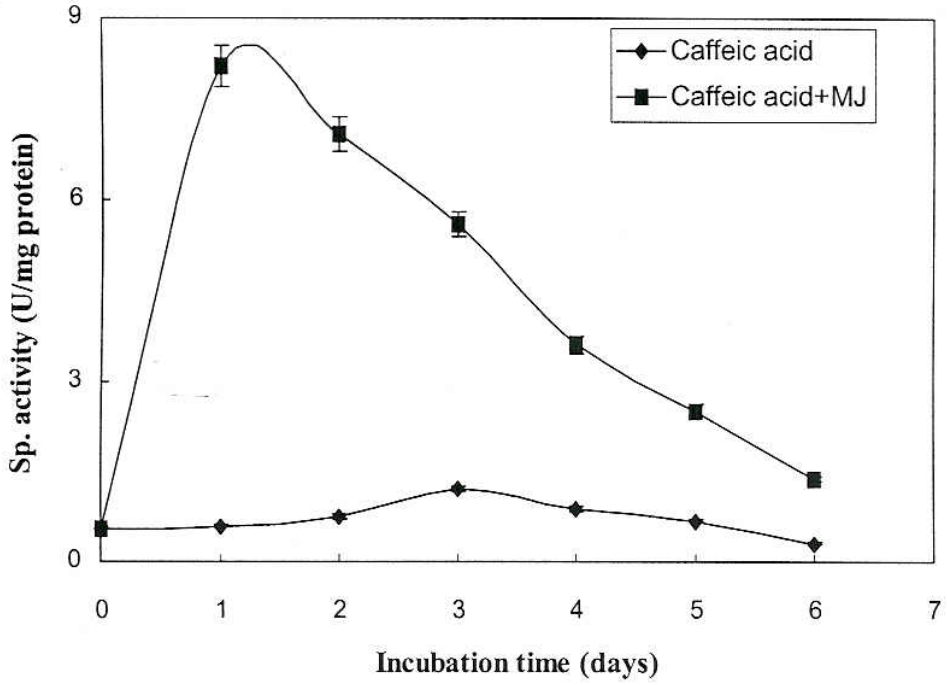


Fig 3.43: Influence of methyl jasmonate on 3-CAOMT activity of caffeic acid fed normal roots of *C. frutescens*

3.2.3.5 Biotransformation of veratraldehyde to vanillin related metabolites

Veratraldehyde was fed at a concentration of 2mM to the root cultures on the 10^{th} day of culture, which inhibited the root growth (Fig 3.39). After 20 days of incubation, the treated cultures showed a 1.8 fold lesser biomass compared to control unfed cultures. Normal roots could successfully bioconvert veratraldehyde to vanilla flavour metabolites *viz*. vanillin, vanillyl alcohol, vanillic acid, parahydroxy benzoic acid and protocatechuic acid whereas control (unfed) cultures did not show any accumulation of these products. The HPLC profile of biotransformed products is shown in Fig 3.44. Maximum vanillin accumulation was recorded after 6 days of precursor addition as 11.875 mg L^{-1}. Protocatechuic acid and vanillic acid were detected after 6 days of precursor addition and the maximum values were recorded as 0.325 and 0.875 mg L^{-1} at 8^{th} and 10^{th} day of precursor addition, respectively. Maximum levels of parahydroxy benzoicacid and vanillic acid were recorded on 4^{th} and 8^{th} day of precursor addition, respectively (Fig 3.45a). The roots accumulated 40% of total vanillin and vanillic acid (Fig 3.45b). Veratraldehyde treatment resulted in maximum vanillin production than any other precursor investigated for this purpose.

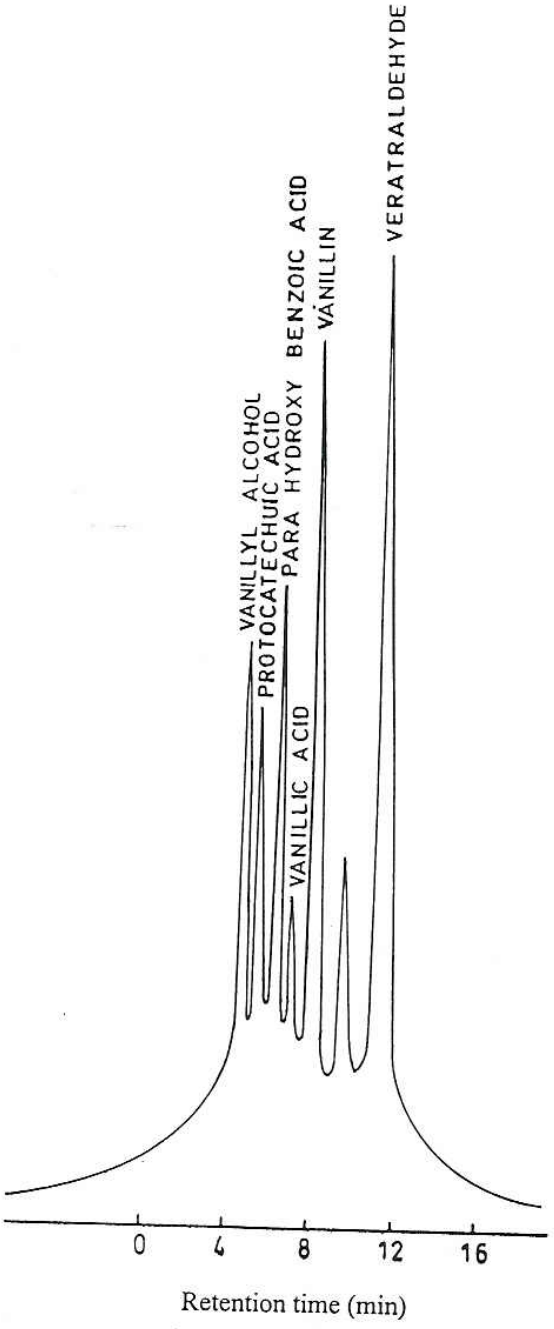


Fig 3.44: HPLC profile of biotransformed products in veratraldehyde fed normal root cultures of *C. frutescens*
Column: C- 18 column (25 cm long, 4 mm i.d.)
Mobile phase: CH_3OH:CH_3COOH:H_20 (25:5:75)
Detector: UV-280 nm

DISCUSSION

4.1 DISCUSSION FOR THE STUDIES USING *B. VULGARIS* HAIRY ROOT SYSTEM

4.1.1 Confirmation of transgenic nature of *B. vulgaris* hairy roots

4.1.2 Growth and betalaine production studies of hairy roots in shake flasks.

4.1.3 Conductivity and osmolarity as indirect methods of biomass estimation

4.1.3.1 Conductivity measurement as a method of indirect estimation of biomass

4.1.3.2 Osmolarity measurement as a better alternative for the estimation of biomass

4.1.4 Bioreactor systems for *Beta vulgaris* hairy roots

4.1.5 Osmolarity and conductivity for indirect estimation of biomass in *Bioreactors*

***4.1.6* Studies on improvement of growth and metabolite production in bioreactors**

4.1.6.1 Effect of polyamines on hairy root growth and metabolite production

4.1.6.2 Influence of Methyl jasmonate (MJ) on growth and metabolite production

4.1.7 Studies on release of pigments under the influence of Cetyl Trimethyl Ammonium Bromide (CTAB).

4.1.8 Accumulation of DOPA and dopamine in hairy root cultures of *B. vulgaris*

***4.1.8.1 Influence of Tyrosine feeding on DOPA & dopamine accumulation in hairy root cultures of* B. vulgaris**

***4.1.8.2 Influence of caffeic acid and catechin on DOPA and dopamine accumulation in hairy root cultures of* B. vulgaris**

In the present study, hairy roots of *B. vulgaris* have been used as production systems for betalaines, which are natural red pigments. Initially the hairy roots were cultured in shake flasks to evaluate the growth and production levels. Conductivity and osmolarity measurements were used to correlate with the biomass values. Various studies on evaluation of bioreactor systems, enhancement of metabolite contents and down stream processing were performed. Studies on the production of DOPA and dopamine, which are the major intermediates in betalaine pathway were also carried out with special emphasis on precursor biotransformation and use of enzyme inhibitors. The results have been discussed in this section.

4.1.1 Confirmation of transgenic nature of *B. vulgaris* hairy roots

The frequency of hairy root induction was more in case of clones obtained from the strain ATCC 15834 (Fig 3.1). Hence, they were used for all further experiments. PCR analysis using the *rol* A primers provided the molecular evidence supporting the transgenic nature of the hairy root cultures of *B. vulgaris* obtained upon *A. rhizogenes* infection (Fig 3.3 a). Primers for *rol A* gene were expected to produce a fragment of 360 bp. With *rol A* primers the expected fragment size was obtained in lanes containing DNA from transformed hairy root cultures where as no bands were observed in lanes containing DNA from untransformed roots. Southern hybridization analysis provided further evidence for the transgenic nature of the hairy roots and integration of the T-DNA into the plant chromosomal genome (Fig 3.3 b).

4.1.2 Growth and betalaine production studies of hairy roots in shake flasks.

The hairy roots exhibited faster growth and higher metabolite production in shake flasks. The specific growth rate recorded was 0.04 day^{-1}. The gradual transformation of pale colored hairy roots into red colour indicates the production of betalaines during the exponential growth phase, as evident from the figure 3.2. The betalaine production was at its peak, reaching a maximum of 160 mg L^{-1} on day 24 of culture (Fig. 3.4). The reduction of both biomass and betalaine contents after 24 days is possibly because of the necrosis of roots finally leading to death of the tissue.

4.1.3 Conductivity and osmolarity as indirect methods of biomass estimation

The progress of growth of hairy roots in culture was accompanied by a decrease in the medium conductivity and osmolarity. Besides this, many other environmental changes such as glucose/sucrose concentration (typical carbon sources for hairy root cultures) and pH were noticeable. Controlled experiments using MS medium without biomass were carried out to evaluate the effect of these environmental changes on medium conductivity and osmolarity. Change in pH from 4 to 6 did not affect the medium conductivity and osmolarity significantly (Table 3.1a). In general, medium pH in a culture system decreases with increase in the culture time. However, from the above results it is understood that this change in pH will not affect the conductivity and osmolarity, thus can be nullified. As sucrose and glucose are non-electrolytes, the medium conductivity had not changed significantly. However, in case of osmolarity, which depends on the number of moles of total solutes rather than ions, increase in glucose and sucrose concentrations resulted in a significant increase in the osmolarity. Increase in temperature from 5 to 35 ^{0}C resulted in an increase in the conductivity from 3.1 to 6.5 mS but had not affected the osmolarity (Table 3.1b,c). However, this effect of temperature on the medium conductivity was minimized by carrying out the experiment under a temperature controlled environment in an incubation room (25±1^{o}C).

