GENETICALLY TRANSFORMED ROOT CULTURES OF RED BEET (*BETA VULGARIS* L.) FOR THE PRODUCTION OF FOOD COLOUR AND PEROXIDASE

Thesis Submitted to the
University of Mysore

for the award of
Doctor of Philosophy
in Biotechnology

by

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MYSORE - 570 020, INDIA
October 2005

.....To my beloved sister
Late Smt. Shivaganga,
Parents and Guide

R. Thimmaraju
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Karnataka, INDIA

DECLARATION

I hereby declare that this thesis entitled **"Genetically transformed root cultures of red beet (*Beta vulgaris* l.) for the production of food colour and peroxidase"** submitted to the University of Mysore for the award of the degree of **Doctor of Philosophy in Biotechnology,** is the result of work carried out by me in **Plant Cell Biotechnology Department,** Central Food Technological Research Institute, Mysore, under the guidance of **Dr. Bhagyalakshmi Neelwarne** during the period Sept 2001- Oct 2005.

I further declare that the results of this work are not previously submitted for any degree or fellowship.

Date: R. Thimmaraju

Place:

Dr. Bhagyalakshmi Neelwarne
Scientist
Plant Cell Biotechnology Department

CERTIFICATE

I hereby certify that the thesis entitled **"Genetically transformed root cultures of red beet (*Beta vulgaris* l.) for the production of food colour and peroxidase"** submitted by **Mr. R. Thimmaraju** to the University of Mysore for the award of the degree of **'Doctor of Philosophy' in Biotechnology,** is the result of work carried out by him in Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore under my guidance during the period Sept 2001- Oct 2005.

Date: **Bhagyalakshmi Neelwarne**

Place: Research Supervisor

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(R. Thimmaraju)

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LIST OF ABBREVIATIONS

ATPE	**Aqueous two phase extraction**
BC	**betacyanin**
BN	**betalaine**
BX	**betaxanthine**
$^{\circ}$C	**degree centigrade**
ca	**approximately**
cm	**centimeter**
D	**dalton**
dA	**rate of change of absorbance**
DOPA	**dihydroxyphenylalanine**
DNA	**deoxyribonucleic acid**
DW	**dry weight**
FW	**fresh weight**
g	**gram**
HPLC	**high performance liquid chromatography**
h	**hour**
IAA	**indole-3-acetic acid**
ISSR	**inter simple sequence repeat**
Kg	**kilo gram**
Kpa	**kilo pascal**
L	**litre**
MS	**Murashige and Skoog**
ml	**milli litre**
mm	**milli metre**
mg	**milli gram**
min	**minute**
mg	**milli gram**
m s	**milli siemens**
µ	**micron**
µl	**micro litre**
µg	**micro gram**
µmol	**micromoles**
µ S	**micro siemens**
ηm	**nanno meter**
NAA	**naphthalene acetic acid**
NPT	**neomycin phosphotransferase**
OD	**optical density**
PCR	**polymerase chain reaction**
%	**per cent**
PMSF	**phenyl methane sulfoate**
POD	**peroxidase**
ppm	**parts per million**
3-PGA	**3-phosphoglycerate**
Ri-plasmid	**root inducing plasmid**
rol	**rooty loci**
rpm	**revolutions per minute**
RZ	**Reinheitszahl constant**
SE	**standard error**
s	**second**
T-DNA	**transfer DNA**
T_R-DNA	**right transfer DNA**
T_L-DNA	**left transfer DNA**
TAE	**tris-aceticacid-EDTA**
TFA	**tri-fluoro acetic acid**
vir	**virulence loci**

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GENERAL INTRODUCTION AND REVIEW OF LITERATURE

CONTENTS

G1.1. Plants as a source of secondary metabolites

Plants produce more than 1trillion 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 based on their biosynthetic pathways (Harborne, 1999). Compared to the primary metabolites like carbohydrates, protein and lipids found in plants, these secondary metabolites are low in abundance, often less than 1% of the total carbon, or the storage usually occurs in dedicated cells or organs (Bourgaud et al. 2001). The secondary metabolites from plants are useful as food additives, flavours, colourants, and pharmaceuticals. The general biosynthetic pathway of plant secondary metabolites is presented in figure G1. 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. 1988) obtained from field grown plants.

Roots are the most important 'hidden half" of the plant system, which not only function as a chief anchorage for the plant but also synthesize many valuable bio-molecules of importance as pharmaceuticals, insecticides, colours, flavours and others. Roots exhibit indefinite growth because of the meristematic activity at the root tip with simultaneous synthesis of important biomolecules. 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; 2002a). Cultured roots are also exploited for their enormous ability to extract soil and water pollutants such as organic compounds, heavy metals, xenobiotics from the contaminated sites – the process generally known 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).

However, plants originating from particular biotypes are difficult to grow outside their local ecosystems and are more prone to pathogen attack. Moreover, the secondary metabolites in plants 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).

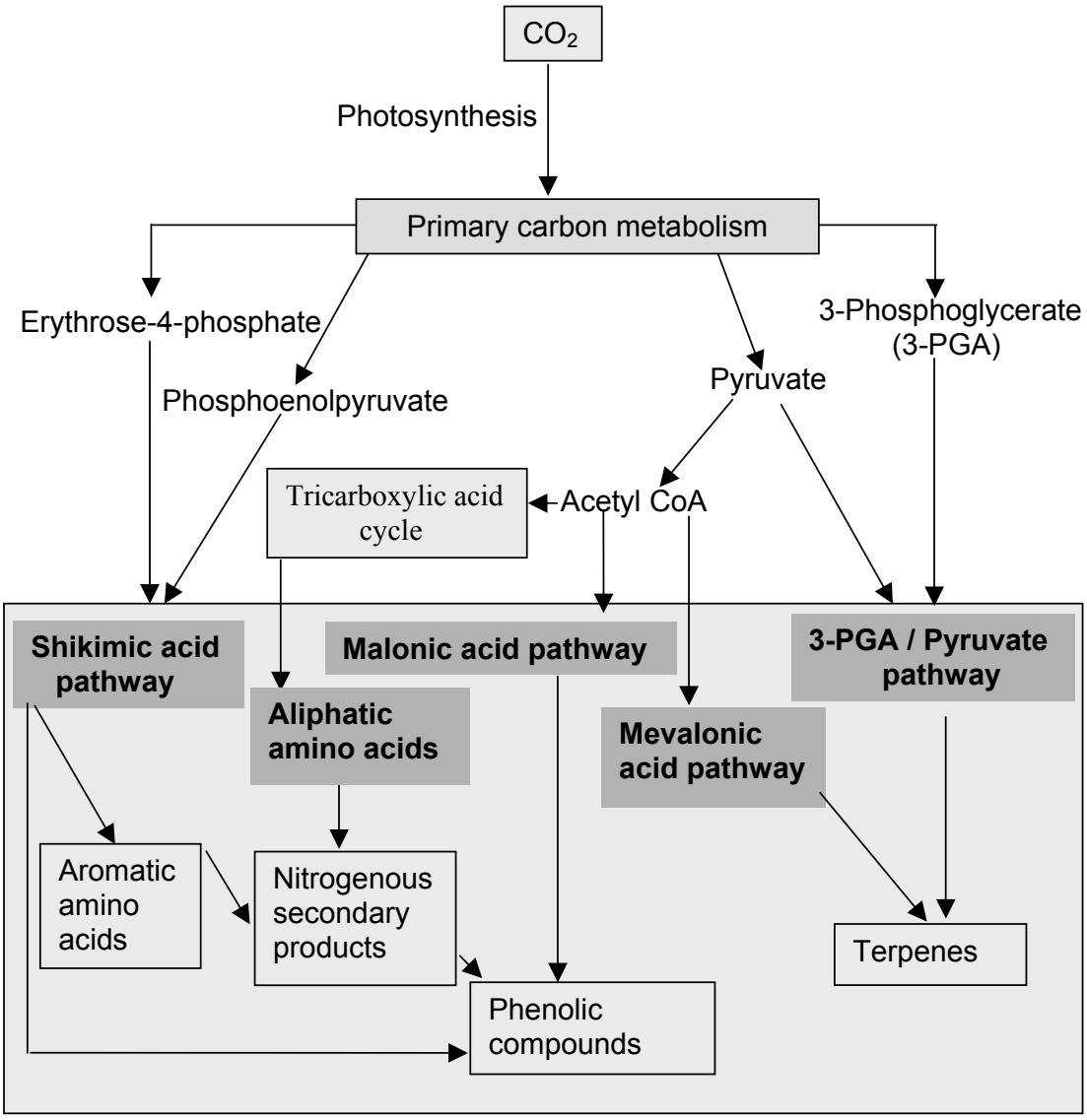

Figure G1. General biosynthetic pathways of secondary metabolites in plants.

With the conceptual beginning made by Haberlandt (1902), a number of scientists around the world subsequently 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 cultured 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 biomass and to a condensed biosynthetic cycle (Dornenburg and Knorr 1995).

G1.2. Plants as a source of enzymes

Apart from various phytochemicals of secondary pathways, plants have been the source of a number of primary metabolites such as enzymes of commercial importance (Biles et al. 1990; Medina et al. 1993). Enzymes are biocatalysts, which initiate and accelerate thousands of biochemical reactions in living cells. They process reactions, which are otherwise not possible under chemical catalysis. In fact, almost all biochemical reactions require one or more enzymes for their completion though a few reactions undergo non-enzymatic conversions.

Enzymes are proteinaceuos in nature; they can be extracted from living tissues, purified and even crystallized. Under controlled conditions of isolation and storage, they retain their original level of activity and in some cases, even exhibit an increased activity (Prabha and Patwardhan 1986). Thus, the purified enzyme can be used to accomplish a specific biochemical reaction outside the cell. This property of enzymes has been employed for laboratory experiments as well as for commercial production of several important biochemical compounds, drugs, clinical reagents, diagnostics and industrial products. Thus some of the enzymes are primary metabolites of high value produced in large quantities in plants.

G1.3. Cultured cells

Cultured plant cells offer various advantages over the field plants for the production of both primary and secondary metabolites. So far, only a small percentage (5-10 %) of all plants has been screened for useful compounds with the aid of modern scientific tools (Schripsema et al. 1996). In the food segment, they are well known as producers of colours, flavours, sweeteners, food additives etc., (Bhagyalakshmi and Ravishankar 1996). Some of the important food additives derived from plant cell cultures are listed in the table G1.

Most of the secondary metabolites are produced during stationary phase of cell cycle rather than during early stages of growth, the reason being the allocation of carbon for primary metabolism (building of cell structures and respiration) during the active growth stage. Once the growth slows down, the 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 (Dornenberg and Knorr 1995) and the problems of scale-up in bioreactors have driven

researchers to look for alternative methods to obtain stable production of secondary metabolites through organ culture. Among the various organ cultures, genetically transformed roots (hairy roots) are gaining tremendous potential in the recent past.

Table G1. Secondary metabolites of food importance from plant cell cultures

Product type	Plant species	Reference
Colours		
Anthocyanins	*Daucus carota*	Rajendran et al. 1994; Narayan et al. 2004
	Perilla frutescens	Zhong and Yoshida 1995
Betalaines	*Beta vulgaris*	Thimmaraju et al. 2003
	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
2 – Acetyl Pyrroline	*Pandanus amaryllifolius*	Thimmaraju et al. 2005
Pungent food additive		
Capsaicin	*Capsicum frutescens,*	Lindsey and Yeoman 1984
	Capsicum annuum	Johnson et al. 1990
Sweeteners		
Stevioside	*Stevia rebaudiana*	Sivaram and Mukundan, 2003
Glycyrrhizin	*Glycyrrhiza glabra*	Hayashi et al. 1988

G1.4. Hairy root cultures

Recently 'hairy root cultures' are gaining importance as production systems for secondary metabolites due to their tremendous potential of higher growth rate, uniform product formation due to their high genetic stability. Being organized, their amenability for scaling-up in reactors is an added advantage (Flores, 1992).

G1.4.1. Mechanism of *Agrobacterium rhizogenes* mediated transformation

A. rhizogenes, a gram- negative soil bacterium, is the causal agent of 'hairy root disease', produces the root-like structures upon infecting the plant. The information responsible for triggering the infection process and the transfer of DNA strand belongs to a circular DNA known as plasmid, which is a symbiont within the *Agrobacterium* cell. The roots that are induced as a result of infection can be grown axenically on hormone-free medium because of their ability to synthesize higher levels of endogenous hormones such as auxins and cytokinins. The process of transformation involves attachment of *Agrobacterium*

to a plant cell in a polar fashion in a two-step process. The first step is mediated by triggering the synthesis of 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 a large number of bacteria at the wound surface (Matthysse et al. 1996). 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 plant cells that give rise to the rooty structures. Such neoformed roots produce opines (psuedo-amino acids 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. G2). The T-DNA of Ri plasmid comprises of two different parts, called the T_L and T_R DNAs. The T_R-DNA harbours the genes responsible for phytohormone production (specifically auxin synthesis) and for biosynthesis of opines (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 *vir* genes does not enter the plant genome but are required for the transfer of T-DNA (Table G2). 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. Once induced, the two-gene products virA and virG mediate the further activation of other *vir* genes. The *vir*A gene product is a membrane – spanning protein that senses external inducer compounds activating the *vir*G. Autophosphorylation of virA protein and the subsequent transphosphorylation of virG protein result in the active transcription of the remaining *vir* genes (*virB, virC, virD*, and *virE*) (Jin et al. 1990). Apparently, the *virD* gene products (virD1 and virD2) recognize the borders of the T-DNA, nick one strand of the Ri plasmid and create, a single stranded copy of the T-DNA by unidirectional DNA replication catalyzed by the cellular repair apparatus. The virD1 protein appears to be the agent responsible for nicking the Ri plasmid and the virD2 protein becomes covalently attached to the 5' end of the T-strand, resulting in a linear, single stranded copy of the T-DNA. The virE2 and virE1 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 result in the initiation of hairy roots at the infected site. It has been shown that the T_L-DNA of Ri plasmid can 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 *rolA, rolB* and *rolC*. 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; 1991b).

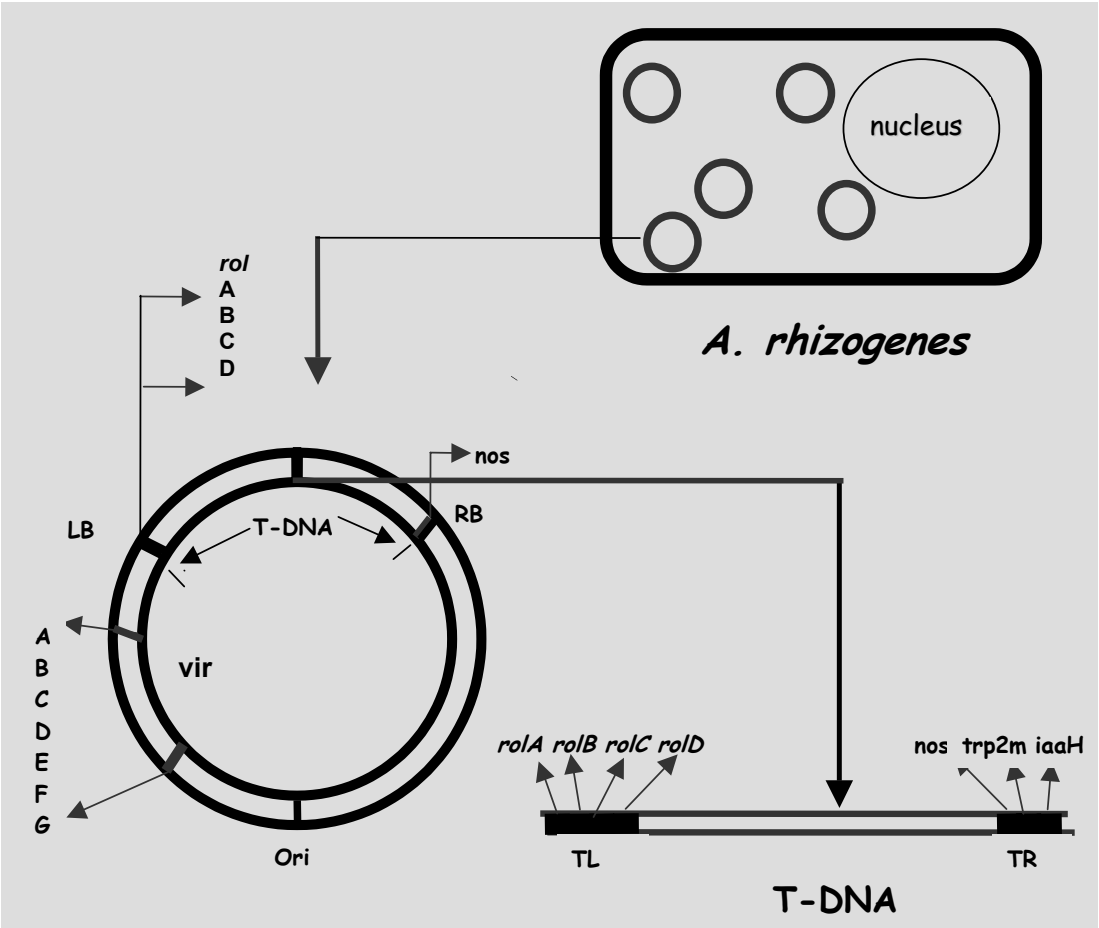

Figure G2. Ri plasmid of *A. rhizogenes*: LB-left border; RB-right border; TL- left transfer DNA; TR- right transfer DNA; genes for auxin synthesis (*aux1*, *aux2* and opine synthase gene (*ops*); *OPCAT*- genes for opine catabolism; Ori- origin of Replication; *Vir* - virulence Region.

Table G2. Functions of *vir* genes of *Agrobacterium rhizogenes* during T-DNA transfer (Source: Winans, 1992)

***vir* locus**	**Function**
A	Regulatory (recognizes plant metabolites, activates *vir* G)
G	Regulatory (transcriptional activator of other *vir* genes)
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

G1.4.2. Hairy root cultures as a source of phytochemicals

Hairy roots are obtained after the successful transformation of a plant with *A. rhizogenes*. They have received considerable attention from plant biologists, both for the basic research and 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; Doran, 1997). They often display higher growth rates due to profuse lateral root growth with doubling time ranging from 1-day (Maldonado-Mendoza et al. 1993; Bhagyalakshmi and Ravishankar 1996) 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 in 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 (Holmes et al. 1997; Bhagyalakshmi et al. 1998). Successful results have been

obtained by modifying the composition of the nutrient medium (Tone et al. 1997) or by applying elicitors (Rijhwani and Shanks 1998). Some of the important secondary metabolites reported from cultured hairy root cultures are listed in the table G3.

Table G3. Secondary metabolites produced by hairy root cultures in various plant species

Plant	**Secondary metabolite**	**Reference**
Aconitum heterophyllum	Aconites	Giri et al. 1997
Atropa belladonna	Atropine	Christen, 1999
Azadirachta indica A. Juss	Azadirachtin	Allan et al. 1999
Beta vulgaris	Betalaine pigments	Thimmaraju et al. 2004
Bidens sp.	Polyacetylenes and thiophenes	Flores et al. 1988
Brugmansia candida	Tropane alkaloids	Pitta-Alvarez et al, 2000
Catharanthus roseus	Indole alkaloids, ajmalicine	Vitali and Ventrone, 2002
Centranthus rubber	Valepotriates	Christen 1999
Cichorium intybus	Esculin, Esculetin	Bais et al. 1999
Cinchona ledgeriana	Quinine	Hamill et al. 1989
Coleus forskohlii	Forskolin	Sasaki et al. 1998
Datura candida	Scopolamine, Hyoscyamine	Christen et al. 1989
Datura stramonium	Hyoscyamine, Sesquiterpene	Payne et al. 1987
Daucus carota	Flavonoids, Anthocyanin	Bel-Rhlid et al. 1993
Digitalis purpurea	Cardioactive glycosides	Saito et al. 1990
Fagra zanthoxyloids Lam.	Benzophenanthridine	Couilerot et al. 1999
Fagopyrum	Furoquinoline alanine	Trotin et al. 1993
Fragaria	Flavanol	Motomari et al. 1995
Geranium thubergee	Polyphenol	Ishimaru et al. 1991
Glycyrrhiza glabra	Tannins	Wei et al. 2001
Gynostemma pentaphyllum	Flavonoids	Fei et al. 1993
Hyoscyamus muticus	Hyoscyamine, lubumin	Singh et al.1998
Lithospermum erythrorhizon	Sesquiterpenes, hernandulcin	Fukui et al. 1998
Lobelia cardinalis	Shikonin, Benzoquinone	Yamanaka et al 1996
Lobelia inflata	Polyacetylene glucoside	Yonemitsu et al. 1990
Lotus corniculatus	Lobeline, polyacetylene	Carron et al. 1994
Nicotiana hesperis	Condensed tannins	Parr et al. 1984
Nicotiana rustica	Nicotine, Anatabine	Hamill et al. 1986
Nicotiana tabacum	Nicotine, Anatabine	Flores and Filner, 1985
Ocimum basilicum	Rosmarinic acid	Bais et al. 2002b
Ophiorrhiza pumila	Camptothecin-related alkaloids	Hiroshi et al. 2002
Panax ginseng	Ginsenosides	Washida et al. 1998
Papaver somniferum	Codeine	Arellano et al. 1996

Perezia cuernavcana	Sesquiterpene quinone	Santos et al. 1998
Pimpinella anisum	Essential oils	Tada et al. 1995
Platycodon grandiflorum	Polyacetylene glucosides	Ishimaru et al. 2003
Pratia nummularia	Polyacetylene glucosides	Sato et al. 1991
Rauwolfia serpentina	Reserpine	Lodhi et al. 1996
Rubia peregrina	Anthraquinones	Sato et al. 1991
Rubia tinctorum	Anthraquinone	Gulham et al. 1999
Rudbeckia sps	Polyacetylenes and thiophene	Uczkiewicz et al. 2002
Rudbeckia hitra L.	Steroidal saponins	Hamill et al. 1987
Solanum lacinialum	Steroidal alkaloids	Yu et al. 1996
Valeriana officinalis L.	Valepotriates	Granicher et al. 1995
Vinca minor	Indole alkaloids (vincamine)	Tanaka et al. 1994
Withania somnifera	Withanoloides	Banerjee et al. 1994

G1.4.3. Strategies for improvement of secondary metabolite / enzyme production in hairy root cultures

Biosynthesis of secondary metabolites / primary metabolite (enzyme) 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).

Table G4. Strategies for the improvement of metabolite production in hairy root cultures

Clone improvement	Screening and selection of a best clone Genetic modification: Addition of *rol* genes
Altering medium composition	Carbon source Phytohormones Precursors Anti-metabolites
Improved culture conditions	Inoculum size pH Temperature Light Culture volume Agitation
Elicitation	Elicitors
Downstream processing	Product release and recovery

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; Rijhwani and Shanks 1998). The possible ways for the improvement of secondary metabolite production in hairy root cultures are listed in the table G4. A large body of literature is available on individual facets of the above listed aspects.

G 1.4.4. Scale up of hairy root cultures

Despite high growth rate and high metabolite production studies on scale-up of hairy roots and design of ideal bioreactor for commercial exploitation are scanty. Since hairy roots grow as tangled mass, and each clone has different branching pattern than the other with continuously changing rheology indicate highly complicated sets of conditions for scale-up studies. 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, and so on (Bhagyalakshmi et al. 1998). 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 adopted 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.

Different types of bioreactors, such as the bubble column, air-lift, rotating drum, stirred tank and mist bioreactors have been employed for hairy root cultures as given in the Table G5.

Table G5. Various types of bioreactors used for the scale-up of hairy root cultures

Bioreactor type	Plant system	Reference
Stirred- tank	*Armoracia rusticana*	Taya et al. 1989 b
	Atropa belladona	Jung and Tepfer 1987
Stirred-tank	*Atropa belladona*	Jung and Tepfer 1987
(impeller isolated)	*Datura stramonium*	Hilton and Rhodes 1990
	Duboisia leichhardtii	Muranaka et al. 1992
Air-lift	*Trigonella foenum-graceum*	Rodriguez-Mendiola et al. 1991
	Panax ginseng	Yoshikawa and Furuya 1987
	Lippia dulcis	Sauerwein et al. 1991
	Beta vulgaris	Sanchez et al. 2002
Bubble column	*Catharanthus roseus*	Toivenon et al. 1990
	Tagetes patula	Buitelaar et al. 1991
	Hyoscyamus muticus	Flores and Curtis 1992
	Beta vulgaris	Mukundan et al. 1998
	Daucus carota	Kondo et al. 1989
	Artemisia annua	Wyslouzil et al. 2000
	Artemisia annua	Kim et al. 2001, Liu et al. 1999
Acoustic mist	*Beta vulgaris*	DiIorio et al. 1992a
	Carthamus tinctorius	-do-

G1.5. The betalaines

Betalaines are a class of pigments comprising of betaxanthines (yellow) and betacyanines (violet). They are vacuolar water-soluble chromo alkaloid pigments found to accumulate in the flowers, fruits and sometimes in vegetative tissues of plants from the order Caryophyllales. These secondary metabolites are also produced in response to insect; pathogen or virus attacks on plants as observed in case of necrotic yellow vein virus. Wohlpart and Mabry first introduced the name “betalain” during 1968 to describe these pigments as derivatives of betalamic acid identified in red beet plant (*Beta vulgaris*), which is the major commercial source of betalaines. Due to their coluorant properties they are recently gaining high importance as colourants in various food and pharmaceutical formulations.

Nomenclature: As early as 1876, Bischoff in his thesis mentioned that certain anthocyanin-like plant pigments that contain nitrogen but are otherwise similar to true anthocyanins. Because of these similarities, the pigments, if red are have been referred to in the literature as 'nitrogenous anthocyanins' or 'betacyanins' and, if yellow, as 'flavocyanins' or 'betaxanthins'. To achieve uniformity in nomenclature, Wyler and Dreiding suggested that the red pigments of this type be known as betacyanines and the yellow as betaxanthines.

The terminal letter 'e' was added by Fischer and Dreiding so that the names confirm with the nomenclature of the I. U. P. A. C. (Mabry and Dreiding 1968). The term 'betalaine' was introduced by Mabry and Dreiding (1968) as a class designation for these pigments.

Structure: Structural characteristic of all the betalaines is the presence of betalamic acid chromophore, a hydropyridine moiety attached via a vinyl group to another nitrogenous group (Miller et al, 1968). The betaxanthines are formed by the condensation of an amino acid or an amine with the aldehyde group of the betalamic acid, resulting in a Schiff-base. This basic structure gives the stronger yellow or yellow – orange colour to betaxanthines with a maximum absorbance at 470-486ηm. In spite of the colour difference betacyanines are also made of a betalamic acid linked to a molecule of cyclo-DOPA (Wyler and Dreiding 1961). The violet colour of the latter is due to an aromatic structure inducing a strong shift of 60ηm in the absorbance maxima (534-554ηm) (Wyler et al. 1963; 1984).

Biosynthesis: Compared to a number of secondary metabolites betalaines have a rather simple biosynthetic pathway requiring only a few steps for betaxanthine and/or betacyanine synthesis. Betalaines are derivatives of tyrosine; the tyrosine is hydroxylated by a tyrosinase to give 3,4-dihydroxyphenylalanine (DOPA). Betalamic acid, the common chromophore of betalaines is formed by the successive enzymatic cleavage of the DOPA aromatic ring at the position 4, 5-, giving the unstable seco-DOPA molecule (Fischer and Dreiding 1972; Terradas and Wayler 1991). Spontaneous condensation of the chromophore with an amino acid or an amine forms the betaxanthines (Schliemann et al. 1999), whereas betacyanine biosynthesis requires the presence of a cyclo-DOPA molecule. The formation of cyclo-DOPA results from the oxidation of DOPA into a dopaquinone intermediate by a polyphenol oxidase, followed by a spontaneous cyclization of this intermediate. The following enzymatic step leading to the formation of betacyanines is still an open question. It is not clear if the betalamic acid molecule condenses with a cyclo-DOPA molecule or with its glucosylated form, depending on the species studied. However, recently Kobayashi et al (2001) concluded that condensation step occurs between a non-glucosylated cyclo-DOPA and a betalamic acid molecule, followed by glucosylation and acylation steps (Fig. G3).