4.1.3.1 Conductivity measuremnt as a method of indirect estimation of biomass

Conductivity measurement has been used as an indirect method of biomass estimation in continuous on line monitoring of plant cell cultures in bioprocess engineering studies due to its accuracy and efficiency (Ryu *et al.,* 1990). It is known that hairy root cultures require different inorganic nutrients and carbon sources for their propagation (Toivonen 1993). Hence the uptake of these ionic materials results in decrease in the medium conductivity. This is evident in the present study wherein the conductivity showed a gradual decrease from an initial value of 5.4 mS to 3.7 mS over a period of time (Fig. 3.5a). Initially the correlations were done taking the initial values of biomass and conductivity in the calculation. But in Fig. 3.5 b, as the values of ΔX and ΔC were calculated from initial values (X_0 and C_0) it is imperative that the

plot has to pass through the origin. Although the fit in Fig. 3.5 b is reasonably good, as pointed out by Kwok *et al.,* (1992), this method has serious limitations. This is mainly due to the dependency of the relationship between ΔX and ΔC on initial biomass. They showed that involvement of these initial values in the calculation forced the line of regression through origin resulting in errors from 35% to 400%. This was verified by plotting ΔX/ΔC against culture time as shown in Fig. 3.6 a. This indicates a need for a more accurate method of correlating biomass with conductivity. An alternate method of correlation indicated by Ballica *et al.,* (1993) was proved to be a reliable method for better correlation which is in y=mX+C form (Fig 3.6 b), as it do not involve initial values in calculation.

4.1.3.2 Osmolarity measurement as a better alternative for the estimation of biomass

Osmolarity method offers more advantages over the conductivity method. Unlike conductivity which depends on the number of moles of ions alone, osmolarity takes into account the total number of moles of all solutes present in the medium and hence is a more reliable method for the estimation of biomass. For instance, the medium contains sucrose, which is a major carbohydrate source and change in its concentration cannot be determined by conductivity as it is a non electrolyte (Madhusudhan *et al.,* 1995). As the biomass increases, the nutrients are taken up by the growing hairy root cultures resulting in decrease in number of moles of media solutes which in turn results in a proportional decrease in osmolarity.

In the present case, the osmolarity initially showed an increase which was due to the hydrolysis of sucrose to glucose and fructose resulting in the increase in number of moles which in turn resulted in an increase in the osmolarity. Subsequently osmolarity decreased as these sugars were eventually taken up by the growing hairy roots (Fig. 3.7 a). As already mentioned for conductivity, in this case also the involvement of initial values in correlation of biomass and osmolarity led to errors as ΔX and ΔO were calculated from initial values and it was obvious that the plot has to go through origin (Fig. 3.7 b). This method has limitations when goodness of fit is

taken into account. One main reason for the poor fit was hydrolysis of sucrose to glucose and fructose resulting in high osmolarity in the initial periods of growth. The poor fit was also because of the dependency of ΔX and ΔO on initial biomass which was verified by plotting ΔX/ ΔO against culture time as already shown in Fig. 3.8 a. Similar to the case of conductivity, here also the equation y=mX+C was proved to be a promising method for correlating the biomass and osmolarity during batch culture of *B. vulgaris* hairy root cultures (Fig. 3.8 b).

Thus from the present study, it was evident that osmolarity is advantageous over conductivity in the estimation of biomass, particularly in case when the roots were growing in bioreactors. As the hairy roots cannot be taken out in reactors for intermittent biomass measurements, the indirect estimation by these methods has relevance.

4.1.4 Bioreactor systems for *Beta vulgaris* hairy roots

The present study provided the suitability of bioreactor system based on mass transfer studies and designs of bioreactors. The hairy roots of *B. vulgaris* were evaluated in three different bioreactor configurations *viz*. bubble column, nutrient sprinkle and acoustic mist. Among all the bioreactors studied for this purpose, the acoustic mist bioreactor (Fig 3.14) proved to be better with high biomass, growth rate (Fig 3.10) as well as betalaine production (Fig 3.12). In the present study these results were corroborated. Further, the new design of acoustic mist bioreactor facilitated a very thin liquid film formation on the growing hairy roots before it coalesced and dripped down.

In the present study with bubble column (Fig 3.9) and nutrient sprinkle bioreactors (Fig 3.13), the hairy roots after 20 days of culture, formed clumps inhibiting the oxygen transfer which probably was the reason for the lesser biomass and metabolite content when compared to acoustic mist bioreactor and shake flask. It may be noted that growth of hairy roots in culture depends on the over all mass (oxygen) transfer, which in turn is a combined effect of gas-liquid (G/L, sparged air to

medium) and liquid - solid (L/S, dissolved oxygen in the medium to the growing roots) mass transfer (Suresh *et al.,* 2001). The relative importance of mass transfer studies depends on the hairy root systems under study. In case of acoustic mist bioreactor, the liquid film around the root was thin allowing the air to pass easily into the cell membrane resulting in a better mass transfer enabling more metabolic activity leading to more products. Moreover, in case of mist bioreactors, as the gas composition in the reactor environment can be closely controlled; oxygen was not a limiting factor (Weathers *et al.,* 1997). Hairy roots in liquid systems grow in approximately spherical clumps but display a high degree of spatial heterogeneity (Bourgaud *et al.,* 2001). In a study conducted on *Atropa belladona* hairy roots in a liquid-dispersed reactor, Williams and Doran (2000) demonstrated the occurrence of both vertical and radial gradients of root density, with higher biomass levels near the top of the root bed, and in the inner part of the root pellets. This can be partially attributed to heterogeneity and mass transfer limitations of the roots, regarding oxygen (Tescione *et al.,* 1999, Shiao and Doran 2000), and nutrients (Williams and Doran 2000).

The solid-liquid mass transfer between root and medium depends on the thickness of the liquid medium around the root. For good oxygen transfer the liquid film should be minimum wherein the mist reactors find their importance (Weathers and Zobel 1992). Oxygen limitation to the roots may lead to anaerobiosis where ethylene is produced retarding the basal root initiation (Weathers *et al.,* 1997). Acoustic mist bioreactors have been used for cultivation of hairy roots (DiIorio *et al.,* 1992 a) providing the opportunities of changing culture conditions *viz* gas flow rate, gas composition, mist cycle and intensity. Some limitations regarding the problems of transducer (as the transducer was in direct contact with nutrient medium) and failure of electrical compounds were observed caused by repeated autoclaving in studies by Woo and Park (1993). Later it was modified to provide an acoustic window (Buer *et al.,* 1996), which still had problems of difficulty in fabrication and use of expensive materials. An improved version of acoustic mist bioreactor replacing the acoustic window by polypropylene vessel was found to be effective in later studies (Chatterjee

et al., 1997). Acoustic mist bioreactors have also been proved to control hyper hydration (Correll and Weathers 2001 a) and for plantlet acclimatization (Correll and Weathers 2001 b) in carnation plants.

Hairy root cultures are generally less submitted to erratic metabolite production than undifferentiated cells. However, a spatial heterogeneity is always possible along the growing organ. In the case of hairy roots, each root tip grows linearly, and the older part of the roots are still present in the culture medium. As a consequence, the total root biomass is always composed of young and older tissues. These tissues present various possibilities of secondary compounds as in the case of flavonoids reported by Bourgaud *et al.,* (1999).

The autoclavable nylon mesh as a support for hairy roots grown in nutrient sprinkle bioreactor and in acoustic mist bioreactor helped in uniform distribution of root biomass. Modifications in bioreactors designs and addition of certain chemicals have also been tried for better mass transfer. To name a few- Kanokwaree and Doran (1998) have used porous polypropylene membrane tubing as a supplementary aeration device and FC-43 perflurocarbon emulsion was added to the medium to improve oxygen transfer in roots. Combined air sparging resulted in better growth in *Atropa belladona* hairy root cultures. Similarly, carbon dioxide (1% V/V) was shown to enhance the growth and metabolite production during cultivation of hairy roots of *Beta vulgaris* and *Carthamus tinctorius* in nutrient mist bioreactors (DiIorio *et al.,* 1992 b).

In the present study, the mist cycle (10 min on and 20 min off), was sufficient to provide nutrients to the roots. There was no dead tissue observed in the reactor indicating better oxygen mass transfer without any limitation. Wyslouzil *et al.,* (1997) have developed aerosol models for mist deposition on hairy root cultures. Their analysis showed that the root-droplet system in a mist bioreactor meets the assumptions for the standard models of particle capture by impaction, interception, and diffusion used in industrial filters. They have found that the peak in the particle

size distribution of the mist generated by the ultrasonic transducer is always close to the minimum in the particle deposition curves. This implies that the mist can penetrate deeply even into a dense root bed. Wyslouzil *et al.,* (1997) also suggested that intermittent misting cycle prevents root clogging by previously captured particles and allows time for liquid to drain from the roots.

From the above results, it is inferred that the acoustic mist bireactor is more suitable one for cultivation of *B. vulgaris* hairy roots. The higher mass transfer rates led to higher production of biomass as well as betalaine contents. The additional features such as de-linking of bioreactor vessel from transducer facilitated repeated autoclaving of the reactor without causing problem to transducer, thus reducing the cost. Further, the simplicity and ease of operation finds great importance in using the bioreactor for scale-up studies. However, to develop a commercially viable large scale mist culture system, it is necessary that the system contains a bioreactor combined with a mistifier in a single unit with multiple ultrasonic transducers (Woo and Park 1996).

4.1.5 Osmolarity and conductivity for indirect estimation of biomass in bioreactors

Based on the shake flask experiments, it was suggested that osmolarity would be a better method to estimate the biomass more easily and promptly (as described in 3.1.4.2). Osmolarity in the present study is important in view of the fact that the major carbohydrate used was sucrose in the medium (3%). As it was not possible to determine the change in concentration of sucrose, which is a major nutrient, by conductivity, osmolarity measurement gained importance. In the present study, the equation y=mX+C was adapted for a better correlation as reported by Ballica *et al.,* (1993). In the present study, the biomass values of different bioreactors employed were predicted using an empirical equation obtained based on the shake flask data (Fig 3.6 & 3.8). The experimental values for biomass were as reported in the present study with different designs of bioreactors. The predicted values of biomass were obtained by substituting the values obtained from shake flask data by the equation y=mX+C. In case of biomass values predicted from conductivity, the m and c values

were –7.64 and 41.29 (Fig 3.6 b), respectively. In case of biomass values predicted from osmolairity, the m and c values were –0.215 and 49.234, respectively (Fig 3.8 b). The experimental and predicted values of biomass calculated by using conductivity and osmolarity measurements for all three bioreactors *viz*. acoustic mist, bubble column and nutrient sprinkle are given in Fig 3.15. From the figure, it is understood that the osmolarity values recorded at regular time intervals predicated the biomass yield in an effective way compared to conductivity.

The results also supported our earlier assumption of the use of osmolarity as a method for the indirect estimation of biomass and could be adapted to predict the biomass levels in bioreactors. Therefore, determination of harvest time to obtain more biomass and product yield was possible. Ramakrishnan *et al.,* (1999) have used osmolairty as a method for monitoring biomass in hairy root cultures of *Hyoscyamus muticus* grown in bioreactors and found that the results were in a good correlation with that of the experimental values.

4.1.6 Studies on improvement of growth and metabolite production in bioreactors

The comparative evaluation of bioreactor system for *B. vulgaris* hairy roots provided valuable information for the selection of a reactor system for further studies. Even though, the acoustic mist bioreactor was proved to be best in prototype model, further experiments have been carried out in a bubble column bioreactor of larger capacity.