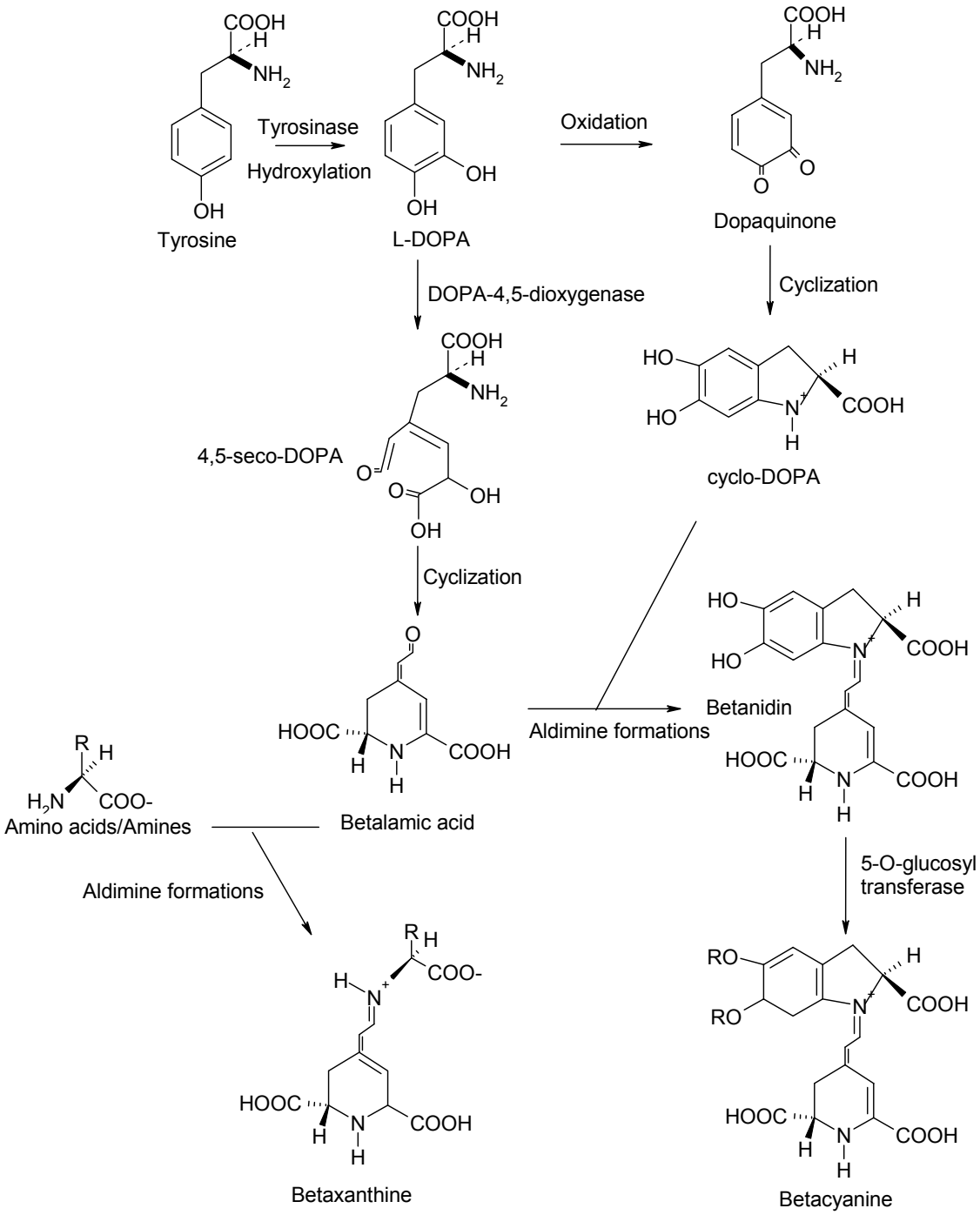

Figure G3. The biosynthetic pathway of betaxanthine and betacyanines (Strack et al. 2003).

G 1.6. Peroxidase (EC 1. 11. 1. 7) (POD)

POD in its widest sense, includes a group of specific enzymes such as NAD-peroxidase, NADP-peroxidase, fatty acid peroxidase, glutathione peroxidase etc., as well as a group of very non-specific enzymes from different sources, which are simply known as 'peroxidase' (H_2O_2 oxidoreductase). POD catalyzes the dehydrogenation of a large number of phenolics, aromatic amines, hydroquinones etc., and thus having an important role in the *in vivo* system. Several substrates are used to assay the activities of PODs. Generally, guaiacol and orthodianisidine are used as a substrate for the assay of POD where the following reaction is involved.

$$\text{Guaiacol} + H_2O_2 \xrightarrow{\text{POD}} \text{oxidized guaiacol} + 2\ H_2O.$$

The resulting oxidized (dehydrogenated) product tetra-guaiacol is a coloured compound. The rate of formation of dehydrogenated product is a measure of the POD activity, which is assayed spectrophotometrically. Since a wide range of chemicals can be modified by catalytic activity of POD, several novel applications have been suggested for this enzyme, including the treatment of waste water containing phenolic compounds, synthesis of various aromatic chemicals and removal of peroxide from materials such as food stuffs and industrial wastes. Thus, POD has also been widely used as an important component of reagents for clinical diagnosis of various laboratory experiments.

G1.6.1. Role in plant physiology

The POD exists in plants and plays crucial role in their growth and development; following are some of the important aspects in which POD has been implicated

- Regarded as a major enzyme involved in the defense and host plant resistance reactions mainly because of its over-production in the challenged tissue either by biotic or abiotic stress (Perera et al. 2004).
- Associated with the hyper sensitive response and proved to play a significant role in plant disease resistance (Lamb et al. 1993)
- Induced systemically in response to herbivore attack in a number of plants (Davis et al. 1987).
- Involved in the regulation of endogenous auxin and abscissic acid levels thus modulating the plant growth processes (Biles et al. 1990; Medina et al. 1993)
- Involved in the differentiation of tracheary elements and lignification in *Picea stichensis* (Richardson et al. 1997).

- Apart from a number of protective functions, POD has been reported to impart UV tolerance and lignin polymerization in plants (Richardson and Mc Dougall, 1997).
- Involved in the turnover and degradation of some of the plant pigments such as anthocyanins (Lim et al. 1989; Calderon et al. 1992).

Most of these physiological functions of POD in plants are associated with the plant's ability to generate hydroxyl radicals through various mechanisms (Fig G4)

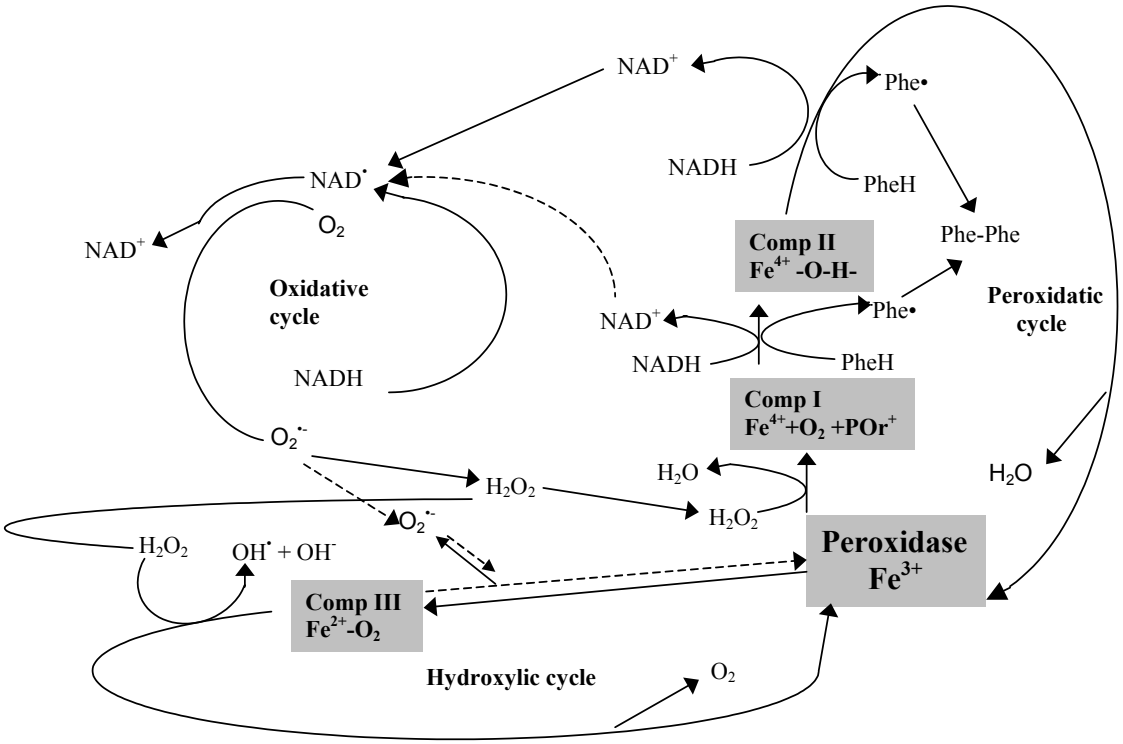

Figure G4. Schematic diagram representing physiological role of peroxidases in plants, which generate hydroxyl radicals through different pathways such as oxidative, hydroxylic and peroxidative pathways. POD mediates the oxidation of phenolic substrates (PheH) by H_2O_2 to phenoxy (Phe•) radicals that polymerize to generate molecules such as lignins and extensins. Reduction of substrates such as NADH (dihydroxyfumerate) initiates the oxidative cycle that reduces O_2 to O_2• and H_2O_2 which is utilized to produce compound III that reduces H_2O_2 to OH in the hydroxylic cycle (adopted after modification from Chen and Schopfer, 1999).

G1.6.2. Applications of POD

Most of the potential applications suggested for commercial horseradish peroxidase (HRP) may also be suggested for PODs from plants. Some of the prospective applications for plant derived PODs are listed below.

1. Reagent in clinical diagnostics (Yamada et al. 1987; Nathan & Joan (1971). POD as a Cell marker in nuroscience (Kristensson and Olsson 1971; La vail and La vail 1972; Mesulam, 1982).
2. POD based biosensors (Clark, Jr 1962).
3. Reagent in enzymatic synthesis of organic compounds (Rannau et al. 1998; Lui et al. 1999; Ikeda et al. 2000; Oguchi et al. 2000; Hutterman et al. 2001; Raitman et al. 2002; Kim et al. 2003; Sakharov et al. 2003; Uyama and Kobayashi 2003).
4. Biotransformation and coupled enzyme assays (Hruby et al. 1997; Kutney et al. 1996; Kutney, 1998; Fang and Barcelona 2003).
5. POD in Bioremediation of contaminated soils (Torres et al. 1997; Ayala et al. 2000).
6. Bio-bleaching and Bio-pulping (Katagiri et al. 1995; Jimenez et al. 1997; Arana et al. 2002; Hatakka et al. 2002; Hatakka et al. 2003; Li et al. 2003).
7. Treatment of wastewaters and industrial effluents (Nicell et al. 1993; Yu et al. 1994; Kennedy et al. 2002; Wagner et al. 2002)

G1.6.3. POD from cultured plant cells and tissues

The cell culture media of numerous plant cell cultures have been shown to contain remarkably high activities of oxidative enzymes, particularly POD (Barz et al. 1990). In many cases, there occurred continuous synthesis and release into the medium of such enzymes during growth phase. The specific plant cultures and the characteristics of respective PODs are listed in the table G6.

G1.6.4. POD from genetically transformed (hairy) root cultures

It is well known that cell cultures are highly recalcitrant leading to periodic fluctuations in their biosynthetic potential where, the enzyme yield may not be uniform. Therefore, to have a system with predictable yield, organ cultures are preferred. Hairy roots from quite a few plant species have been reported to produce appreciable levels of PODs. An overview of the different plant cell and hairy root cultures, which are reported to produce POD, have been listed (table G6) along with the physicochemical properties of respective POD.

Table G6. Physico-chemical properties of peroxidases from different plant sources

Source	Mol. weight (KDa)	pI	Optimum		Substrate	Km	Reference
			pH	Temp (^oC)			
Carrot hairy roots	-	-	-	-	Guaiacol	-	Kim and Yoo, 1996
Horse radish hairy roots	-	8.6	-	-	Guaiacol/ o-dianisidine	-	Flocco et al, 1998
Turnip hairy roots	39.3	9.6	5.0	50	o-dianisidine	144 µM	-
	36.0	7.2	4.5	-	ABTS	0.7 mM	Agostini et al, 2002
Poplar xylem	46-54	3.1-3.8	-	-	ABTS/3'3'diaminobenzidine	-	Duarte- Vazquez et al, 2001
Haevea brasilensis bark	50	3.5	-	-	o-dianisidine / H_2O_2	18.6 µM	
Carrot roots	45	>9.3	6.0	25	Guaiacol	-	Wititsuwannakul et al, 1997
Tomato	34-37	9.0	7.0	25	Extensin	-	Nair and Showalter, 1996
Barley coleoptiles	31-34	9.3 & 7.3	5.5	25	ABTS	-	Brownleader et al, 1995
Raphanus sativus roots	45	-	5.0	40	ABTS	4.78 mM	Kristensen et al, 1999
Beta vulgaris root	-	4.5 - 5.7, 9.0	5.0	25	4 - methoxy α – napthol	0.14 mM	Aruna and Lali, 2001
Chinese cabbage	-	4.83-4.78	-	-	-	-	Escribano et al, 2002
Sycamore maple cell suspension culture	-	3.68	-	-	-	-	Wang et al, 1999
Tobacco	-	4.5-8.4	-	-	-		-
Velvet leaf (*Abutilon theophrastis*)	-	4.03-8.66	5.0	25	Guaiacol / H_2O_2	-	Shinshi and Nagochi, 1976
Sandal wood somatic embryo culture	-	-	6.0	50	-	10.91mM	Pal et al, 2003
Prunus brassica callus culture	30	6.0, 6.8 & 10	6.4	25	Guaiacol / H_2O_2	-	Zhau et al, 2002
Carrot compact cell aggregates			6.0	25	Guaiacol / H_2O_2	-	Xu et al, 1998
Cotton ovule culture	-	-	5.5-6	4 - 60	Guaiacol	-	Mellon, 1986
Cow pea suspension cultures	-	-		70	Guaiacol / H_2O_2	0.483	Moreno et al, 1990
Peanut cell cultures	-	-	5.0	-	-	-	Xu et al, 1987
Horse radish cell cultures	-	-	-	-	orthoaminophenol	-	Yamada et al, 1987
Cassava (*Manihot esculenta*) cell suspension	-	9, 9.5 & 3	6.0	-	3,5-dichloro-2-hydroxy-benzene sulfonic acid	-	Gomez-Vasquez et al, 2004
	-	3.1- 8.8	6.0				

With the available literature and background information one would very well appreciate the commercial potential of hairy root cultures for the production of various primary and secondary metabolites. Since red beet (*Beta vulgaris* L) is found to contain both betalaines (Von-Elbe, 1979) and peroxidase enzyme (Thimmaraju et al, 2005) this study has been focused on developing process for improved production of betalaines and peroxidase using red beet hairy roots by fine-tuning various parameters and elucidating the possible mechanisms for which the following objectives were considered:

- To establish different clones of hairy roots using different strains of *Agrobacterium rhizogenes* and double transform the superior clone with additional *rol* genes and characterize the hairy root clones so obtained for growth, morphology and pigment synthesis.
- To study the effect of different culture volumes on the performance of hairy root cultures.
- To study the growth and pigment formation in a bioreactor for improved mass-transfer.
- To study off-line and on-line recovery of pigments.
- To screen hairy root clones for the production of peroxidase enzyme and select the best clone for further elicitation of production and partial purification and characterization of the enzyme.
- To integrate biomass production and product recovery.

The work done is presented in the following chapters

1. Establishment and characterization of clones
2. Optimization of Growth conditions and scale-up
3. Studies on peroxidase enzyme
4. Downstream processing

CHAPTER 1

ESTABLISHMENT AND CHARACTERIZATION OF CLONES

CONTENTS

SUMMARY

*T*en clones of red beet (*Beta vulgaris*) hairy roots were induced using different strains of *Agrobacterium rhizogenes* viz., A4, A 2/83, A 20/83 and LMG-150, accordingly the clones were named as A4(1), A4(2), A4(3), A 2/83(1), A 2/83(2), A 2/83(3), A 20/83(1), A 20/83(2), A 20/83(3) and LMG-150. Further, the clones were characterized based on their growth performance, capacity for pigment synthesis, endogenous auxin content, T-DNA copy number and ISSR genetic marker. The clone LMG-150 that produced highest biomass and betalaine pigment was selected as the better performing or best clone and was used in all further studies. However, significant differences in morphological and physiological characteristics were observed among the ten clones that depended mainly on the *A. rhizogenes* strain. The growth and pigment synthesis in all the clones were directly proportional to the endogenous auxin level but not to T-DNA copy number, despite the fact that T-DNA harbors the *rol* genes, which influence endogenous phytohormone levels. In the clone obtained from LMG-150 that produced highest biomass and pigments, it was found that the enzyme peroxidase was involved in regulating the endogenous auxin pool. In addition an attempt was also made to re-transform the best clone for insertion of additional *rol* genes. Two double transformed clones were obtained, one for *rolABC* and the other for *rolC* gene of *A. rhizogenes*. The double transformed clones when studied for the growth and pigment synthesis it was found that the LMG-150 double transformed for *rolABC* genes (clone *LMG-ABC*) produced highest biomass and betalaine when compared to LMG-150 and double transformed clone for *rolC* gene.

Publication

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1.1. INTRODUCTION AND REVIEW OF LITERATURE

The enormous potential offered by hairy roots for the production of various secondary metabolites has been well documented (Flocco et al. 1998; Carrizo et al. 2001; Wang and Tan 2002). Hairy root cultures of red beet have been studied extensively for the production of natural colorants, the betalaines (Thimmaraju et al. 2003a; 2003b; 2004; Suresh et al. 2004) that are water-soluble having a vast application in food and pharmaceutical formulations. A few studies published recently on red beet hairy roots involve product efflux, recovery and elicitation (Suresh et al. 2004, Savitha et al. 2005) to improve the productivity. Most of these studies were done either with the use of a single clone (if selected?) or may be cultures comprising of a mixture of clones. Selected clones of hairy roots are both genetically and biochemically stable when compared to cell suspension cultures and hence have gained better attention over the years (Carrizo et al. 2001; Wang and Tan 2002) than cell cultures (Doran, 2002). However, each hairy root clone established by *Agrobacterium rhizogenes* mediated transformation is expected to behave differently from the other clones in terms of growth, morphology and secondary metabolite production mainly due to changed physiological status of the host cell (Doran, 2002). Hence characterization of several clones would result in the selection of a best clone in terms of growth and secondary metabolite production (Doran 2002). Only a few studies focused on the characterization of individual root clones. Shanks and Bhadra (1989) evaluated 5 hairy root clones established by using different cultivars of *Catheranthus roseus* for growth and production of alkaloids such as ajmalicine, catheranthine and serpentine and found that all the clones produced alkaloids to expected levels. Endogenous auxin levels and polyamines were found to influence growth and coumarin production in hairy root clones of chicory (Bais and Ravishankar, 1999). Apart from the biochemical and genetic stability, the performance of such cultures depended largely on the type of carbon source (Bhagyalakshmi et al. 2004), temperature, endogenous IAA (Cardarelli et al. 1987) and number of T-DNA copies (Doran 2002).

Therefore, the present chapter was focused on two major objectives; first to establish several clones of hairy roots using different strains of *Agrobacterium rhizogenes*, confirm the integration and copy number of T-DNA, to characterize root clones based on morphology, ability to synthesize betalaine pigments leading to the selection of a best clone for pigment production; second to study the biochemical status such as endogenous levels of IAA and peroxidase in each clone. In addition, the sensitivity of each clone to exogenous

auxin and the translation of such sensitivity to the turnover of betalaine productivity have also been addressed.

1.2. *MATERIALS AND METHODS*

1.2.1. Plant material and culture method

Certified seeds of red beet variety 'Ruby Queen' were obtained from Indo-American hybrid seed company, Bangalore, India. The seeds were surface disinfected using 0.15% aqueous mercuric chloride solution for 5 min followed by five rinses with sterile double distilled water, then placed aseptically on hormone-free Murashige and Skoog's (1962) (MS) medium containing 3% sucrose adjusted to pH 5.8 before autoclaving at 121°C for 15 min and solidified with 2.5g L^{-1}gelrite. Seeds were incubated at 25 ± 1°C for 16h photoperiod with 40 μmol m^{-2} s^{-1} until two-leaved seedling stage. Various explants from these aseptic plants were used for transformation and induction of hairy root clones.

1.2.2. Growth of *Agrobacteria* and transformation

Agrobacterium rhizogenes strains A4, A 2/83, A 20/83, ATCC 15834 and LMG-150 were cultured on YMB medium (YMB medium; 1L containing 10g of glucose, 10g of yeast extract, 1g of ammonium sulphate, 0.25g of potassium biphosphate, 15g of agar with pH adjusted to 6.8 before autoclaving) and activated for co-cultivation. Different explants obtained from aseptic seedlings of red beet such as cotyledonary leaf, hypocotyl and mature leaf were placed on MS solid medium and then inoculated with different strains of *A. rhizogenes* separately by gently wounding with a needle dipped in *A. rhizogenes* culture and incubated in dark. About two weeks after infection, several hairy roots appeared at the wounded sites. Individual hairy root clone arising from a single transformation event in different explant was excised aseptically and cultured in hormone – free MS liquid medium containing 500mg L^{-1}cefotaxime (Sigma-Aldrich, USA) and 200mg L^{-1}augmentin (Sigma-Aldrich), USA for 1 week and the process was repeated three times to completely eliminate *A. rhizogenes*. Further, the hairy root stock cultures of individual clone were grown in 150ml Erlenmeyer flasks containing 40ml liquid MS medium with 3% sucrose and maintained on a rotary shaker at 90 rpm in dark at 25 ± 1°C unless mentioned otherwise.

1.2.3. Confirmation of Ri T-DNA integration

1.2.3.1. PCR analysis

The polymerase chain reaction (PCR) was used to detect the Ri T-DNA integration into the plant genome. The bacteria-free roots grown in MS basal medium were harvested, blotted gently on sterile filter paper and quickly frozen in liquid nitrogen. Thereafter, genomic DNA from putative transformants and normal roots was extracted by using plant

genomic DNA preparation kit GenElute^{TM} supplied by Sigma, USA as per the manufacturer's instruction. PCR was performed using *rolA* gene specific primer sets (forward – 5' AGA ATG GAA TTA GCC GGA CTA 3'- and reverse – 5' GTA TTA ATC CCG TAG GTT TGT TT 3'–) (Sigma USA), which were designed using Primer3 software. The PCR mixture (25µl) contained 50ηg of DNA prepared from normal and hairy roots respectively as the template, 1X PCR buffer, 25ρmoles of each primer, 2.5mM of dNTPs and 1 unit of Taq DNA polymerase (MBI fermentas, Lithuania). PCR was carried out by amplifying with initial denaturation at 94°C for 5min followed by 35 cycles each of 1min denaturation at 94°C, 1min annealing at 55°C and 1min extension at 72°C with a final extension of 72°C for 10min using Eppendorf (Model: 22331) thermal cycler. The PCR products were separated on a 1.5% agarose gel prepared in 1X TAE buffer (pH 8.0), visualized by staining with 0.1% ethidium bromide and documented by using gel documentation system (Herolab, Germany).

1.2.3.2. PCR-Southern analysis

The PCR products were separated on 1.5% agarose gel prepared in 1X TAE buffer (pH 8.0), transferred to nylon membrane (BioBond^{TM} – Plus, Sigma, USA) using standard protocols (Sambrook et al, 1989) and probed using a psoralen biotin (Ambion Inc, USA) labeled 308 bp fragment of the *rolA* Gene obtained from *A. rhizogenes* strain ATCC 15834. The probe was prepared after purification using Qiagen PCR purification kit (MinElute^{TM}) and labeled with psoralen biotin. The labeling, hybridization and detection were done with Ambion Bio detect kit (Ambion Inc, USA) according to the manufacturers instructions.

1.2.4. ISSR fingerprinting of hairy root clones

The ISSR primer, $(GA)_8C$ was used for ISSR fingerprinting. The amplification was carried out in 1X PCR buffer with 1.5mM $MgCl_2$, 2.5mM of dNTPs and 1 unit of Taq DNA polymerase (MBI fermentas, Lithuania), 200ηM of primer (Sigma, USA). PCR was performed with a temperature programme of, initial denaturation at 94°C for 1min, annealing at 50°C and 1min extension at 72°C with a final extension of 72°C for 10min. The amplification products were size separated on 1.5% agarose gel and stained with ethidium bromide, visualized and documented. PCR reaction was repeated at least twice to ensure the reproducibility of results.

1.2.5. Genomic Southern analysis

The genomic DNA prepared from each clone as explained earlier was purified using Clean Genei Kit obtained from Bangalore Genei India. Approximately 10ηg of purified DNA was subjected for restriction digestion with *HindIII*, the restricted fragments were separated on a 1.5% agarose gel, transferred to nylon membrane (BioBond^{TM} –Plus, Sigma, USA) using standard protocols (Sambrook et al. 1989) and probed using a psoralen biotin (Ambion Inc, USA) labeled 308bp fragment of the *rolA* Gene obtained from *A. rhizogenes* strain ATCC 15834. The probe was prepared after purification using Qiagen PCR purification kit (MinElute^{TM}) and labeled with psoralen biotin. The labeling, hybridization and detection were done with Ambion Bio detect kit (Ambion Inc, USA) according to the manufacturers instructions.

1.2.6. Treatment of hairy roots with auxins and tryptophan

To study the effects of different auxins on growth and production of betalaine in hairy root clones, 10mg each of different clones were cultured in 50ml conical flasks containing 15ml of MS liquid medium with 0.5 and 1.0ppm of either Indole acetic acid (IAA) or Naphthalene acetic acid (NAA) and incubated in dark on an orbital shaker kept at 90 rpm maintained at 25°C, however control were without any addition of auxin. The total biomass (fresh weight in g L^{-1}) and betalaine content (mg L^{-1}.) were determined at the end, i.e., on 20th day and betalaine productivity was expressed as milligrams produced per litre of medium input.

1.2.7. Extraction and estimation of endogenous auxin content

Endogenous IAA was extracted by following a modified method of Stossel and Venis (1970) and Knegt and Bruinsma (1973). Briefly, about 1g of hairy root was ground to fine powder using liquid nitrogen, suspended in 10ml methanol and filtered through a sintered glass filter (G2) under suction. The residue on the filter was re-extracted twice each with 10ml of methanol and the total filtrate was evaporated to an aqueous residue on a rotary evaporator (BUCHI Rotavapor R – 205). pH of the aqueous residue was brought to 8.5 by adding 10ml cold 0.5M K_2HPO_4. The contents were transferred to a separating funnel and shaken with 10ml petroleum ether twice; the aqueous layer was collected and again shaken with 10ml diethyl ether. The lipid fraction was discarded and the pH of the aqueous layer was adjusted to 3.0 by adding 3ml phosphoric acid (2.8M). IAA was extracted using another 10ml diethyl ether, which was again extracted with 10ml of 50mM K_2HPO_4. The pH of the solution was adjusted to 3 using phosphoric acid (0.28M). Lastly, IAA was extracted into a

final 10ml diethyl ether. The ether was evaporated to dryness under reduced pressure and the residue was dissolved in 2ml cold redistilled methanol.

The estimation was based on the principle that IAA reacts with tri-fluoro-acetic acid (TFA) and acetic anhydride resulting in indole-α-pyrone, which can be measured by spectro-fluorimetry. A known volume of the IAA extract was taken in a clean test tube and dried using nitrogen gas, about 0.2ml of ice cold TFA – acetic anhydride (1.1) reagent mixture was added and mixed thoroughly. The tubes were kept on ice exactly for 15min to ensure the complete conversion of IAA into indole-α-pyrone, the reaction was stopped by adding 3ml triple distilled water. Similarly, a blank was prepared by adding 3ml triple distilled water to a test tube with 0.2ml TFA – acetic anhydride. The IAA content was estimated by recording fluorescence (excitation at 440ηm and emission 490ηm) using a spectro-fluorimeter (Spectro-fluorophotometer, Shimadzu, RF 5301 PC). The values were expressed as ηg g^{-1} FW referring to a standard graph prepared by using standard IAA (Sigma-Aldrich, USA).

1.2.8. Extraction and assay of peroxidase enzyme

Peroxidase enzyme was extracted by homogenizing 50mg fresh tissue of hairy roots, in 1ml sodium phosphate buffer of pH 6.0 containing 1mM DTT and 0.1mM PMSF. After homogenization the homogenate was centrifuged at 12,000rpm twice and the supernatant was assayed for POD activity by following the method of Agostini et al (1997). Briefly, 1ml assay mixture was prepared which consisted 100µl of 1% H_2O_2, 100µl of 0.25% ortho-di-anisidine hydrochloride, 10µl of enzyme extract and 790µl of sodium phosphate buffer (pH 6.0). The change in OD per minute (dA min^{-1}) at 460ηm was recorded using kinetic programme in UV – visible spectrophotometer (Shimadzu UV – 160A) at 25°C. The results were expressed as units per gram fresh weight (U g^{-1} FW), using standard graph prepared by using standard horseradish POD obtained from ICN biochemicals, USA (RZ >3.0, Cat# 36451; Lot# 3117E). To quantify the extra cellular POD in the medium, the spent medium was taken and centrifuged twice at 12000rpm at 4°C to remove cell debris if any and the supernatant was assayed for enzyme activity.

1.2.9. Estimation of betalaines

Roots were homogenized (1g fresh weight in 100ml) in acidified methanol (adjusted to pH 5.2 using 0.1N HCl) using a Sorvoll cell homogeniser and centrifuged at 10,000×g at 4°C for 10min. The supernatant was analysed spectrophotometrically (Shimadzu, double beam) for Betacyanine and Betaxanthine (Nilsson, 1970). Betacyanine was monitored at

540ηm and betaxanthine at 480ηm and the total betalaine was quantified by adding the value of betacyanine with betaxanthine (Abeysekere et al. 1990; Nilsson, 1970). The suitability of this method was checked by analysing typical samples by HPLC.

1.2.10. High performance liquid chromatography (HPLC)

Analysis of betalaines was done by using appropriately dissolved (Thimmaraju et al. 2003a; 2003b) lyophilized hairy root extract in methanol at pH 5.2. Betaxanthine and betacyanine were separated on sephadex C-25 using citric acid-acetic acid (0.1% of each) aqueous solution. The fractions were lyophilized, re-dissolved in 1ml methanol (pH 5.2), passed through Millipore membrane (0.45μm) and C_{18} cartridge (Waters, USA) and subjected to HPLC analysis under the following conditions: Column: C_{18} Bondapak (4.6mm x 300mm), mobile phase; methanol-water (9:1), flow rate: 0.5mlmin^{-1}, column temperature: 25°C, detected at 480ηm for betaxanthine and 540ηm for betacyanine, with retention time of 5 and 7 min for betaxanthine and betacyanine respectively.

For analysis and quantification of sugars (glucose, fructose or sucrose) in the spent medium, HPLC was done using aminopropyl column (Shimadzu) with RI detector using HPLC-grade acetonitrile - water (7:3) at a flow rate of 1mlmin^{-1} (Prabha and Bhagyalakshmi 1998).

1.2.11. Growth and morphological observations

The growth of different clones in various studies was done gravimetrically and expressed as gram fresh weight produced per litre medium. The morphological changes were routinely observed under a stereomicroscope. The detailed record on root length, number of laterals per cm root, length of laterals, length from tip to branch point, thickness of the root and hairiness etc., were recorded.