4.1.6.1 Effect of polyamines on hairy root growth and metabolite production

In the present study, addition of polyamines (spermidine and putrescine) enhanced the biomass growth as well as betalaine production as evident from Fig. 3.16. Polyamines are known to enhance the primary root length and extended branching as reported by Bais *et al.,* (2000 a) in shake flask experiments on hairy roots of *B. vulgaris*. Further more, they observed that *B. vulgaris* hairy root cultures treated with PAs such as spermine, spermidine and putrescine developed more tertiary

branching. Maximum number of tertiary roots were obtained with putrescine and spermidine (0.75 mM each) which was 2.07 fold higher than that of control.

The increased biomass upon treatment with polyamines enhanced the productivity of betalaines. The enhanced growth and betalaine production in this study could be attributed to the increased free titers of endogenous polyamines especially spermine as observed in shake flask experiments by Bais *et al.,* (2000 a). They have reported that there was a 1.64 fold higher free spermine level compared to control when speermidine and putrescine (0.75 mM each) were fed to the cultures reflecting its important role in root growth and enhanced secondary metabolite production. Similarly, increased free endogenous polyamines mainly spermine was observed in fast growing tumors of plants (Bagni *et al.,* 1978) and animals (Rusell 1973). Similarly, Hiatt and Malmburg (1988) have reported 43% more growth in cultures of *Nicotiana sylvestris* with 90 µM putrescine. Chatterjee *et al.,* (1983), working with *Phaseolus mungo,* observed increased free spermine levels and decrease in putrescine and spermidine concentration associated with an increase in the root growth which was linked to the inter-conversion of free forms of these amines followed by their utilization for cellular differentiation.

4.1.6.2 Influence of Methyl jasmonate (MJ) on growth and metabolite production

MJ enhanced the metabolite production in the present study using shake flask cultures, though it inhibited the growth (Fig. 3.17a,b). MJ at a concentration of 40µM resulted in higher production of betalaines as 32.36 mg g^{-1} DW, which was 1.35 fold higher when compared with control unfed cultures. As reported by Gundlach *et al.,* (1992), Jasmonic acid and its ester, methyl jasmonate have been shown to positively effect secondary metabolite production in over 36 plant species. Sharan *et al.,* (1998) have reported that MJ along with fungal elicitor activated the enzyme phenyl ammonia lyase (PAL) and resulted in higher production of scopoletin and scopoline in tobacco cell cultures. Jasmonic acid and its derivatives have an integral role in the cascade of events that occur in the elicitation process leading to activation of genes of secondary metabolism (Gundlach *et al.,* 1992).

In case of studies in a 3L- bubble column bioreactor, MJ at a concentration of 40μM led to higher production of pigment betalaine (36.13 mg g^{-1} DW) which is 1.4 fold higher when compared to un-fed control (Fig 3.18). Although it was shown to reduce the biomass content it has not shown any immediate inhibition or necrosis of roots. It was also shown to enhance the metabolite contents. It is reported to enhance the production in hairy roots of *Catharathus roseus* (Rijhwani and Shanks 1998), hairy roots of *Brugmansia candida* (Spollansky *et al.,* 2000), hairy roots of *Tanacetum parthenium* L (Stojakowska *et al.,* 2002), *Datura stramonium* (Zabetaki *et al.,* 1999) and normal roots of *Hyoscymus muticus* (Singh *et al.,* 1998). Further studies would involve the use of MJ after exponential phase of growth that would probably lead to further increase in betalaine production with more biomass increasing the overall productivity. Thus the use of elicitors in bioreactors would be a valuable biotechnological strategy for enhanced product formation, reducing the costs of down stream processing and making the process an economically feasible one (Spollansky *et al.,* 2000).

4.1.7 Studies on release of pigments under the influence of Cetyl Trimethyl Ammonium Bromide (CTAB).

Recent work in our laboratory on the efflux of betalaines from hairy roots of *B. vulgaris* in shake flasks provided the basis for the selection of a suitable effluxing agent and the optimum concentration for better pigment release (Bhagyalakshmi *et al.,* 2002, Raju *et al.,* 2003 a,b). Among various surfactants (Tween-20, Triton X-100 and CTAB) studied, CTAB was found to be very effective in release of the pigments maintaining the viability of roots for further use (Bhagyalakshmi *et al.,* 2002).

CTAB at a concentration of 0.002% resulted in the gradual release of betalaine pigments from the hairy roots which reached a maximum of 17.3 mg L^{-1} after 48 hours of treatment (Fig. 3.20a). Moreover, the colour was stable which was evident from the hue values which have not shown immediate degradation unlike with other surfactants (Raju *et al.,* 2003 a). At higher concentration of CTAB, even though

the efflux was more, the roots were affected which were not able to revive growth when subcultured into fresh MS medium. Thus it was found that, with CTAB treatment at 0.002%, the roots could grow well when subcultured into fresh medium as shown in table 3.3. However, the pigment release at CTAB levels below 0.002% was also very less (Fig. 3.20a). The increase in 'a' (redness) and 'b' (yellowness) with incubation time with CTAB indicates that the color was stable and CTAB at 0.002 % has not affected the color (Fig 3.20 b).

CTAB being a surfactant would probably permeabilize the cell membrane, facilitating the release of betalaines in this case. Many researchers have worked on the release of pigments from various hairy root systems aiming for reuse of root cultures. In typical vacuolar pH conditions, betalaines exist in ionized state and would not diffuse out of the vacuole. But at lower pH, the zwitterionic nature permits diffusion and release from the vacuole (Mukundan *et al.,* 1998 b). The release of betalaines in the present study cannot be attributed to pH change as there was no change in pH after the addition of CTAB. Taya *et al.,* (1992) demonstrated the release of pigments from hairy roots of beet by continuous shaking for 48 hours in shake flasks and attributed the release to oxygen deprivation as oxygen starvation leads to acidification of vacuoles (Drew 1997), altering the membrane permeability finally leading to efflux of pigments. As oxygen starvation leads to the death of the tissue which cannot be reused, in the present study, efflux was carried out at normal oxygen supply rate (2 SLPM) which helped not only for preventing the browning of tissue but also for the viability of roots. DiIolrio *et al.,* (1993) attributed the release of pigments to heat treatment (42 ^{0}C for 45 min), which increased the membrane permeability. Pigment release was observed to be primarily from partially lysed and fragile pigment containing cells which were susceptible for oxygen starvation (Kino-Oka *et al.,* 1992). More recently Yang *et al.,* (2003) used low-level electric currents and voltages to extract betalaines from *B. vulgaris* cell suspension cultures. In their studies, using Electrophoretic Tubular Membrane Reactors (ETMR), they have found that oscillatory application of electric field led to more product formation retaining the viability of the cells. They have attributed the release of pigments to temporary and

reverse permeabilization of cell membrane caused by externally imposed electric field. The cells treated in ETMR with electric potential up to 10 V did not lose their viability.

Based on the results, it was suggested that CTAB at 0.002% concentration was optimum for better betalaine release without affecting the root cultures. This study provides an insight for the use of bioreactors for continuous process of product accumulation. Moreover, reuse of root cultures would provide additional benefit to the process.

4.1.8 Accumulation of DOPA and dopamine in hairy root cultures of *B. vulgaris*

L-Dihydroxyphenylalanine (L-DOPA) is an important precursor in betalaine biosynthesis. From the earlier studies it was evident that betalaine accumulation in hairy roots is growth associated. Thus it can be proposed that there would be a possibility of a reaction converting DOPA to betalaines over a period of time. In the present study, maximum pigment production was recorded as 24 mg/ g DW on day 20 of culture from an initial 7.5 mg/g DW recorded as on the day 5^{th} of culture. From figure 3.22 it is evident that, in the initial stages of growth, more DOPA than dopamine was accumulated which probably was converted to betalaines.

Fluorescence detector was adopted to detect very low concentration of metabolites in the culture and contents in the range of pico moles were resolved in the HPLC chromatogram (Fig. 3.23). There was more quantity of dopamine accumulation compared to DOPA in the present study which indicates the faster conversion of DOPA to further metabolites such as *cyclo* DOPA and betalamic acid. Alain (2001) reported the accumulation of various metabolites in the extracts of *Beta vulgaris* cell cultures *viz*., tyrosine, DOPA, dopamine, tyramine, *cyclo* DOPA glucoside (CDG) and betanine. Further, recent studies using beet seedlings fed with dopamine revealed the experimental proof for formation and occurrence of dopamine derived betacyanin (Kobayashi *et al.,* 2001). In their experiments with hairy root cultures of *Beta vulgaris*

(yellow beet), they found that the main betaxanthin (miraxanthin V) and the major betacyanin (2-descarboxy-betanidin) are dopamine derived; the occurrence of similar structures for the minor betacyanins was also suggested. The results were confirmed by feeding dopamine to beet seedlings and analyzing by LC-MS and NMR. It was hypothesized that the condensation step between 2-descarboxy-*cyclo*-Dopa and betalamic acid is the decisive reaction followed by glucosylation and acylation. Concerning dopamine, it could be speculated that most of this precursor in hairy root cultures of yellow beet has to be transported directly into the vacuole for the formation of the main betaxanthin, miraxanthin V, whereas a minor portion is oxidized by tyrosinase to 2-descarboxy-*cyclo*-Dopa, leading to the dopamine derived betacyanins (Fig 4.1) (Kobayashi *et al.,* 2001).

4.1.8.1 Influence of Tyrosine feeding on DOPA & dopamine accumulation in hairy root cultures of **B. vulgaris**

The function of tyrosine in forming DOPA as an end product, which occurs in various plant tissues (Teramoto and Komamine 1988), or as an intermediate metabolite, e.g. in the biosynthesis of benzylisoquinoline alkaloids (Rueffer and Zenk 1987), has been well reported. In the present study, feeding of tyrosine (1mM) to the hairy root cultures enhanced the production of DOPA and dopamine on day 8 and 11 of culture as 48.8µg g^{-1}DW and 1.62 mg g^{-1} DW, respectively (Fig 3.25). There was more quantity of dopamine rather than DOPA in the present studies indicating the faster conversion of DOPA to further metabolites such as *cyclo*-DOPA and betalamic acid.

Similar studies on feeding of amino acids to beet hairy roots have resulted in formation of corresponding betaxanthins (Schliemann *et al.,* 1999). There was neither aminoacid specificity nor a stereoselectivity observed in this process. They concluded that the decisive step i.e formation of betanidin by condensation of betalamic acid with amino acids (possibly including *cyclo*-Dopa or amines) is spontaneous which do not involve any enzymatic activity. This was proved by feeding *cyclo*-Dopa to beet seedlings, which resulted in the formation of betanidin. This was also confirmed by

feeding betalamic acid to seedlings of *Vicia faba* L. (which is known to accumulate high levels of DOPA, but does not synthesize betaxanthins) resulting in the formation of dopaxanthin.