1.2.12. Double transformation of the clone LMG-150

1.2.12.1. Establishment of kanamycin sensitivity of the clone LMG-150

MS solid medium containing filter sterilized kanamycin sulphate (Hi media Chemicals, India) @ 50, 100, 200, 300, 400ppm was prepared and about 100 pieces hairy roots of clone LMG 150 were cultured in each treatment. The cultures were continuously monitored for the growth. Observations were recorded at the end of 3rd and sixth week and expressed as per cent mortality.

1.2.12.2. Growth of *Agrobacterium tumefaciens* and transformation

Agrobacterium tumefaciens strain GV3101 harbouring plasmid constructs such as pPCV002 containing *rolABC* with CAMV 35S promoter, pPCV002 *rolB* with own promoter

and pPCV002 containing *rolC* under CAMV 35S promoter respectively having a gene for carbenicillin resistance as bacterial selection marker and *NPT*II as plant selection were kindly donated by Prof. Thomas Schmulling, Freie Universitat Berlin, Angewandte Genetik, Albrecht-Thaer-Weg 6, 14195 Berlin. The constructs containing different rol genes were as in presented in the fig 1.1. The bacterial cultures were activated by growing overnight in LB broth (NaCl-10g L^{-1}; Tryptone - 10g L^{-1}; Yeast extract - 5g L^{-1} pH adjusted to 7.5 before autoclaving) containing filter-sterilized carbenicillin (100ppm) added to the autoclaved medium cooled to 30˚C. The activated culture having OD of about 0.5 at 665ηm was used for co-cultivation of hairy root clone LMG-150. The co-cultivated cultures were further handled as described in the section 1.2.2, for initiation of hairy root clones. The antibiotic washed co-cultivated hairy root pieces were placed on MS solid medium containing 100-ppm of kanamycin. All antibiotics were filter-sterilized using 0.45µm Sortorius™ filters.

Figure 1.1. The ready to use constructs present in *A. tumefaciens* (GV3101) containing *rol* gene used for double transformation of the clone LMG-150

1.2.13. Statistical analysis

All the experiments involved at least six replicates unless mentioned otherwise. Fresh weight of hairy roots, their branching frequency on different media and pigment data were averaged from two separate experiments and further analyzed for variance using Microsoft Excel 2000 version. The results were presented as the mean ± SE (standard error)

1.3. RESULTS AND DISCUSSION

1.3.1. Transformation, initiation and establishment of different clones

Among the different explants used, cotyledonary leaf explant showed highest response in terms of transformation and initiation of hairy root clones with the transformation frequency ranging from 5-20% for all the strains of *A rhizogenes* (Table 1.1).

Table 1.1 Effect of ex plant type and *Agrobacterium* strain on induction of hairy roots

Strain	Total No. of explants	No. of explants showing HR* induction	% Transformation
Mature leaf			
LMG-150	100	-	-
A 20/83	100	-	-
A 2/83	100	10	10
A4	100	-	-
A15834	100	-	-
Hypocotyl			
LMG-150	100	-	-
A 20/83	100	-	-
A 2/83	100	8	8
A4	100	-	-
A15834	100	-	-
Cotyledonary leaves			
LMG-150	100	5	5
A 20/83	100	15	15
A 2/83	100	20	20
A4	100	15	15
A15834	100	10	10

* Hairy roots

The other explants such as mature leaf and hypocotyl showed 10 and 8% transformation respectively, for only one strain of *A rhizogenes* i.e., A 2/83 and no response to other strains. Among the cotyledonary leaf explants, the strain A 2/83 showed the best result with 20% transformation frequency. Of the several hairy root clones induced from different strains only ten clones obtained from cotyledonary leaf explants survived during selection on medium with antibiotic. Among the ten clones, one was that from the strain LMG-150 and three each from the strain A4 such as A4(1), A4(2), A4(3), three were from A 2/83 *viz*., A 2/83(1), A 2/83(2), A 2/83(3) and three from the strain A 20/83 *viz*., A 20/83(1),

A 20/83(2), A 20/83(3) (Fig 1.2). None of the clones induced by strain A15834 survived during selection process. Among the different explants of red beet and strains of *A. rhizogenes* used for induction of hairy root clones, cotyledonary leaf explants gave the best response with highest transformation frequency irrespective of the strain used. Such an observation has also been made in another study (Santarem et al, 1998).

1.3.2. Confirmation of Ri T-DNA integration and molecular characterization of hairy root clones with ISSR fingerprinting

The bacteria-free hairy root clones were confirmed for transformation by PCR analysis using a *rolA* specific primer. All the ten clones showed the presence of a 308bp *rolA* specific amplicon, which was reproduced when, tested twice confirming the stable integration of the Ri T-DNA (Fig 1.3A). Further, it was confirmed again by PCR southern analysis using 308bp *rolA* gene specific probe (Fig 1.3B). The clones were subjected for PCR analysis using an ISSR primer, which produced similar banding pattern in all the clones except for the presence of an additional faint band, which was specific for the clone A 2/83 (1) (Fig. 1.4). This test further validated the integrity of the clones with respect to ISSR loci and there were no major detectable differences among the clones with respect to the ISSR loci.

1.3.3. Growth pattern of different clones on hormone free MS-liquid medium

Fig 1.5 clearly indicates that there were clear cut differences among the different clones of hairy roots, where LMG-150 showed a typical sigmoid growth pattern with maximum biomass production on 20^{th} day of the culture period with an exponential phase ranging from 5^{th} to 20^{th} day reaching a stationary phase thereafter. The clones A 2/83(1), A 2/83(3), A 20/83(2) and A 20/83(3) produced maximum biomass on 15^{th} day reaching a stationary phase thereafter. Growth of the other four clones steadily increased from 5^{th} day and reached maxima on 25^{th} day indicating that these clones were able to continue the growth phase for longer duration than the rest of the clones (Fig 1.5).

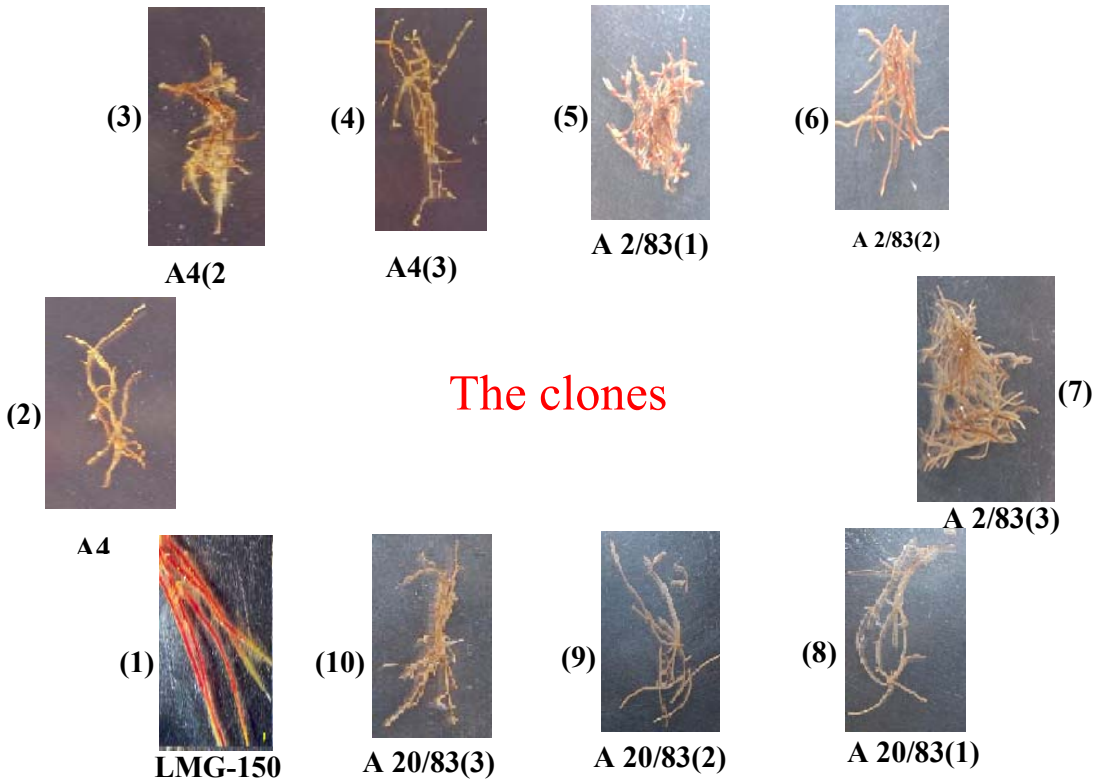
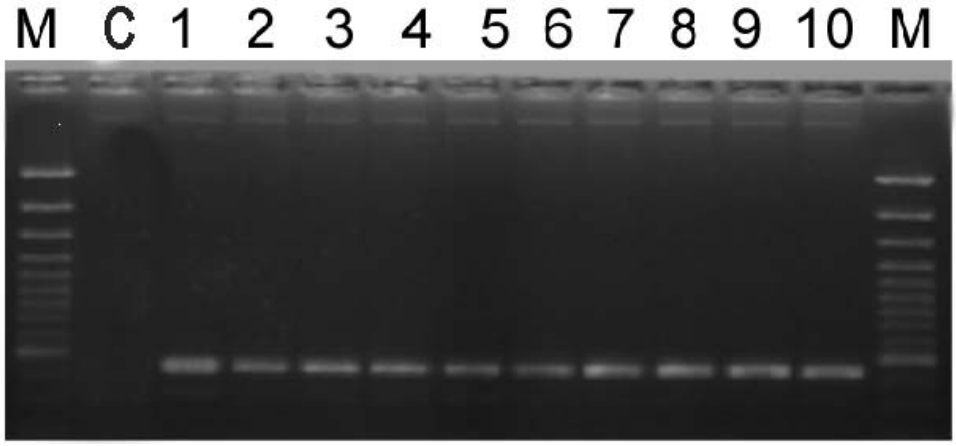


Figure 1.2. Ten different clones of hairy roots established using different *A. rhizogenes* strains such as LMG-150; A4; A 2/83; A 20/83. The clones grown on MS liquid medium were photographed on 20th day of the growth period showing variations among the clones with respect to pigment synthesis and other morphological properties.

A]



B]

Figure 1.3. Confirmation of transgenic nature of hairy root clones by PCR (A) and Southern analysis (B). Lanes: 1= LMG-150; 2=A4(1); 3=A4(2); 4=A4(3); 5=A 2/83(1); 6=A 2/83(2); 7=A 2/83(3); 8=A 20/83(1); 9=A 20/83(2); 10=A 20/83(3); M = Marker i.e., 100bp ladder; C = untransformed control from seedling explants. PCR was performed using *rol*A gene specific primer sets (forward- 5' AGA ATG GAA TTA GCC GGA CTA 3' and reverse- 5' GTA TTA ATC CCG TAG GTT TGT TT -3') (Sigma, USA), which were designed using Primer3 software. The amplified DNA was run on 1.5% (w/v) agarose gel to separate the DNA fragments, transferred to nylon membrane (BioBond^{TM}-Plus, Sigma). Southern analysis (B) was performed by using a 308bp psoralen biotin (Ambion Inc, USA) labelled fragment of *rol*A gene.

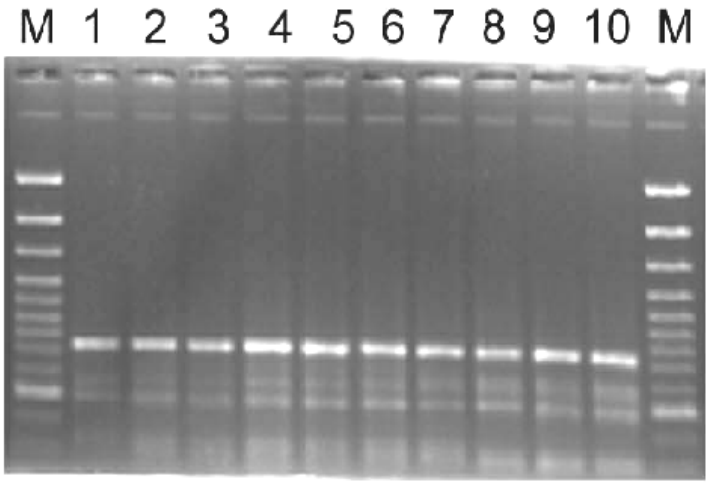


Figure 1.4. ISSR fingerprint for different clones of hairy roots amplified by using the primer $(GA)_8C$. Lanes: 1=LMG-150; 2=A4(1); 3=A4(2); 4=A4(3); 5=A 2/83(1); 6=A 2/83(2); 7=A 2/83(3); 8=A 20/83(1); 9=A 20/83(2); 10=A 20/83(3); M = Marker i.e., 100bp ladder.

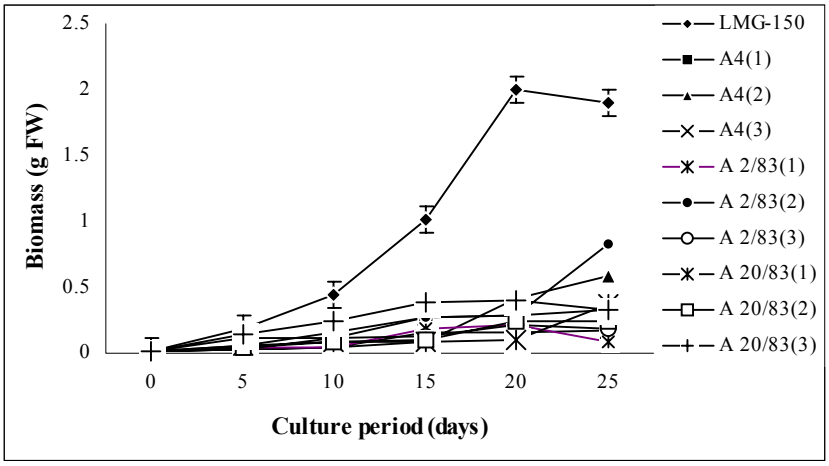


Figure 1.5. Growth curves showing biomass accumulation in different clones of hairy roots grown in 50ml conical flasks containing 15ml MS liquid medium. The values are the average of six replicates of two independent experiments.

1.3.4. Biomass accumulation and pigment synthesis

The pattern of biomass accumulation and pigment synthesis significantly varied from one clone to another. Growth and pigment synthesis among the different clones showed high variation. The clone LMG-150 produced highest biomass of more than 130g L^{-1} FW in MS liquid medium without any hormones. Upon supplementation of 0.5 and 1.0ppm IAA to the same clone a significant reduction in biomass was observed accounting to less than 15g L^{-1} whereas other auxin NAA supported a higher biomass of 100g L^{-1} (Fig. 1.6A). The clone A4(1) was the next best clone in terms of biomass production where a highest of about 80g L^{-1} FW on MS with 1 ppm of NAA was recorded which was followed by the clone A 2/83(2) where a highest biomass was 60g L^{-1} FW on medium with IAA. Rest of the clones produced poor biomass accounting for less than 40g L^{-1} on different media with significant variations in growth performance. None of the clones showed any significant improvement in growth when grown on MS liquid medium containing 2ppm of tryptophan (trp) -a precursor for IAA synthesis (Fig. 1.6A).