Constabel and Haala (1968) observed higher phenol oxidase activities in betanin-producing callus cultures of *Beta vulgaris* var. crassa than in non-producingcultures. The effects of enzyme co-factors and inhibitors on betacyanin accumulation in callus cultures of *Portulaca grandiflora* (Endress 1979) suggested the involvement of a tyrosinase in betacyanin biosynthesis. This assumption has been supported by the detection of PPO transcripts which correlate with betacyanin accumulation in fruits of *Phytolacca americana* (Joy *et al.,* 1995) as well as by the description of a tyrosinase from the betalaine –accumulating pileus of *Amanita muscaria* (Mueller *et al.,* 1996). Steiner *et al.,* (1999) indicated that a specific tyrosinase is involved in the biosynthesis of betalaines and both tyrosinase reactions (hydroxylation and oxidation) are involved in the formation of a low-molecular weight plant product. However, presence of tyrosinase activity would not be the sole prerequiste for betalaine biosynthesis. The absence of betalaines despite the presence of tyrosinase activity in white petals of *P. grandiflora* could be explained by the lack of DOPA-dioxygenase. Mueller *et al.,* (1997) were successful with their experiments wherein the gene for DOPA-dioxygenase from *Amanita muscaria* was introduced into the white petals of *P. grandiflora* by particle bombardments, resulted in the formation of betalaines.

From the present study it is understood that the hairy roots could bioconvert externally fed tyrosine to DOPA and dopamine, even though it did not improve the betalaine content (Fig. 3.24, 3.25). This reduction in betalaine content can be attributed to growth inhibition by tyrosine which further led to lesser pigment productivity. It is also evident that the enzymes tyrosinase and DOPA decarboxylase are very active which were responsible for the biotransformations to occur.

4.1.8.2 Influence of caffeic acid and catechin on DOPA and dopamine accumulation in hairy root cultures of **B. vulgaris**

Based on the above studies it was proposed to carryout experiments by blocking the enzyme activities for enhanced DOPA and dopamine production. One of the approached would be to inhibit further conversion of DOPA and dopamine to their latter metabolites. In this context, it was proposed to use caffeic acid and catechin which are well known as oxygenase inhibitors (Catherine *et al.,* 1997, Kerry and Evans 1999) to the cultures. It is known from the previous studies that DOPA and dopamine accumulated to a maximum extent during the early stages of growth. Thus these inhibitors were added on the day 12 of culture. Both the compounds enhanced the DOPA and dopamine levels in hairy root cultures as evident from Fig. 3.27. However, caffeic acid enhanced the metabolite levels than that of catechin. Both the compounds inhibited the biomass growth. DOPA dioxygenase is a central enzyme of biogenesis of betalaine which converts DOPA to betalanic acid. The higher production of DOPA and dopamine under the influence of caffeic acid and catechin might be because of the inhibitory action of these chemicals on tyrosinase, preventing further conversions.

Till now there is no report on the isolation of DOPA dioxygenase from plant source. But this enzyme from mushroom *Amantita muscaria* has been isolated and tested for its activity on DOPA in model systems (Girod and Zryd 1991, Schliemann *et al.,* 1998). Mueller *et al.,* (1997) have proved that the recombinant enzyme DOPA dioxygenase has both 2, 3 and 4, 5- dioxygenase activities forming betalamic acid and muscaflavin, respectively. The higher dopamine production throughout the study than DOPA would be because of the decarboxylation reactions of tyrosine (after hydroxylation) or DOPA. Studies on dopamine derived betacyanins (Kobayashi *et al.,* 2001) and *in vitro* models using enzyme extracts of red beet cell cultures (Terradas 1989) suggest the action of DOPA decarboxylase strengthening the results of the present study.

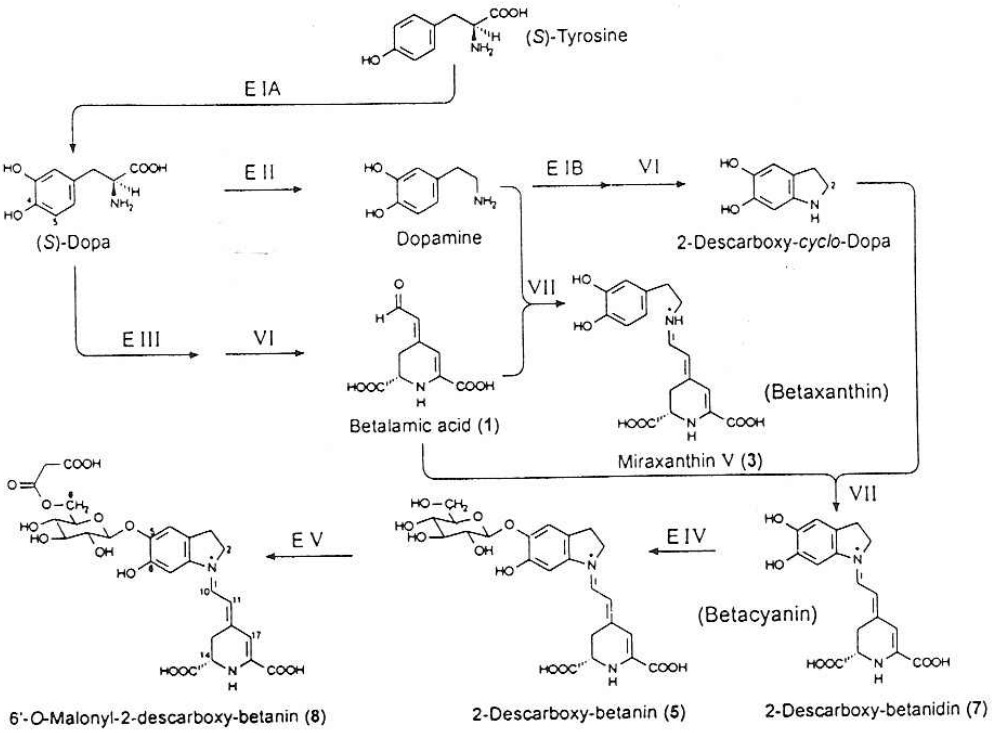


Fig 4.1: Proposed biosynthetic pathway to dopamine derived betalaines in *Beta vulgaris* hairy root culture.
Enzymatically catalyzed steps: E IA: hydroxylating activity of tyrosinase, E IB: Oxidizing activity of tyrosinase, E II: DOPA decarboxylase, E III: DOPA 4,5-dioxygenase, E IV: glucosyl transferase, E V: malonyl transferase.
Spontaneous reactions: VI: cyclization reaction, VII: condensation reaction (aldimine formation)

Further work would involve the studies on metabolic block of enzymes such as DOPA dioxygenase to produce more DOPA, which is an important pharmaceutical compound. One of the approaches would be to use anti-sense for this enzyme, which probably would lead to enhanced production of DOPA. DOPA dioxygenase can be used as a marker gene in the transformation of betalaine containing plants. Work is

also required to study the betanidin degrading enzymes, as ultimately the stability of betalaine is the key factor for their usage. The first substantial evidence for the enzymatic decolourisation of betalaines came from the studies with protein fractions from red beet (Soboleva *et al.,* 1976). A membrane–associated fraction degraded betalaines in an oxygen-dependent reaction; its inhibition by metal chelators suggested the involvement of a Polyphenol Oxidase (PPO). On the other hand, cell wall fractions from red beet catalyzed the H_2O_2-stimulated decolourisation of betanin suggesting the participation of a peroxidase (Wasserman and Guilfoy 1983, 1984). It was shown that the betalaine decolorizing enzyme activity of callus cultures of *Portulaca grandiflora* was inhibited by ascorbic acid, which blocks the activity of PPOs, as well as the β-glucosidase inhibitor β-gluconolactone. Thus it was proposed that a hitherto uncharacterized betacyanin decolorizing enzyme is composed of a betalanine hydrolyzing β-glucosidase and a PPO.

The results throw light on understanding the betalaine biosynthetic pathway with special reference to methodology for over production of DOPA and dopamine by precursor feeding and use of enzyme inhibitors. The hairy root cultures of *B. vulgaris* can thus be exploited for the production of other important compounds in addition to betalaines.

4.2 DISCUSSION FOR THE STUDIES USING *C. FRUTESCENS* NORMAL ROOT SYSTEM

4.2.1 Growth and phenylpropanoid accumulation in normal root cultures

4.2.2 Biotransformation studies using normal roots of *C. frutescens*

4.2.2.1 Influence of feeding ferulic acid on vanillin related metabolite accumulation

4.2.2.2 Biotransformation of protocatechuic aldehyde to vanillin related compounds

4.2.2.3 Biotransformation of caffeic acid to vanillin related metabolites

4.2.2.4 Biotransformation of veratraldehyde to vanillin related compounds

In the present study, normal roots of *C. frutescens* have been used to check their ability to bioconvert externally supplied phenylpropanoid precursors to vanillin and related metabolites. Earlier studies on *C. frutescens* cell cultures have shown that they have the necessary enzymatic machinery to perform certain biotransformation reactions such as oxidation, reduction, methylation and demethylation (Ramachandra Rao 1998). Attempts were made to use bioreactors for biotransformations and also to study the enzyme activities to understand the mechanism of biotransformation. The results have been discussed here in detail.

4.2.1 Growth and phenylpropanoid accumulation in normal root cultures:

The normal root cultures of *C. frutescens* grown in MS medium with 0.5 mg L^{-1} NAA have shown maximum growth of 6.5 g FW 40 mL^{-1}. The roots started forming callus like structures after 20 days of culture, as reported by Chavez-Moctezuma and Lozoya-Gloria (1996) (Fig 3.28). The callusing nature increased with culture time. Even in cultures grown in MS basal medium the callusing was observed indicating that this is not because of the externally added NAA. However, the root growth in MS basal medium was not proper. Fresh root cultures obtained from seedlings were used for all the experiments.

It was found that phenylpropanoid intermediates such as cinnamic acid, caffeic acid, coumaric acid and ferulic acid along with vanillylamine accumulated in root cultures (Fig 3.30). From this observation, one might infer that the respective enzymes of phenyl propanoid pathway are metabolically active. The non-existance of capsaicin in root cultures could be because of lack of capsaicin synthase activity in root cultures, which performs the condensation reaction between vanillylamine and 8-methylnonenoic acid (Sukrasno and Yeoman 1993). These observations are in accordance with those reported by Ishikawa *et al.,* (1998) in *C. annum* root cultures. The lower concentrations of these phenylpropanoids and other metabolites in normal roots might be because of their rapid conversion to other metabolites – e.g. lignin-like substances- as reported by Sukrasno and Yeoman (1993) in the case of *C. frutescens* fruits.

4.2.2 Biotransformation studies using normal roots of *C. frutescens*

4.2.2.1 Influence of feeding ferulic acid on vanillin related metabolite accumulation

Ferulic acid, which is the nearest precursor to vanillin, when fed at concentrations of 1 and 2 mM led to the accumulation of vanillin related metabolites, vanillin being the major one. In cultures treated with 1 and 2 mM ferulic acid, maximum vanillin accumulation of 1.88 mg L^{-1} and 2.5 mg L^{-1} was observed, on day 6 after precursor addition, respectively (Fig. 3.33, 3.34). From these observations it is understood that ferulic acid, at higher concentrations of precursor addition (2 mM), led to more biotransformed product accumulation. But when the efficiency of bioconversion is taken into account, only less quantity of ferulic acid contributed to the formation of vanillin related metabolites. The percentage conversion was low, amounting to 5-7% of total ferulic acid fed in all the studies. This could be because of the utilization of ferulic acid in a different biosynthetic pathway, thereby limiting its role in the production of flavour metabolites. Yeoman *et al.,* (1990) reported variation in vanillin accumulation in cell lines derived from a single plant of *C. frutescens* fed with ferulic acid, which they attributed to different patterns of enzyme activity. Sukrasno and Yeoman (1993) suggested that the lower capsaicin levels in cell suspension cultures could be due to the rapid bioconversion of phenolic metabolites to lignin. In their experiments with *Capsicum* fruits, they found that the production of capsaicinoids takes place after the increase in fruit size ceases. Moreover, the onset of capsaicinoid accumulation is paralleled by the disappearance of the three cinnamoyl glycosides and two flavonoids, together with the active accumulation of lignin-like substances. In the present study, no capsaicin production was observed in roots, even after precursor addition. But the production of various vanilla flavour metabolites in precursor fed cultures, but not in the unfed cultures, is an interesting observation.

Feeding of ferulic acid (2 mM) and β-cyclodextrin (BCD) complex (1:1) enhanced the accumulation of biotransformed products. Moreover, vanillin

accumulation was recorded as 3.75 mg L^{-1} on day 6 after precursor addition, which was 1.5 times higher than in cultures fed with ferulic acid (2 mM) alone (Fig. 3.35). Ferulic acid being sparingly soluble in water was made more available to the root culture upon complexation with BCD resulting in higher production of metabolites. Similarly, using β -cyclodextrin complexation, Ramachandra Rao *et al.,* (2002) have reported higher biotransformation rates of digitoxin to digoxin, as well as isoeugenol and ferulic acid to vanillin related compounds in cell cultures of *C. frutescens*. Cyclodextrins not only increase the solubility of the lipophilic substrates but also significantly alleviate their inhibitory influence (Bar, 1989). However, there was no improvement of growth observed in the present study after complexation of ferulic acid with BCD.

Ferulic acid being a cheaper compound could be a better substrate for the bioconversion reactions to form vanillin. Not only higher plant cell cultures, but also algal cultures such as *Spirulina platensis* and *Haematococcus pluvialis* were able to bioconvert externally fed ferulic acid to vanillin and other related metabolites (Ramachandra Rao 1998, Usha *et al.,* 2002). Falconnier *et al.,* (1994) reported the biotransformation of ferulic acid to vanillin, vanillic acid and vanillyl alcohol by the white rot fungus *Pycnoporus cinnabarinus*, which also showed oxidative decarboxylation of vanillic acid to 2-methoxyhydroquinone. Labuda *et al.,* (1993) reported that *Corynebacterium glutamicium* produced a mixture of vanillin and vanillic acid from ferulic acid. Lesage-Meessen *et al.,* (1996) showed a two-step bioconversion process for the production of vanillin from ferulic acid. In the first step, *Aspergillus niger* converted ferulic acid to vanillic acid. In the second step, vanillic acid was reduced to vanillin by *Pycnoporous cinnabarinus*.

Yeoman *et al.,* (1990) showed that, different cell lines of *C. frutescens* derived from a single plant behave differently and observed that cell line 122 accumulated vanillin and not capsaicin when treated with ferulic acid , while cell line 121 accumulated more p-coumaric acid and capsaicin than vanillin. They have concluded

that the differences in the production of vanillin or capsaicin depend on the pattern of enzymatic activity. Johnson *et al.,* (1996) observed that when *C. frutescens* callus cultures were fed with ferulic acid, there was an increased capsaicin production along with vanillin accumulation. In this case also a very small amount of ferulic acid was converted to vanillin (0.304 mg L^{-1} vanillin from 2.5 mM ferulic acid after 15 days of culture), which further supports the channeling of precursors into other biosynthetic pathways. It would be logical to attribute the distribution of externally fed precursors into two related pathways, *viz*. the capsaicinoid pathway and the phenylpropanoid pathway. Knuth and Sahai (1991) have used cultured cells of *Vanilla planifolia* for the production of vanillin which is a direct approach for the production of vanillin. More recently, Podstolski *et al.,* (2002) reported the existence of vanillin precursors such as 4-hydroxybenzaldehyde in addition to vanillin in cell cultures of *Vanilla planifolia*.

When ferulic acid was fed along with β-cyclodextrin (1:1) to cultures growing in a three-liter bubble column bioreactor (working volume of 1.75 L), maximum vanillin production of 1.63 mg L^{-1} was obtained; other vanillin related metabolites were also formed after 9 days of precursor addition (Fig. 3.33). From the table 3.3 it is clear that the roots in bioreactor after harvesting had very less amount of the flavour compounds. The main advantage of the use of bioreactors may be in cultures which secrete secondary metabolites into the medium. In the present case most of the biotransformed products were found in the medium rather than in cells. Thus it would be appropriate to use a bioreactor wherein the medium can be taken out and extracted for products of interest. In this regard, use of compounds which would permeabilize the cells also can be useful but keeping in mind the viability of root cultures. Recently, Stentelaire *et al.,* (2000) have reported the production of vanillin from vanillic acid using a fungus *Pycnoporus cinnabarinus* in mechanically agitated and air-lift bioreactors. They have found that vanillin production was high in mechanically agitated bioreactor i.e 1.26 g L^{-1}. However, vanillic acid bioconversion to vanillin occurred under the conditions of reduced dissolved oxygen concentration, gentle agitation, high carbon dioxide production and low specific growth rate. Addition of XAD-2 resin to the culture medium helped in limiting the toxicity of vanillin towards the fungal biomass as

well as the formation of unwanted by-products such as methoxyhydroquinone and allowed the concentration of vanillin produced to reach 1.5 g L^{-1}. There were no significant differences in efficiencies of bioconversion rates in shake flasks, when compared to bioreactors. However, an improved design of bioreactor specially for this system would help in better biotransformation process for continuous operations.

The first part of the reaction sequence in capsaicin biosynthesis comprising the aromatic pathway is shared with other pathways of general phenylpropanoid metabolism and is common to all higher plants. This leads to the formation of a wide range of phenolic compounds, including cinnamates, benzoates, flavonoids, coumarins, tannins, saponifiable cell wall phenolics, and lignin-like substances (Stafford 1974, Fry 1982) . However, the latter part of the reaction sequence from ferulic acid through vanillin and vanillylamine to capsaicin is only found in fruits and cell cultures of *Capsicum frutescens* synthesizing capsaicinoids.

The formation of vanillin related metabolites involves several reactions. The formation of vanillin from ferulic acid might be via feruloyl-CoA through a reaction similar to □-oxidation of fatty acids (Funk and Brodelius, 1990 a). Vanillic acid was formed by further oxidation of vanillin, whereas reduction yielded vanillyl alcohol. The demethylation of ferulic acid led to the formation of p- coumaric acid, which further underwent □-oxidation to form p-hydroxybenzoic acid. Funk and Brodelius (1990a) have reported that demethylation of 4-methoxycinnamic acid led to formation of p-coumaric acid, which was then converted to p-hydroxybenzoicacid by □-oxidation. The probable biotransformation pathway of ferulic acid to different vanillin related metabolites is shown in Fig. 4.2.

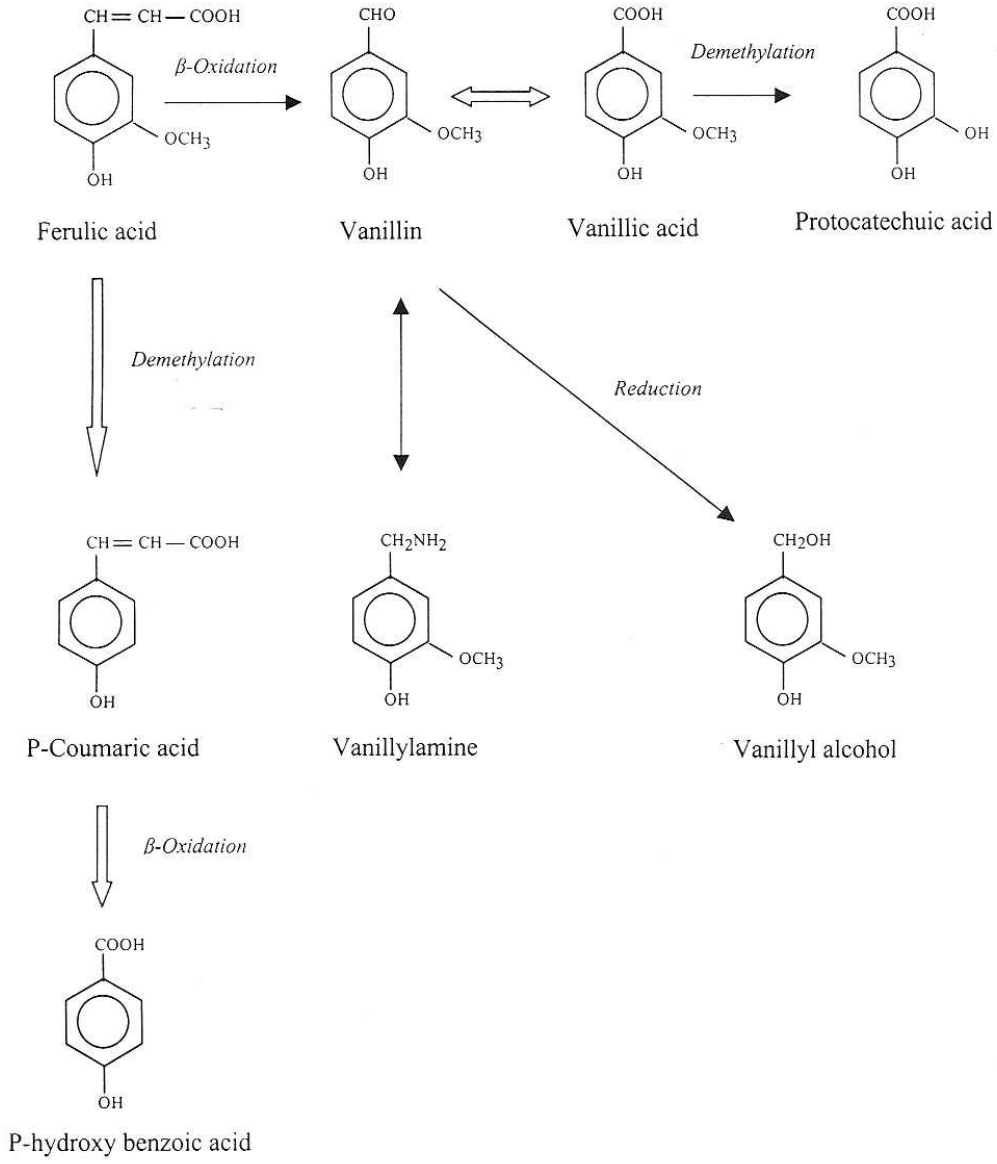


Fig 4.2: Probable biosynthetic pathway of biotransformation of ferulic acid to vanillin related metabolites in *C. frutescens* normal roots

4.2.2.2. Biotransformation of protocatechuic aldehyde to vanillin related compounds

Root cultures could biotransform externally added protocatechuic aldehyde wherein a maximum vanillin production of 1.2 mg L^{-1} was recorded on day 6 after precursor addition (Fig. 3.38). But in this case, the cultured roots also have shown significant contents ranging from 30-50% of the total production. The lesser bioconversion rate (3.2 %) in the protocatechuic aldehyde fed cultures could be attributed to the same reason of diversion of pathway similar to ferulic acid fed cultures. Ramachandra Rao (1998) reported the biotransformation of protocatechuic aldehyde in free and immobilized cells of *Capsicum frutescens*. It was shown that feeding protocatechuic aldehyde apart from increasing the vanillin content also helped in higher production of capsaicin. The formation of vanillin from protocatechuic aldehyde involved *O*-methylation reaction and oxidation to produce vanillin and protocatechuic acid respectively (Fig 4.3).