Pigment synthesis varied significantly in each hairy root clone on different auxin containing media. The highest biomass producing clone, LMG-150, produced highest pigments on hormone-free MS liquid medium amounting for nearly 200mg L^{-1} betalaine. This was followed by A 2/83(1), which produced over 90mg $L^{-1}$of betalaine however in the presence of 1ppm NAA. In most of the other clones the pigment synthesis was completely suppressed. The auxin NAA enhanced pigment synthesis in a few clones such as A 2/83(1) A4(3) when compared to other medium whereas the other auxin, IAA, appeared to completely suppress pigment synthesis in almost all the clones. The clone A 2/83(3), as an exception, produced highest betalaine content of about 20m $gL^{-1}$when grown in MS liquid medium containing tryptophan (2-ppm) - a precursor of IAA though rest of the clones did not show any response to similar treatment (Fig 1.6B).

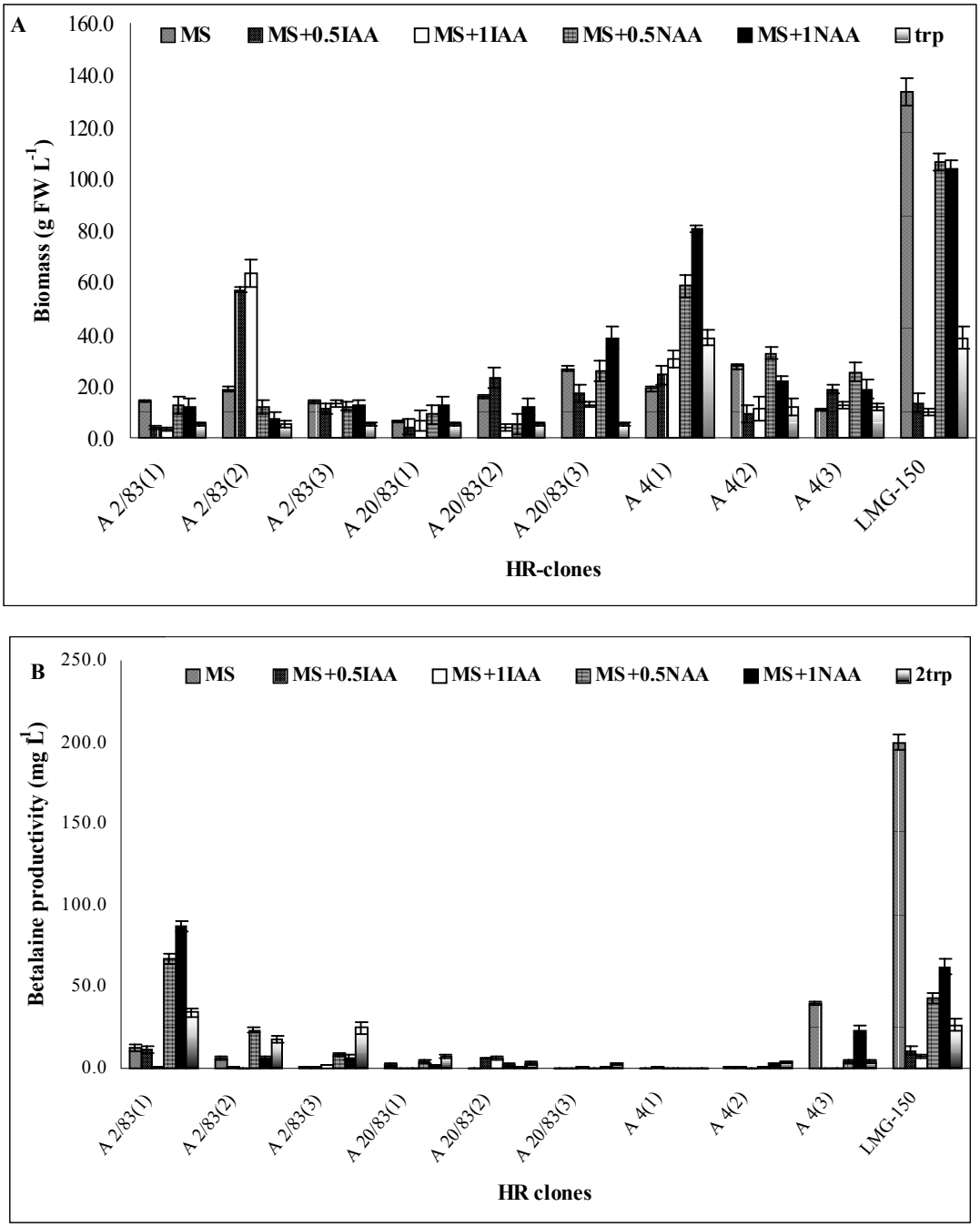


Figure 1.6. Biomass accumulation (A) and the productivities of betalaines (B) in different clones of hairy roots grown on Hormone free MS liquid medium (MS) and MS with 0.5 and 1ppm IAA, MS with 0.5 and 1ppm NAA and MS with 2ppm tryptophan. The data is the mean of six replicates of two independent experiments vertical bars represent SE (standard error of means).

1.3.5. Morphological response

All the ten clones (Fig 1.2) were characterized for different morphological characters such as colour, thickness, hairiness, length of the primary root, the length from tip upto first branch point, number of laterals per cm length, length of the laterals, etc.,. The different clones on MS liquid medium without any hormone showed significant morphological variations. The clone LMG-150 produced highest number of laterals of about 9.20 per cm length of the primary root with red pigmentation. Lesser degrees of these qualities in other clones - A 20/83(3), A4(1), A4(2), A4(3) were observed. The clone A 2/83(2) produced a highest number of about 8.0 lateral branches per centimeter length of the root followed by A 2/83(3) and A 20/83(2). The number of laterals produced by rest of the clones ranged from 2.25 to 4.50. Except the clone LMG-150 that was deep red in colour, the appearance in other clones ranged from white to pale yellow to dull red due to difference in pigment synthesis. Almost all the clones, except LMG-150, A 2/83(2) and A4(2), produced very thin roots. Most of the clones had dense hairs distributed all over the surface except for the clones derived from A 2/83 (Table1.2).

Supplementation of natural auxin (IAA) into culture medium (MS) generally suppressed the growth and pigment synthesis in clone LMG-150. Contrarily, the other clones showed slight improvement in terms of overall growth resulting in increase in root length, number and length of the lateral roots. There was also a considerable improvement in the synthesis of pigment in most of the clones at an IAA level of 0.5 ppm and a higher concentration was suppressive (Table 1.3).

Table 1.2 Growth and morphology of hairy root clones on hormone free MS liquid medium (20 days after inoculation)

HR –Clone	Root* Length (cm)	Length from tip to Br. point (cm)	No. of laterals cm^{-1}	Length of the Laterals (cm)	Colour	Thickness	Hairiness
MS							
LMG-150	7.2 ± 0.04a	2.90 ± 0.003a	9.20± 0.05a	2.36 ± 0.01a	Red	Thick	Dense
A 2/83(1)	1.2± 0.01d	0.25± 0.001d	2.25± 0.03d	0.35± 0.02e	Yellow	Thin	Dense
A 2/83(2)	1.1± 0.01d	0.35± 0.01d	8.25± 0.04a	0.95± 0.01c	Slight red	Thick	Sparse
A 2/83(3)	1.0± 0.01d	0.30± 0.001d	6.00± 0.04b	0.80± 0.03d	Slight red	Thin	Sparse
A 20/83(1)	1.6± 0.01d	0.50± 0.002c	3.50± 0.03c	0.63± 0.01d	White	Very thin	Sparse
A 20/83(2)	0.9± 0.005	0.30± 0.01d	5.25± 0.04b	0.35± 0.01e	White	Thin	Dense
A 20/83(3)	2.7± 0.02b	1.03± 0.02b	4.00± 0.03c	0.23± 0.001e	White	Thin	Dense
A4(1)	3.0± 0.02b	1.15± 0.01b	2.75± 0.03d	0.80± 0.001d	Yellow	Thin	Dense
A4(2)	2.1± 0.02c	0.67± 0.001c	4.50± 0.03c	0.93± 0.02c	White	Thick	Dense
A4(3)	2.2± 0.02c	1.08± 0.01b	3.50± 0.03c	0.43± 0.01e	White	Thin	Dense

Table 1.3 Effect of IAA on growth and morphology of hairy root clones (20 days after inoculation). The Values are the mean ± SE (standard error) of six replicates and the values represented by same letter with in the same column are not significant.

HR Clone	Length (cm)	Length from tip to Br. point (cm)	No. of laterals / cm	Length of the Laterals	Colour	Thickness	Hairiness
0.5ppm IAA							
LMG-150	5.30 ± 0.04a	1.00± 0.01a	5.10± 0.03c	0.9± 0.001a	Red	Thick	Dense
A 2/83(1)	1.18 ± 0.02d	0.23± 0.001c	2.50± 0.01d	0.25± 0.002d	Red	Slender	Sparse
A 2/83(2)	1.10 ± 0.01d	0.28± 0.002c	8.50± 0.04b	0.48± 0.001c	Purple	Thick	Dense
A 2/83(3)	1.00 ± 0.01d	0.20± 0.001c	7.00± 0.04b	0.40± 0.001c	Red	Thick	Dense
A 20/83(1)	2.20 ± 0.02c	0.55± 0.003b	2.75± 0.01d	0.83± 0.001b	White	Slight thick	Dense
A 20/83(2)	2.00 ± 0.03c	0.55± 0.004b	11.00± 0.04a	0.50± 0.002c	Purple	Thick	Dense
A 20/83(3)	2.70 ± 0.01b	0.93± 0.001a	2.25± 0.02d	0.23± 0.001d	Light red	Slender	Dense
A4(1)	3.10 ± 0.01b	0.55± 0.002b	4.75± 0.03c	0.95± 0.001a	Yellowish red	Thick	Dense
A4(2)	3.08 ± 0.02b	0.40± 0.003b	2.25± 0.01d	0.43± 0.01c	Yellowish	Slight thick	Dense
A4(3)	3.20 ± 0.01b	1.10± 0.01a	2.50± 0.01d	0.93± 0.02a	White with red spots	Thick	Dense
1ppm IAA							
LMG-150	5.50± 0.04a	1.20± 0.01a	4.60± 0.03c	0.40± 0.01b	White	Thin	Dense
A 2/83(1)	1.05± 0.01d	0.30± 0.001f	2.25± 0.01e	0.25± 0.02c	Yellowish red	Thin	Dense
A 2/83(2)	1.10± 0.02d	0.38± 0.001e	5.25± 0.01b	0.48± 0.01a	Red	Thick	Dense
A 2/83(3)	1.11± 0.02d	0.40± 0.02e	5.20± 0.02b	0.50± 0.03a	Red	Thick	Dense
A 20/83(1)	1.12± 0.01d	0.30± 0.01e	2.10± 0.01e	0.61± 0.02a	slight red	Thin	Dense
A 20/83(2)	2.80± 0.03c	0.77± 0.01c	2.25± 0.01e	0.50± 0.03a	White	Thin	Dense
A 20/83(3)	2.80± 0.02c	0.93± 0.01b	2.25± 0.03e	0.35± 0.01b	White	Thin	Dense
A4(1)	3.03± 0.03b	0.93± 0.01b	6.50± 0.03a	0.55± 0.01a	Yellowish red	Thick	Dense
A4(2)	1.72± 0.01d	0.83± 0.02c	3.00± 0.02d	0.23± 0.02c	White	Thin	Dense
A4(3)	2.73± 0.02c	0.53± 0.03d	4.50± 0.02c	0.50± 0.03a	Yellowish red	Thick	Dense

Table 1.4 Effect of NAA on growth and morphology of hairy root clones (20 days after inoculation). The Values are the mean ± SE (standard error) of six replicates and the values represented by same letter with in the same column are not significant.

HR -Clone	Length (cm)	Length from tip to Br. point (cm)	No. of laterals / cm	Length of the Laterals (cm)	Colour	Thickness	Hairiness
0.5ppm NAA							
LMG-150	5.40± 0.03a	1.00± 0.01b	5.60± 0.03a	1.00± 0.01a	Yellowish red	Thick	Dense
A 2/83(1)	1.20± 0.02c	0.43± 0.001c	2.25± 0.01d	0.35± 0.002c	Purple	Thick	Dense
A 2/83(2)	1.75± 0.01c	1.12± 0.02b	1.75± 0.02d	0.50± 0.02b	Yellowish	Thin	Dense
A 2/83(3)	0.90± 0.01d	0.30± 0.001d	3.50± 0.01c	0.25± 0.001d	Slight purple	Thick	Dense
A 20/83(1)	1.35± 0.02c	0.28± 0.002d	6.25± 0.01a	0.30± 0.003c	White	Very thin	Dense
A 20/83(2)	0.98± 0.01d	0.13± 0.003e	5.00± 0.03b	0.35± 0.001c	Slight red	Slight thick	Dense
A 20/83(3)	3.60± 0.03b	1.48± 0.01a	3.75± 0.03c	0.48± 0.003b	Slight red	Slight thick	Dense
A4(1)	2.02± 0.01c	0.20± 0.001d	5.25± 0.03b	0.37± 0.001c	Yellowish red	Thick	Dense
A4(2)	2.05± 0.02c	0.28± 0.002d	2.25± 0.01d	0.25± 0.003d	Yellowish	Thick	Dense
A4(3)	2.05± 0.01c	0.33± 0.003d	2.00± 0.01d	0.38± 0.001c	Purple	Thick	Dense
1ppm NAA							
LMG-150	5.70± 0.04a	1.20± 0.03a	2.10± 0.02c	0.40± 0.01c	Yellowish red	Thick	Dense
A 2/83(1)	1.35± 0.02d	0.40± 0.01b	2.25± 0.01c	0.35± 0.02d	Purple	Thin	Dense
A 2/83(2)	1.35± 0.02d	0.45± 0.02b	1.50± 0.03d	0.75± 0.03b	Yellowish	Very thin	Dense
A 2/83(3)	1.18± 0.03d	0.30± 0.01c	4.00± 0.04b	0.28± 0.001e	Purple	Thick	Dense
A 20/83(1)	0.98± 0.01e	0.13± 0.001d	4.75± 0.05b	0.30± 0.01d	White	Very thin	Dense
A 20/83(2)	2.45± 0.03c	0.35± 0.001c	6.80± 0.04a	0.35± 0.002d	Purple	Thick	Dense
A 20/83(3)	3.10± 0.03b	1.40± 0.002a	4.50± 0.03b	1.18± 0.03a	Dark purple	Thick	Dense
A4(1)	1.98± 0.01d	0.13± 0.001d	7.25± 0.04a	0.33± 0.001d	Yellowish red	Thick	Dense
A4(2)	1.45± 0.01d	0.30± 0.02c	4.50± 0.03b	0.40± 0.01c	Yellowish red	Thick	Dense
A4(3)	2.03± 0.03c	0.46± 0.01b	4.25± 0.02b	0.48± 0.02c	Purple	Thin	Dense

In the presence of NAA at two different concentrations a slight improvement in growth, morphology and pigment synthesis was observed in clones A 20/83(3) and A 20/83(2) with variable effects in other clones (Table 1.4). The results were almost similar when tryptophan (2-ppm) was supplemented (Table 1.5).