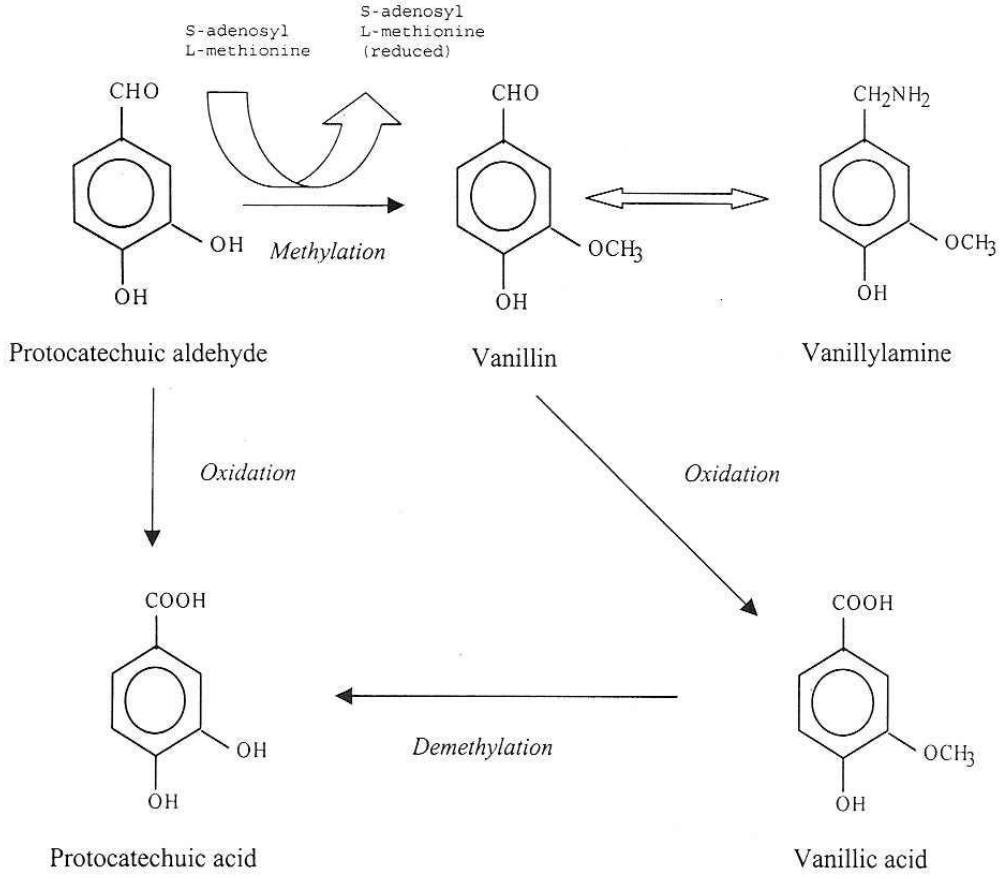


Fig 4.3: Probable biosynthetic pathway of biotransformation of protocatechuic aldehyde to vanillin related metabolites in *C. frutescens* normal roots

4.2.2.3. Biotransformation of caffeic acid to vanillin related metabolites

Caffeic acid at a concentration of 1.5 mM has shown some inhibitory effect on biomass growth which is evident from the Fig. 3.39. Caffeic acid being a phenolic compound might have led to the lesser biomass accumulation. Caffeic acid at further higher concentrations severely affected root growth and the roots turned black. However, roots could successfully biotransform caffeic acid to various vanilla flavour metabolites (Fig. 3.41). In the initial days of culture, ferulic acid was formed in

greater amounts than vanillin. After 24 hours of precursor feeding the contents of ferulic acid and vanillin were recorded as 685 and 467.5 mg L^{-1}, respectively. With increase in incubation time the ferulic acid content gradually decreased, and there was an increase in vanillin production. Similarly Ramachandra Rao (1998) reported the accumulation of vanillin in free and immobilized cells of *C. frutescens* wherein caffeic acid influenced the formation of capsaicin rather than vanillin. When MJ was added along with caffeic acid the rate of biotransformation increased, leading to higher production of vanilla flavour products (Fig. 3.42).

To study the influence of MJ on the efficiency of biotransformation, experiments were carried out to measure the specific activity of CAOMT which catalyses the conversion of caffeic acid to ferulic acid by transferring a methyl group from S-adenosyl-L-methionine to the 3-hydroxyl group. The sp. Activity of CAOMT has shown an increase as shown in Fig. 3.43. This increase in activity can be correlated to the increased ferulic acid accumulation in the initial stages after precursor addition which later led to more production of vanillin accumulation by oxidation reactions. Sharan *et al.,* (1998) reported MJ mediated elicitation of Phenylalanine ammonia lyase activity in cell cultures of tobacco which in turn enhanced the accumulation of scopoletin and scopolin. They have observed a transient increase in accumulation of PAL mRNA when MJ was fed to the cultures. It is understood from these findings that MJ treatment results in activating various enzymes of biosynthetic pathways. Ochoa-Alezo and Gomez-Peralta (1993) have reported a 6.3 fold higher activity of CAOMT in fruit tissue compared to callus cultures. Xue and Brodelius (1998) have reported increased CAOMT activity when suspension cultures of *V. planifolia* were treated with kinetin. Two CAOMTs *viz*. 3-*O*-CAOMT which is involved in biosynthesis of lignin precursors and 4-*O*-CAOMT which is involved in formation of vanillic acid from isoferulic acid were studied wherein kinetin induced the activity of 4-*O*-CAOMT by 100 fold to that of 3-*O*-CAOMT which ultimately led to the production of vanillic acid. Xue and Brodelius (1998) have even isolated the cDNAs of CAOMT from kinetin induced cell cultures of *Vanilla*.

Some enzymes of this type (CAOMT) also catalyse the methylation of 5-hydroxyferulic acid to sinapic acid. These cinnamic acids are intermediates of the phenyl propanoid pathway. Ferulic acid and sinapic acids are further metabolized to lignin precursors via a pathway involving 4-hydroxycinnamoyl:CoA ligase (4CL), cinnanoyl-CoA reductase and coniferyl alcohol dehydrogenase. Lignin biosynthesis plays an important role in cell wall construction during regular growth and differentiation in plants, as well as in the response of plants to stress such as elicitor stimuli and pathogen attack. Therefore 3-CAOMTs from many plants have been studied (Dumas *et al.,* 1988, Funk and Brodelius 1992, van Doorsselaere *et al.,* 1993). The relevance of 3-CAOMTs in the plant defense response has also been investigated and it was shown that expression of this enzyme is stimulated transiently by elicitor treatment.

In the present study, ferulic acid formation was higher in the early stages after caffeic acid feeding as reflected by *O*-methylation reaction and when MJ was fed along with Caffeic acid, it probably enhanced the CAOMT activity leading to increased biotransformed product. Further, ferulic acid upon oxidation probably led to vanillin production along with other metabolites (Fig. 4.4). Ramachandra Rao (1998) has reported more vanillin production when SAM was added along with protocatechiuc aldehyde and attributed the increase to CAOMT activity. Xue and Brodelius (1998) were of the opinion that 3-*O*-CAOMTs express some 4-*O*-CAOMT activity and vanilla CAOMTs show high homology to CAOMTs from other plants such as tobacco and chilies. Ramachandra Rao (1998) has reported more production of capsaicin than vanillin when caffeic acid was fed to *Capsicum* callus cultures. But in the present study with *Capsicum* roots not producing capsaicin, the addition of caffeic acid resulted in more vanillin production. However, the formation of vanillin and other flavor compounds was less in this case also, indicating the possible channeling of caffeic acid to other biosynthetic pathways such as lignin.

4.2.2.4. Biotransformation of veratraldehyde to vanillin related compounds

Veratraldehyde even though inhibited the growth of roots (Fig. 3.36) was bioconverted to vanillin related metabolites. Maximum vanillin production was recorded on day 6 of precursor addition as 11.875 mg L^{-1} (Fig 3.45). Among all the precursors studied for the biotransformations, *Capsicum* root cultures could biotransform veratraldehyde very well leading to the highest production of vanillin than any other precursors. However, the best conversion of ferulic acid to vanillin was reported in *Corynebacterium* which produced 76 mg L^{-1} in the presence of $_{DL}$-dithiothreitol. Veratraldehyde has structural similarity with vanillin, moreover, *Capsicum* cultures are known to perform the demethylation reactions very well (Ramachandra Rao 1998). This could be the reason for the higher bioconversion rates. The results of the present study are in accordance with the studies by Ramachandra Rao (1998) wherein biotransformation of veratraldehyde to different vanilla flavour metabolites was achieved using *Capsicum* callus cultures as well as alga-*Spirulina*

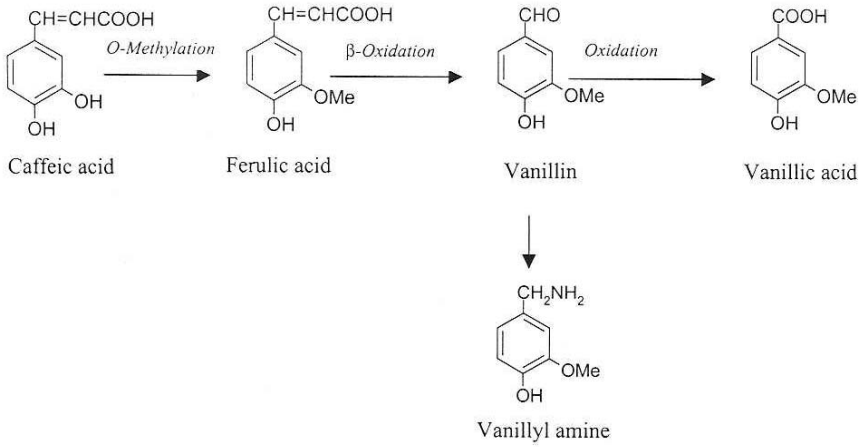


Fig 4.4: Probable biosynthetic pathway of biotransformation of caffeic acid to vanillin related metabolites in *C. frutescens* normal roots