All in all, the external supplementation of auxin or IAA precursor (tryptophan) did not cause any significant improvement in terms of growth morphology and pigment synthesis. None of the hairy root clones functioned better than clone LMG-150 in terms of growth, morphology and pigment synthesis under various treatment conditions. Bais et al (1999) made similar observations in chicory hairy root cultures where the clones produced by LMG-150 showed highest growth rate, biomass and metabolite production.

Table 1.5. Effect of tryptophan on growth and morphology of hairy root clones (20 days after inoculation). The Values are the mean ± SE (standard error) of six replicates and the values represented by same letter with in the same column are not significant.

HR -Clone	Length (cm)	Length from tip to Br. point (cm)	No. Of laterals/ cm	Length of the Laterals	Colour	Thickness	Hairiness
2ppm tryptophan							
LMG-150	6.50± 0.04a	1.60± 0.03a	6.00± 0.04a	1.50± 0.01a	Yellowish red	Thick	Dense
A 2/83(1)	0.75± 0.01c	0.30± 0.001c	2.50± 0.02c	0.28± 0.001e	Yellowish red	Very thin	Sparse
A 2/83(2)	1.80± 0.01b	1.33± 0.02a	1.25± 0.01d	0.65± 0.002c	Yellowish	Very thin	Sparse
A 2/83(3)	0.75± 0.02c	0.48± 0.01a	3.00± 0.03c	0.38± 0.001d	Purple	Thick	Dense
A 20/83(1)	1.30± 0.03c	0.30± 0.003a	2.25± 0.02c	0.38± 0.003d	White	Very thin	Sparse
A 20/83(2)	1.30± 0.01c	0.18± 0.001c	5.00± 0.04b	0.10± 0.001f	White	Thin	Sparse
A 20/83(3)	3.03± 0.03b	1.05± 0.02a	1.75± 0.01d	0.90± 0.003b	White	Very thin	Sparse
A4(1)	2.30± 0.03	0.43± 0.002a	5.25± 0.03b	0.45± 0.001d	Yellowish red	Thick	Dense
A4(2)	2.03± 0.02 b	0.40± 0.001a	4.75± 0.03b	0.25± 0.002e	Yellowish red	Thick	Dense
A4(3)	1.40± 0.01c	0.47± 0.001a	4.25± 0.02b	0.38± 0.001d	Yellowish red	Thick	Dense

1.3.6. Endogenous IAA level

Fig 1.7 shows the endogenous IAA level on 15th day of the growth period. Among the different clones, LMG-150 showed highest endogenous IAA of about 245ηg g^{-1} FW followed by A4(3), A4(1) and A 20/83(3) whereas the other clones A4(2), A 2/83(2), A 20/83(1), A 2/83(3), A 20/83(2) and A 2/83(1) showed the same in a decreasing order. In general higher endogenous IAA level positively correlated with the biomass and not with pigment synthesis. It is well documented that the endogenous IAA content is largely the determinant of growth and development of transformed root cultures (Tanaka et al. 2001). The *rol* genes present on the T-DNA of *A. rhizogenes* in turn are known to influence endogenous IAA content (Schmulling et al. 1993). The endogenous auxin content estimated for individual clones showed positive correlation and seemed to directly affect the growth performance in medium without exogenous auxin in hairy root lines of Egyptian clover (Tanaka et al. 2001).

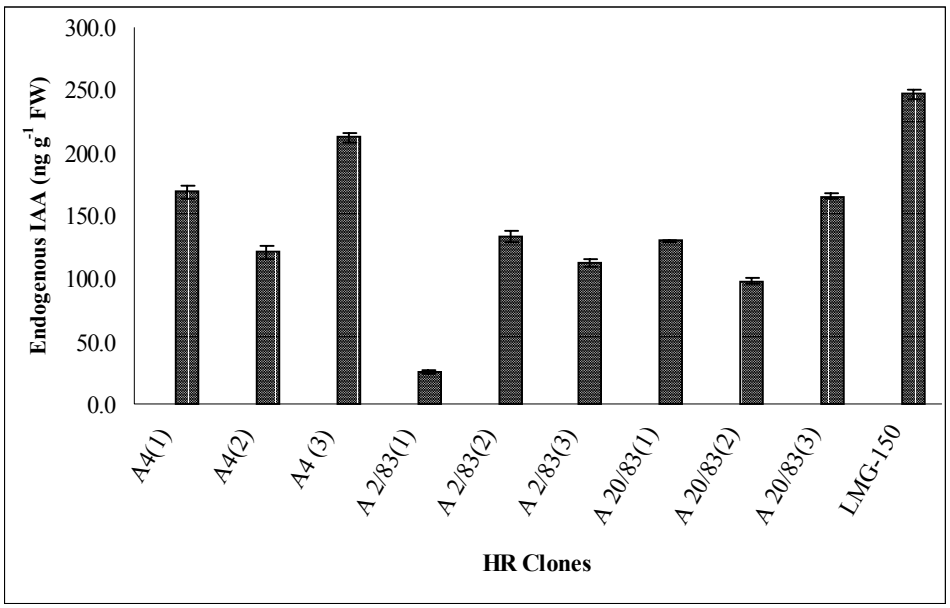


Figure 1.7. Endogenous auxin content in different clones estimated on 20th day of culture period. The values are the average of six replicates of two independent experiments. Vertical bars indicate standard error of mean.

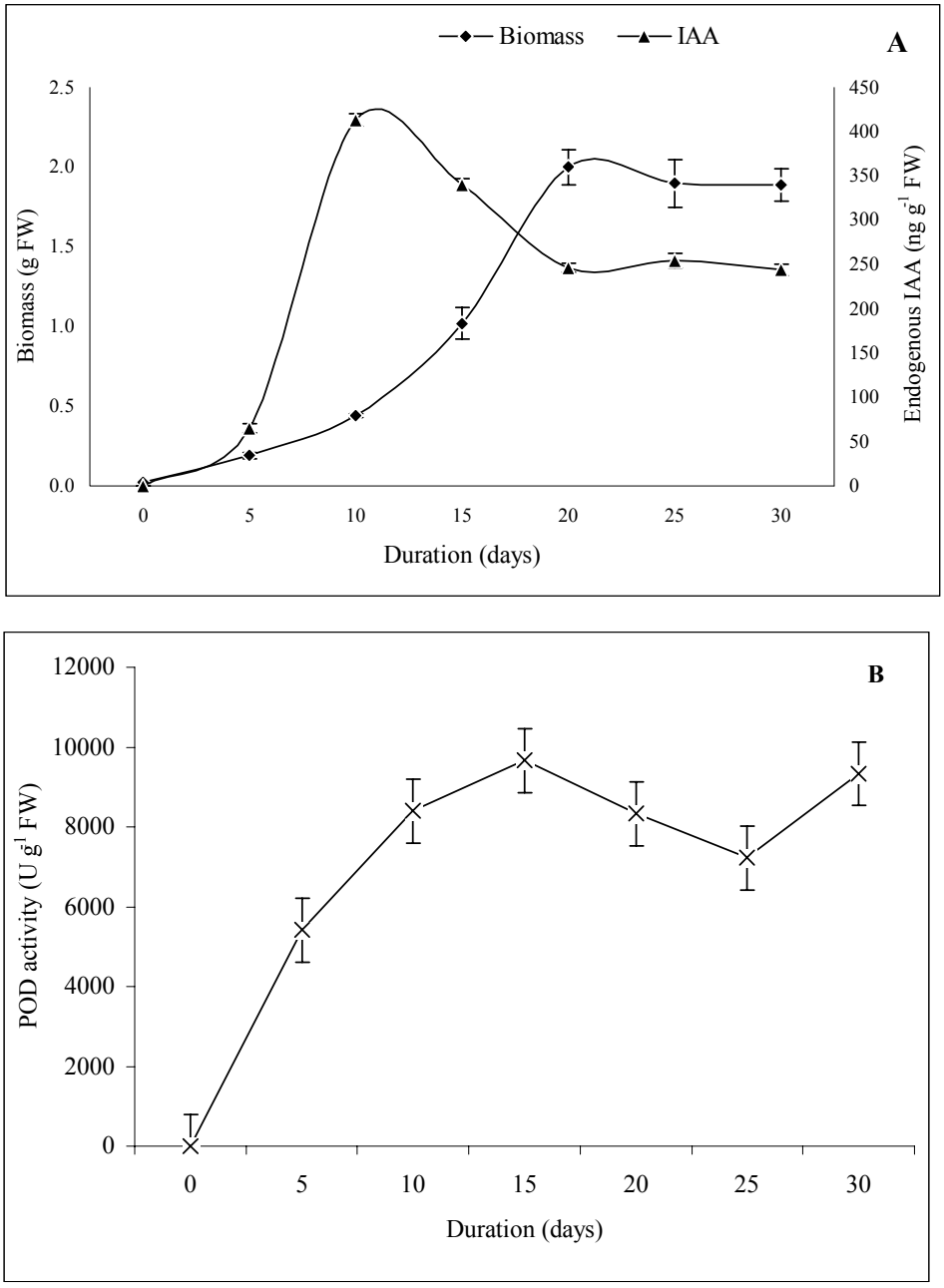


Figure 1.8. Level of endogenous IAA content, Biomass accumulation (A) and peroxidase enzyme (B) estimated at different growth stages in the clone LMG -150. The values are the mean of six replicates of two independent experiments. Vertical bars indicate standard error of mean.

1.3.7. Endogenous IAA and peroxidase activity at different growth stages in LMG-150

The level of peroxidase enzyme and endogenous auxin content in the best growing clone LMG-150 studied over a growth period of 30 days is presented in the Fig 1.8A & B. The IAA content increased rapidly in the active growth stage form 5^{th} day and reached maximum on 10^{th} day (> 400ηg g^{-1} FW). The IAA content decreased further and reached the plateau on 20^{th} day during which time the biomass reached the peak (on 20^{th} day) followed by a slight decrease reaching a plateau thereafter. The activity of peroxidase, on the other hand, increased steadily showing an activity of 9000U g^{-1} FW on 15^{th} day and increased further on 20^{th} day reaching 10000U g^{-1} FW with a decline further and there was again an increase at the end of the growth phase (Fig 1.8 A & B).

It is well known that peroxidases are a set of key enzymes that are involved in the degradation of endogenous IAA (Pressey, 1990). The assay for peroxidase in different clones on 20^{th} day of the culture showed an inverse correlation between the endogenous IAA pools, indicating its possible role in the degradation of IAA. The clone LMG-150 was characterized for endogenous IAA, level of POD and biomass production at various growth stages. The observations indicated that endogenous IAA pools increased steadily from the beginning reaching a peak on 10^{th} day with a decrease thereafter correlating well with peroxidase levels whereas during subsequent period the latter reached maximum on 15^{th} day of the growth period. With these results one can suspect that the endogenously accumulated IAA might be subjected to degradation by increased POD causing a down-regulation of endogenous IAA. However the results also indicate that the amount of IAA produced upto 10^{th} day may be sufficient to propel the hairy root cultures further towards growth and metabolism resulting in the maximum biomass production on 20^{th} day. These results also indicate the possible role of POD in the regulation of endogenous IAA levels in red beet hairy roots as observed in other such studies (Liu et al. 1996; Lagrimini, 1996).

1.3.8. Southern blot analysis of hairy root clones

Southern hybridization of the *Hind-III* digested genomic DNA of individual clones was carried out using a psoralen-biotin labeled 308bp *rolA* gene specific probe. The results shown in the figure 1.9 revealed that most of the clones, except A 2/83(1), had at least two hybridization signals indicating the presence of at least one T-DNA copy insert whereas, the clone A 2/83(1) (lane 5 Fig 1.9) showed 4 hybridization signals indicating the presence of at least two copies of the T-DNA inserts.

The figure 1.9 also showed a significant variation in the size of the hybridization signals indicating the possible occurrence of independent transformation events in each clone.

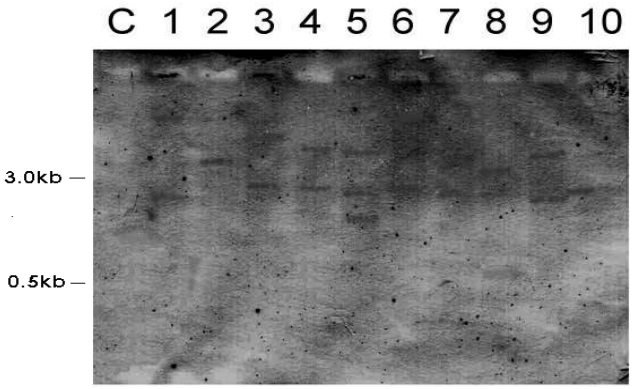


Figure 1.9. Molecular analysis of T-DNA in the genomic DNA of different hairy root clones transformed with *A. rhizogenes*. The DNA was digested with *Hind*-III to provide a single restriction within the T-DNA. The blot was probed with non-radioactive alkaline phosphatase - labelled 308bp PCR amplified *rolA* gene fragment, where presence of two bands equals to one copy. Lanes 1-10; 1=LMG-150; 2=A4 (1); 3=A4(2); 4=A4(3); 5=A 2/83(1); 6=A 2/83(2); 7=A 2/83(3); 8=A 20/83(1); 9=A 20/83(2); 10=A 20/83(3); M = Marker i.e., 100bp ladder; C = untransformed control from seedling explants.

Higher the number of T-DNA copies meaning harboring of more number of genes involved in the synthesis of IAA (Inze et al, 1984; Camilleri and Jouanin 1991) where one can expect changed growth and metabolic pattern in such hairy root clones. However in the present study southern analysis of different clones, though showed at least one copy of the T-DNA insert in all the clones and two T-DNA insert in clone A 2/83(1), there was no positive correlation with the growth, metabolite and endogenous auxin level. This observation made for the first time clearly indicates that mere presence of higher number of T-DNA copy inserts would not cause any increase in growth and production of secondary metabolites.

1.3.9. Double transformation of LMG-150

The *rol* genes of *A. rhizogenes* are known to cause varied effects on plant morphology and metabolism (Schmulling et al, 1988; 1989) including secondary metabolite production. An attempt was made to re-transform already transformed hairy root clone LMG-150 for pyramiding with additional *rol* genes. Initially the clone was screened for the sensitivity to various levels of kanamycin to select a best concentration for the selection of

double transformants. The results presented in the table 1.6 indicate that the kanamycin at 100ppm is ideal for selection of transformants. Therefore MS medium with 100ppm kanamycin was used for further screening of the transformants.

Table 1.6. Sensitivity of the clone LMG-150 to different levels of kanamycin

Kanamycin (ppm)	No. of HR pieces inoculated	No. Survived	% Mortality	Remarks
3 Weeks after inoculation				
50	100	20	80	Red
100	100	1	99	Red
200	100	0	100	Dark
300	100	0	100	Dark
400	100	0	100	Dark
6 Weeks after inoculation				
50	100	8	92	All the roots turned dark
100	100	0	100	
200	100	0	100	
300	100	0	100	
400	100	0	100	

The results indicated that the hairy roots can be double transformed and few putative double transformed clones for additional *rolABC* and *rolC* genes were obtained (Fig 1.10). Many double transformation experiments for hairy root cultures reported so far involved the transformation of more than one gene to the seedling explants and generating hairy root cultures from the transformed explants (Zhang et al. 2004**).** But in the present study the transformed root cultures were directly used to incorporate additional *rol* genes.