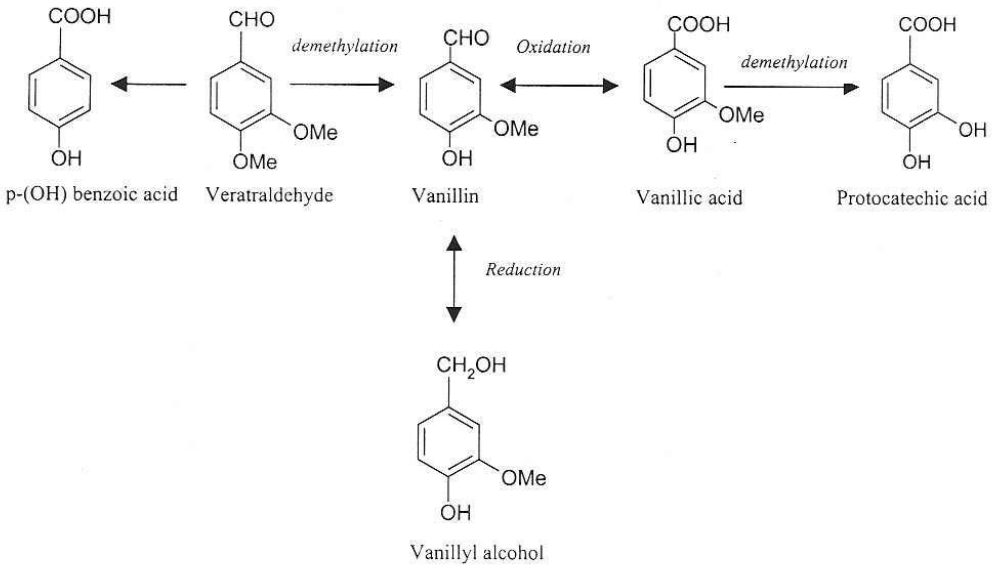


Fig 4.5: Probable biosynthetic pathway of biotransformation of veratraldehyde to vanillin related metabolites in *C. frutescens* normal roots

platensis cultures. The probable biosynthetic pathway of veratraldehyde biotransformation is shown in Fig 4.5, wherein the precursor veratraldehyde was demethylated to vanillin and parahydroxy benzoic acid. Vanillin upon oxidation converted to vanillic acid, which in turn was demethylated to protocatechuic acid. The formation of vanillyl alcohol was by reduction of vanillin.

In all the studies, it was found that root cultures without precursors and autoclaved roots with precursors have not shown any accumulation of vanillin related metabolites. It clearly shows that the bioconversions were mediated by the enzymatic machinery in the root cells and not by any other factors such as temperature, pH and light. Further work would involve studies on biosynthetic pathways wherein the precursors are diverted, resulting in accumulation of other compounds such as lignin. Since the root cultures do not produce capsaicin, the phenylpropanoid precursors may be channeled to vanilla flavour metabolite pathways by inhibiting lignin biosynthesis.

SUMMARY

Secondary metabolites from plant cell cultures have found great importance in many ways ranging from food to medicine (Ravishankar and Venkataraman 1990). But the disadvantages of cell cultures such as slower growth and instability forced the researchers to explore newer systems with high growth as well as production capabilities. In this regard, hairy root cultures, which offer various advantages such as faster growth and stable metabolite production have gained importance as culture systems for the production of secondary metabolites (Toivenon 1993). They are more amenable for scale-up in bioreactor than cell cultures for the production of high value secondary metabolites. However, the selection and design of bioreactors for a particular system depends on mass transfer. The liquid dispersed bioreactors have problems of mass transfer which can be overcome by the use of acoustic mist bioreactors wherein the hairy roots are in constant touch with the gas phase (Weathers *et al.,* 1989). Various methods for enhancement of metabolites have been tried in hairy root cultures including use of fungal elicitors (Bais *et al.,* 2000a), polyamines (Bais *et al.,* 2000b) and Methyl jasmonate (Sharan *et al.,* 1998, Rijhwani and Shanks 1998). Most of the work has been limited to shake flask experiments only. Once the metabolite is accumulated, it is very important to extract the product out into the medium. Many compounds such as surfactants (Bhagyalakshmi *et al.,* 2003), oxygen stress (Kino-Oka *et al.,* 1998), heat (DiIorio *et al.,* 1993), pH (Mukundan *et al.,* 1998b, Raju *et al.,* 2003a) have been used for this purpose. In most of the cases the viability of hairy roots was affected upon metabolite release, hence were not able to grow when subcultured into a fresh medium.

Biotransformation is an emerging field of biotechnology and encompasses both enzymatic and plant/microbial biocatalysis. The production of high value food metabolites, fine chemicals and pharmaceuticals can be achieved by biotransformations using biological catalysts in the form of enzymes and whole cells. From an industrial point of view, biotransformations performed by plant cell culture systems can be desirable when a given reaction is unique to plant cells and the product of the reaction has a high value. Vanillin (4-hydroxy-3-methoxy

benzaldehyde) a very well known flavour compound is an important intermediate of phenylpropanoid pathway. Exogenous application of ferulic acid to suspension cultures of *Vanilla planifolia* resulted in the formation of coniferyl aldehyde, along with traces of vanillylamine as reported by Funk and Brodelius (1990 a, b). Biotransformation studies of phenyl propanoid intermediates such as ferulic acid, vanillylamine, protocatechuic aldehyde were reported with *Capsicum* free as well as immobilized cultures (Johnson et al 1996, Ramachandra Rao 1998) as well as with algae like *Spirulina* (Ramachandra Rao 1998) and *Haematococcus* (Tripathi et al 2001). Certain fungal elicitors and chelating agents like β-cyclodextrin were used to enhance the biotransformation rates for enhanced production of vanilla flavour compounds (Ramachandra Rao 1998). Jasmonic acid and its methyl ester, methyl jasmonate have also been studied widely as elicitors of secondary metabolites particularly with hairy roots (Rijhwani and Shanks 1998, Spollansky *et al.,* 2000, Stojakowska *et al.,* 2002, Zabetaki *et al.,* 1999) as well as normal roots (Singh *et al.,* 1998).

In this investigation, studies were focused on two culture systems; *Beta vulgaris* hairy roots for the production of betalaines and *Capsicum frutescens* root cultures for biotransformation of phenylpropanoid intermediates to vanillin related metabolites. The specific objectives of the thesis are given below.

Using *Beta vulgaris* hairy roots it was proposed:

1. To study the growth and betalaine production in *B. vulgaris* hairy roots cultured in shake flasks.
2. To develop a bioreactor system suitable for hairy root cultures of *B. vulgaris* such as bubble column, nutrient sprinkle and acoustic mist. The osmolarity and conductivity studies performed with shake flasks to be corroborated for cultures in bioreactors for biomass estimation.
3. To study the influence of polyamines and methyl jasmonate on growth and betalaine production of hairy roots in bioreactors and also studies on down stream processing of betalaines using surfactants such as CTAB.

4. To study the production of DOPA and dopamine in hairy root cultures and to optimize conditions such as precursor addition (tyrosine) and use of oxygenase inhibitors (caffeic acid and catechin) for over production of these pharmaceutically important compounds.

Using *Capsicum frutescens* normal roots it was proposed:

1. To study the biotransformation capability of normal roots to biotransform using externally fed phenylpropanoid intermediates- ferulicacid, caffeic acid, protocatechuic aldehyde and veratraldehyde to produce vanilla flavour compounds.
2. To use various compounds such as β-cyclodextrin and methyl jasmonate for enhancement of biotransformation rates.
3. To scale-up the developed biotransformation process, especially the use of a bioreactor for this purpose
4. To correlate the biotransformation capability with enzyme activities such as caffeic acid-*O*-methyl transferase (CAOMT) in normal roots of *C. frutescens*.

The highlights of the results

***Beta vulgaris* hairy root system as production systems of betalaines**

***Establishment and confirmation of hairy root cultures of* B. vulgaris**

Hairy root cultures of *B. vulgaris* were obtained upon infection with *Agrobacterium rhizogenes*. Among various strains tried for this purpose such as LMG 150 and A_4, strain ATCC 15834 resulted in high frequency of hairy root initiation and their subsequent growth (Fig 3.1). The transgenic nature of the hairy roots was confirmed by PCR and Southern blot analysis (Fig. 3.3 a,b). The hairy root clones showed the presence of *rol* A gene on PCR amplification. For all further experiments of the present thesis, the hairy roots obtained from strain ATCC 15834 were used.

Growth and betalaine production studies of hairy roots in shake flasks

The hairy roots showed faster growth and stable metabolite production. Maximum biomass of 14.5 g DW L^{-1} was obtained on the day 24 of culture from an

initial inoculum of 0.3 g DW L^{-1}. Similarly, betalaine content has also shown an increase with culture time, which reached a maximum value of 160 mg L^{-1} on the day 24 of culture (Fig 3.4). Biomass and betalaine values showed a decrease after 24 days, which might probably because of the necrosis of the roots.

Estimation of biomass using medium conductivity and osmolarity

Hairy root biomass growth is accompanied by a decrease in the medium conductivity and osmolarity, which gradually decreased with culture time (Fig. 3.5a, 3.7a). The decrease can be attributed to the nutrient uptake by the growing hairy roots from the medium. Osmolarity method offers more advantages than conductivity as it depends on the number of moles of total solutes rather than ions as in the case of conductivity. Attempts were made to correlate the increase in biomass (ΔX) with decrease in conductivity and osmolarity (ΔC and ΔO). However, this type of correlation led to errors as the initial values (X_0 and C_0 or O_0) were involved in calculation (Fig 3.6a). To overcome these limitations, a much simpler and more reliable relationship in y=mX+C form was used (Fig. 3.6 b, 3.8b). The m and C values in case of conductivity studies were found to be –7.64 and 41.29, respectively. The m and C values in case of osmolarity studies were found to be -0.215 and 49.234, respectively. These values were used further to calculate the biomass values in bioreactor systems and compared with the experimental values.

Bioreactor systems for *Beta vulgaris* hairy roots

Three different configurations of bioreactors *viz*. bubble column, nutrient sprinkle and acoustic mist were used for culture of hairy roots (Fig. 3.9, 3.13 and 3.14). Hairy roots cultivated in acoustic mist reactor showed more biomass as well as specific growth rate when compared with that of bubble column and nutrient sprinkle reactor (Fig 3.10, Table 3.2). The betalaine content was high (31.9 mg/g dry weight) in case of acoustic mist reactor and also in bubble column reactor (31.36 mg/g DW) (Fig. 3.12). However, acoustic mist reactor was more effective since the maximum production of betalaines was achieved in 15 days when compared to bubble column and nutrient sprinkle, which took 24 days.

The osmolarity and conductivity values were well correlated with biomass production. The predicted values of biomass obtained from osmolarity measurements were more or less similar to that of experimental (Fig. 3.15).

These results are very much useful for understanding the best reactor configuration for *B. vulgaris* hairy root system for scale-up of hairy roots for the production of betalaines.

Studies on improvement of growth and metabolite production in bioreactors

The present study has been focused on the betalaines production in 3L bubble column bioreactor where the growth pattern and betalaines synthesis under the influence polyamines. A combination of spermidine and putrescine fed to the roots each at levels of 0.75 mM efficiently increased the growth and pigment production resulting in 1.23 fold higher growth (39.2 g FW L^{-1}) and 1.27 fold higher betalaine content (32.9 mg g^{-1} DW) than control (Fig. 3.16).

To standardize the MJ concentration for enhanced betalaine production in bioreactors, pilot experiments were carried out in shake flasks with varied concentrations of MJ. The biomass accumulation was inhibited with an increase in the concentration of MJ. Maximum biomass (6.3 g FW/40 ml culture medium after 20 days) was recorded in control cultures without MJ which was 1.05, 1.1, 1.17, 1.54, and 1.61 fold higher than 10, 20, 40, 70 and 100 µM methyl jasmonate fed cultures respectively (Fig 3.17a). At levels beyond 100 µM the growth was severely affected. Contrarily, the MJ treatments enhanced betalaine formation to extents significantly higher than control cultures (Figure 3.17 b). Among various concentrations studied, 40 µM MJ resulted in maximum betalaines content of 32.36 mg g^{-1} DW on 20th day of culture which was 1.35 fold higher than control cultures on the same day. The overall productivity of betalaines (mg L^{-1}) was higher in 40 µM MJ fed cultures.

Based on shake flask results, it was proposed to carry out further experiments in a 3L- bubble column bioreactor with MJ at 40 µM concentration. The final

biomass concentration in MJ treated cultures was 28.17 g FW L^{-1} on day 18 of culture. The betalaine content was recorded as 36.13 mg g^{-1} DW which was 1.4 fold higher than control (Fig 3.18).

Pigment release under the influence of Cetyl Trimethyl Ammonium Bromide (CTAB).

Based on shake flask data it was proposed to study the effect of CTAB on pigment efflux in *B. vulgaris* hairy roots in a 3L- bubble column bioreactor. At a concentration of 0.002 % the betalaine release was steady with an increase of incubation time. Maximum betalaine content effluxed was observed at 48th hour, which was 17.3 mg L^{-1}. It was found that the lightness "l" values reduced from 91 to 70 recorded on 12 and 48 hours respectively, after CTAB (0.002%) addition. However, the redness and yellowness 'a' and 'b' values increased with incubation time in CTAB effluxed pigments (Fig 3.20b).

Further, the roots from the bioreactor after 48 hours of treatment with CTAB (0.002%) when subcultured into fresh MS medium showed normal growth and metabolite production indicating that they were physiologically active and viable (Table 3.3).

Studies on the accumulation of L-dihydroxyphenylalanine (DOPA) and dopamine in hairy root cultures of B. vulgaris

Beta vulgaris hairy root cultures were extracted for the accumulation of L- DOPA and dopamine at different phases of growth. Maximum DOPA and dopamine accumulations were recorded as 30 µg and 0.975 mg g^{-1}DW on 5th and 15th days of culture time, respectively (Fig 3.22). A fluorescence detector was used in HPLC analysis which enables to detect compounds at very low quantities (Fig. 3.23). Tyrosine which is a major precursor in DOPA mediated betalaine biosynthesis, was fed at a concentration of 1mM. Maximum DOPA and dopamine accumulation was recorded on day-8 and 11 of culture as 48.8 µg and 1.62 mg g^{-1}DW, respectively (Fig. 3.25). However, biomass and betalaine contents showed a decrease compared to

unfed control cultures. DOPA and dopamine levels increased following the addition of oxygenase inhibitors - caffeic acid and catechin at a concentration of 50μM. Maximum DOPA content was recorded on 18th day of culture as 50 and 41.7 μg/g DW in cultures fed with caffeic acid and catechin, respectively. Dopamine content reached a maximum on 16th day of culture as 1.9 and 1.67 mg/g DW in caffeic acid and catechin fed cultures, respectively (Fig. 3.27).

II. *Capsicum frutescens* normal root cultures for biotransformations

Growth and phenylpropanoid metabolism in normal roots of C. frutescens

The roots were pale colored and succulent initially and with subsequent increase of culture time turned into light brown colour and formed callus (Fig. 3.28). The normal roots of *C. frutescens* cultured in MS medium with 0.5 mg L^{-1} NAA have shown maximum biomass of 6.5 g FW 40 mL^{-1} on the day-20 of culture (Fig 3.29). HPLC analysis of normal root extracts revealed the presence of phenylpropanoid intermediates such as cinnamic acid, caffeic acid, coumaric acid and ferulic acid along with vanillylamine; caffeic acid being the major one (Fig 3.30). However, there was no capsaicin accumulation in normal roots.

Biotransformation of ferulic acid to vanillin related compounds

Ferulic acid, which is the nearest precursor to vanillin, when fed at concentrations of 1 and 2 mM led to the accumulation of vanillin related metabolites, vanillin being the major one. In cultures treated with 1 and 2 mM ferulic acid, maximum vanillin accumulation of 1.88 mg and 2.5 mg L^{-1} was observed, on day 6 after precursor addition, respectively (Fig. 3.33, 3.34). Feeding of ferulic acid (2 mM) and β-cyclodextrin complex (1:1) enhanced the accumulation of biotransformed products. Moreover, vanillin accumulation was recorded as 3.754 mg L^{-1} on day 6 after precursor addition, which was 1.5 times higher than in cultures fed with ferulic acid (2 mM) alone (Fig. 3.35). When ferulic acid was fed along with β-cyclodextrin (1:1) to cultures growing in a three-liter bubble column bioreactor the maximum vanillin production of 1.63 mg L^{-1} was obtained; other vanilla flavour metabolites were also formed after 9 days of precursor addition (Fig. 3.36).

Biotransformation of protocatechuic aldehyde to vanillin related metabolites

Root cultures could also biotransform protocatechuic aldehyde wherein maximum vanillin (1.2 mg L^{-1}), vanillylamine (0.5 mg L^{-1}), and protocatechuic acid (0.57 mg L^{-1}) accumulation was recorded on the day 6 after precursor addition (Figure 3.38a). However, in this case, 30-50% of total metabolite content was accumulated in the roots (Fig. 3.38b).

Biotransformation of caffeic acid to vanillin related metabolites

Ferulic acid and vanillin and started accumulating after 24 hrs of caffeic acid addition which was recorded as 685 and 467.5 µg L^{-1}, respectively. Maximum vanillin accumulation was recorded as 2.097 mg L^{-1} on 4^{th} day of addition. Other vanilla flavour metabolites *viz*. vanillic acid and vanillylamine also accumulated. Maximum vanillic acid (840 µg L^{-1}) and vanillylamine (532.5 µg L^{-1}) contents were recorded on day-4 and 5 after caffeic acid addition, respectively (Fig. 3.41a). The bioconversion of caffeic acid to further metabolites *viz*. vanillin, vanillylamine, vanillic acid was shown to be elicited by treating the cultures with 10 µM MJ. Root cultures treated with MJ accumulated more vanillin (3.075 mg L^{-1} on the day-3) than untreated ones showing 1.37 fold higher accumulation (Fig. 3.42a). Concomitant increase in enzymatic activity of Caffeic acid *O*-methyl transferase (CAOMT) was obtained in MJ treated cultures, which was higher than in untreated cultures. There was 13.7-fold increase in CAOMT activity recorded after 24 hours of MJ treatment in root cultures of *C. frutescens* (Fig. 3.43).

Biotransformation of veratraldehyde to vanillin related metabolites

Cultures treated with veratraldehyde accumulated maximum vanillin than caffeic acid fed cultures showing 11.875 mg L^{-1} vanillin accumulation after 6 days of precursor addition. Protocatechuic acid and vanillic acid were detected after 6 days of precursor addition and maximum values were recorded as 0.325and 0.875 mg L^{-1} at 8^{th} and 10^{th} day of precursor addition, respectively (Fig 3.45a). Among all the

precursors studied, cultures treated with veratraldehyde resulted in maximum vanillin production.

In all the above studies, root cultures without precursors and autoclaved roots did not show any biotransformations.

CONCLUSION

The study throws light on the production of betalaines from hairy roots of *B. vulgaris* and biotransformations using *C. frutescens* normal roots for the production of vanillin related metabolites. Various aspects viz. polyamines, MJ and down stream process methodologies have been studied to enhance the metabolite production.

It may be concluded from the present study that hairy roots can be used as a potential system for the production of betalaines. The organized nature and stable metabolite production makes them more amenable for bioreactor cultivation and scale-up studies. Among all bioreactors studied, acoustic mist bioreactor performed well with high growth and metabolite production. There was no mass transfer limitation observed in the acoustic mist bioreactor unlike others such as nutrient sprinkle and bubble column reactors. Osmolarity measurements proved reliable than conductivity for indirect estimation of biomass when the hairy roots were cultivated in bioreactor systems.

The studies on product enhancement (polyamines and MJ) and on-line extraction of pigment (using CTAB) provided an insight for integrating unit operations and developing a process for continuous operation and higher production of phytochemicals in bioreactor systems. In the present study it was found that the roots were still viable and showed normal growth even after CTAB treatment (0.002%) which would be an important observation to make this process a continuous one.

This study has shown the production of DOPA and dopamine, which are very important pharmaceutical compounds from hairy roots of *B. vulgaris*. Further studies with tyrosine feeding led to higher production of DOPA and dopamine. This study is of importance in understanding the biosynthetic pathway of betalaines derived from DOPA and dopamine. Use of oxygenase inhibitors such as caffeic acid and catechin formed the basis to envisage the enzymatic pathways in betalaine production. The higher production of DOPA and dopamine under the influence of caffeic acid and

catechin might be because of the inhibitory action of these chemicals on tyrosinase preventing further conversions.

Future work would be oriented to the studies on metabolic block of enzymes such as DOPA dioxygenase to produce more DOPA, which is an important pharmaceutical compound. Antisense RNA methodology for the enzyme DOPA dioxygenase would also be helpful probably leading the higher production of DOPA and dopamine.

The studies on biotransformation of phenylpropanoid compounds to various vanillin related metabolites using *C. frutescens* normal roots led to understand the biosynthetic pathways involving various chemical reactions.

The higher biotransformation rates in cultures fed with ferulic acid complexed with BCD would be because of the increased solubility and availability of precursor. The formation of vanillin from ferulic acid might be via feruloyl-CoA through a reaction similar to □-oxidation of fatty acids. Vanillic acid was formed by further oxidation of vanillin, whereas reduction yielded vanillyl alcohol. In the present study, biotransformation resulted in higher concentrations of vanilla flavour compounds in the medium than in roots. Thus, it would be appropriate to use a bioreactor system for down stream processing of the biotransformed products. The formation of vanillin from protocatechuic aldehyde involved *O*-methylation reaction and oxidation to produce vanillin and protocatechuic acid respectively.

It was observed that ferulic acid formation was more in the early stages after caffeic acid feeding as reflected by *O*-methylation reaction. When MJ was fed along with caffeic acid, it probably enhanced the CAOMT activity leading to increased biotransformed product. Further, ferulic acid upon oxidation probably led to vanillin production along with other metabolites. Veratraldehyde fed cultures showed maximum vanillin accumulation than other precursors. Veratraldehyde was demethylated to vanillin and parahydroxy benzoic acid.

There was no capsaicin accumulation recorded in the studies indicating the non-existence of capsaicinoid synthase that is considered as a key enzyme in the synthesis of capsaicin. The efficiencies of biotransformation in all the biotransformation studies ranged from 2-9% suggesting the possible channeling of precursors resulting in the accumulation of other compounds such as lignin.

Further work would involve the use of inhibitors of lignin biosynthetic pathway to divert the phenylpropanoid precursors to vanillin related metabolite pathways.

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