The double transformation in clones with additional *rolABC and rolC* genes were confirmed by PCR analysis for *NptII i.e.,* the gene which confer kanamycin resistance to the clones (Fig 1.11). Further the clones were also studied for growth and pigment synthesis in comparison with single transformant LMG-150 clone. The results presented in the figure 1.12 showed that the growth and pigment synthesis was higher in LMG-150 retransformed with additional *rolABC* genes. This improvement in growth and pigment production may be because of the increased length of the primary roots and increased branching resulting in overall improvement in growth and pigment production.

Control (kan100) **Putative transformants**

Figure 1.10. Putative double transformants of LMG-150 with additional *rolABC* genes growing on MS medium with 100ppm kanamycin. (Petri dish of 90mm dia.)

However, the results were not encouraging when the clone was re-transformed with an additional *rolC* gene as there was decrease in growth and pigment synthesis when compared to control LMG-150 and *rolABC* transformed clones. Hence, The *rol A, -B, -C*, genes when present together might act coordinately to bring about improved biomass and pigment synthesis whereas the *rolC* might antagonize the function of already existing *rol*ABC of LMG-150, resulting in the suppression of growth and performance. Increased tropane alkaloid production was observed when hairy root cultures of *Brugmansia candida* were re-transformed with additional *rol* genes (Zhang et al. 2004).

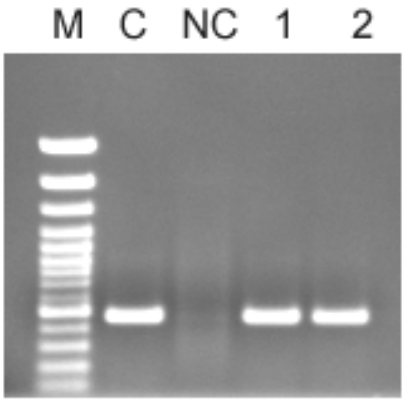


Figure 1.11. Confirmation of double transformation and integration of additional *rol* genes in to the clone LMG-150. PCR analysis was done by using *NPT-II* specific primer, Lanes: M= Marker; C= Vector control; NC = LMG-150 (single transformant); 1= LMGABC (LMG-150 transformed with additional *rol*ABC genes); 2= LMGC (LMG-150 transformed with additional *rol*C gene)

Table 1.7. Growth and morphology of single and double transformed hairy root clones on hormone free MS liquid medium (20 days after inoculation)

HR -Clone	Root* Length (cm)	Length from tip to Br. point (cm)	No. of laterals cm^{-1}	Length of the Laterals (cm)	Colour	Thickness	Hairiness
MS							
LMG-150	7.2 ± 0.04	2.90± 0.003	9.20± 0.05	2.36± 0.01	Red	Thick	Dense
LMG-ABC	8.6± 0.06	3.10 ± 0.004	13.1± 0.006	2.51± 0.006	Red	Thick	Dense
LMG-C	6.6± 0.03	1.90± 0.004	8.3± 0.005	1.91± 0.006	Red	Thick	Dense

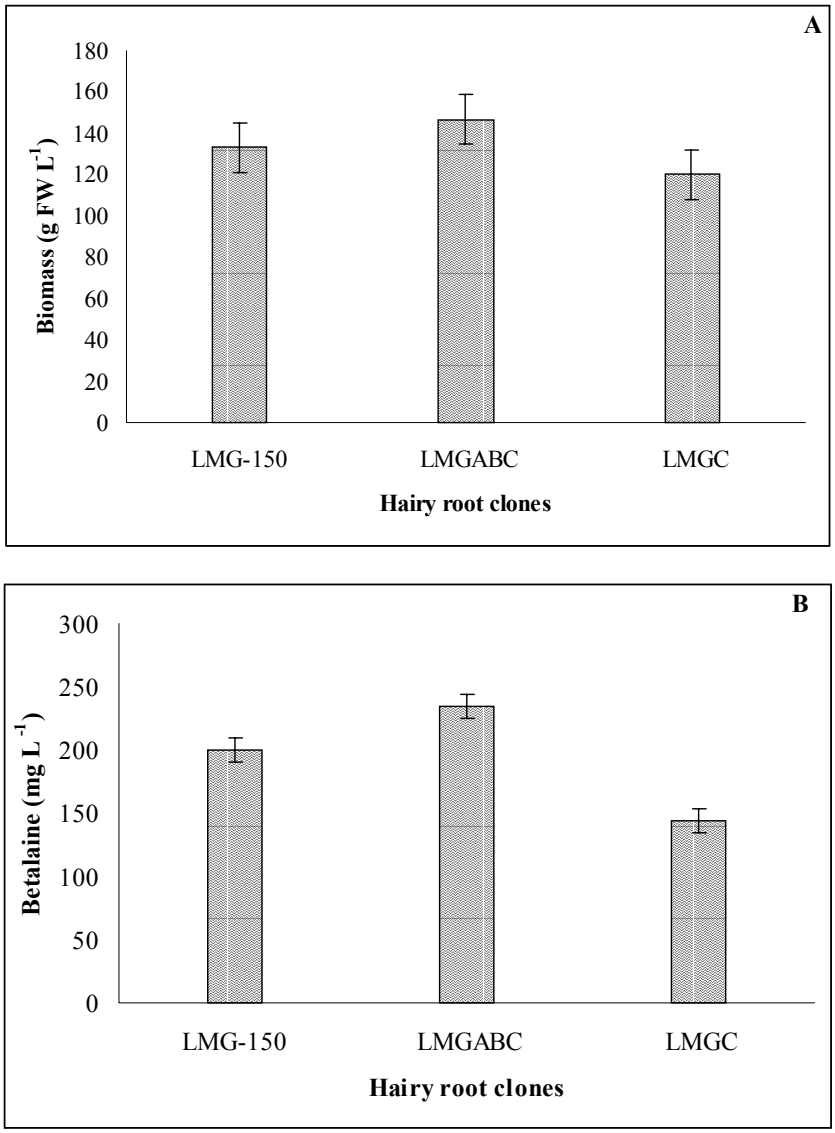


Figure 1.12. Biomass (A) and betalaine production (B) in hairy root clone (LMG-150) re-transformed with *rol*ABC (LMG-ABC) and *rol*C genes (LMG-C). Data is the average of six replicates and the vertical bars represent the standard error of mean.

In conclusion, defferent transformed clones were established and evaluated for various growth and physiological parameters to select a best clone for the production of betalaines. The chapter also considered studies on various other parameters such as endogenous auxin, peroxidase levels, T-DNA copy number and their influence on growth and metabolism of hairy root cultures. In addition, the clones were evaluated for their growth and metabolism in various auxin-containing medium, apart from the tryptophan-

supplemented medium. The double transformation of the hairy root clones was also demonstrated and the influence of additional *rol* genes on growth and pigment production was documented for the first time. Since this clone showed high variability after several subcultures probably due to its high sensitivity to temperature, it could not be considered for further studies. Instead, the single transformant clone LMG-150, that showed stable growth-rate and betalaines production on hormone-free medium was finally selected for further work on various process parameters that are dealt in detail in subsequent chapters.

Chapter highlights

- **Ten clones of genetically transformed roots were established**
- **The clones were characterized for growth morphology and pigment synthesis, selected LMG-150 as the best clone for growth and pigment synthesis**
- **The role of endogenous IAA in growth and development of hairy root clones was established and the role of peroxidase in regulating the endogenous IAA pools was also established**
- **It has been shown that the hairy roots can be double transformed**

CHAPTER 2

OPTIMIZATION OF GROWTH CONDITIONS AND SCALE UP

CONTENTS

SUMMARY

The superior clone LMG -150 was used for further studies on optimizing the important growth conditions such as culture volume, carbon source and scale up using bioreactor for improved mass transfer. Among the different culture volumes studied, the hairy root growth was highest in 250ml flask containing medium with decrease in biomass production in higher volumes. The hydrodynamic pressure exerted on hairy roots at different position in the flask was calculated which indicated that there was increase in hydrodynamic stress with increase in culture volume the results are same with increase in shaker speed indicating the influence of hydrodynamic stress acting on the roots on the growth and development. Upon subjecting to treatments containing different sugars (3% w/v) it was found that sucrose was rapidly utilized, followed by maltose with a very limited use of glucose, but the other hexoses – fructose, lactose, xylose and galactose or glycerol totally suppressed both growth and betalaine synthesis. Therefore, the sucrose was selected as best carbon source for the growth and metabolism. The studies on adaptability, uptake and the response of hairy roots to hexoses in the presence of sucrose showed the signaling role of sucrose in addition to acting as nutrient. Red beet hairy roots appear useful as a model system to study sugar metabolism / signaling due to their sensitivity, to different sugars that may directly link to morphological changes and betalaine synthesis. Scale up studies using different bioreactors showed that the bubble column reactor with an attached medium-containing vessel for air enrichment gave better growth and biomass production when compared to bubble column reactor alone.

Publication

Bhagyalakshmi N, Thimmaraju R and Narayan MS (2004). Various Hexoses and di-hexoses differently influence growth, morphology and pigment synthesis in transformed root cultures of red beet (*Beta vulgaris* L.). Plant Cell Tissue and Organ Culture 78: 183 -195.

2.1. INTRODUCTION AND REVIEW OF LITERATURE

The dynamic environment within the large culture vessel (bioreactor) is known to significantly influence the performance of cells, and more so for organ cultures such as hairy roots due to their continuously changing rheology. The existing complications, particularly the use of impeller for agitated cultures in a bioreactor, will worsen the situation. When higher culture volumes are considered, the decrease in growth rate is linked to high initial substrate concentration causing high ionic strength, osmotic pressure or overloading of membrane transport system (Clark and Blanch 1999). In addition, metabolites that may not be toxic at normal substrate levels may accumulate causing either growth suppression or inhibit the transport of required metabolite. To understand such behavior, downscaling the experiments to simple models become essential. Such models planned for step-wise increase in volume, similar to fed batch cultivation, can simplify the complex set of conditions involved in costly pilot-scale experimentation aimed towards process development. In shake-flask experiments, cells/tissues/organs are exposed to spatially uniform environment so that the product formation does not deviate drastically within the container. These assumptions are often adequate for describing the behaviour of large-scale industrial reactors, even though there may be deviations from the ideal mixing and uniform substrate distribution (nutrient and air) within the vessel (Meijer et al. 1994). Thus gyratory flask can be a versatile tool for studying long-term effects of hydrodynamic stress on plant cells. Also, imposing stress by steadily increasing volumes (hydrodynamic stress) may result in shear-tolerant clones.

In higher plants the nutrients function as substrates for growth and also act as signals triggering cascades of both primary and secondary metabolic events (Koch, 1996; Sheen et al. 1999; Smeekens, 1998, 2000; Gibson, 2000). Many plant genes are controlled by sugars that are involved in a variety of processes such as photosynthesis, storage of protein/starch/lipid and production of homo- and hetero-polysaccharides (Nakamura et al. 1991; Karrer and Rodriguez, 1992; Krapp et al. 1993; Mc Laughlin and Smith, 1994; Sheen et al. 1999; Gibson, 2000; Koch et al. 2000; Winter and Huber, 2000). Sugars are also known to interact with several growth regulators (Lazzeri et al. 1988) leading to the changes in the array of morphological events (Kraemer et al. 2002). Sucrose metabolism is the first step in carbon assimilation in the majority of the carbon importing cells (Koch et al. 2000) in autotrophic and also sucrose dependent heterotrophic cell cultures.

One plant system suited for the study of sugar response would be the cultured root due to its heterotrophic nature free from the interference of photosynthesis. Among root systems, red beet hairy root cultures appear ideal for several reasons, the main ones being their hormone independent growth, easily identifiable morphological changes, uniformity in growth pattern as well as pigment synthesis.

Sugar sensing pathways do not operate in isolation but are part of cellular regulatory networks (Gibson, 2000). Recent results clearly show cross-links between different signaling systems, especially those of sugars, phytohormones and light (Smeekens, 2000). While sucrose is sensed via system acting at transcriptional and translational levels (Rook et al. 1998a & b), hexose sensing can occur via a separate hexokinase independent or hexokinase dependent systems (Smeekens, 2000). Sucrose may readily be hydrolyzed at the cell surface into glucose and fructose, (Street, 1969; Last and Brettell 1990; Schripsema et al. 1991; Kraemer et al. 2002) and these hexoses in turn act *via* a hexokinase or other kinases-fructokinase / glucokinase mediated step. The cell cultures which were able to hydrolyze sucrose to glucose and fructose were also able to utilize externally supplied glucose / fructose (Street, 1969; Kino-Oka et al. 1992; Kraemer et al. 2002). In a sucrose sensing system, it is the influx of sucrose into the cell that is sensed rather than the actual cytosolic sucrose concentration (Gibson, 2000; Winter and Huber 2000).

The present chapter has been focused on generating information on several kinetic parameters that may influence large-scale cultivation in bioreactors where the best clone selected as described in the first chapter has been used. Using shake-flask cultivation system several kinetic parameters such as growth and biomass accumulation, effect of inoculum density, effect of larger volumes gas/liquid - liquid/solid mass transfer and product accumulation were studied. It is well known that hairy roots have a continuously changing rheology as a result of continuous growth and repeated branching. There are also reports on change in medium viscosity due to leaching of certain metabolites from cultured cells of *Beta vulgaris* into the medium (Rodriguez-Monroy and Galindo 1999). For these reasons, as well as to keep a track of nutrient uptake during the cultivation period, medium osmolarity, conductivity and pH were continuously monitored. Sugar being a major input for hairy root cultivation, the capability of hairy roots to sense and utilize different sugars was investigated. Effects of different sugars on morphology, betalaine synthesis and physical conditions of the medium have also been addressed. The basic information thus developed has been effectively applied to develop a bioreactor model with improved mass transfer.

2.2. MATERIALS AND METHODS

The hairy root clone LMG-150 was used for all the experiments of the present chapter for which growth and culture conditions have been explained in chapter 1.

2.2.1. Influence of culture conditions on performance of hairy roots

2.2.1.1. Biomass accumulation and inoculum density

Initial set of experiments had inoculum densities (fresh weight) of 0.75mg ml^{-1}, 25mg ml^{-1} and 75mg ml^{-1} grown in 40ml medium in 150ml flasks. Based on the results of this trial, 25mg ml^{-1} (1g $40ml^{-1}$) was selected and the effect of constant inoculum on two different medium volumes of 40 and 80ml (latter in 250ml flasks) was tested. The rationale behind this is to provide higher levels of nutrients to a fixed inoculum size so that during the entire batch cultivation period one set of culture would not be deficient of substrate/nutrient at a given period. On the other hand, effect of different inoculum densities for the same amount of substrate was also found, so that the information would be useful for obtaining the specific biomass in a fixed period. The cultures were maintained under identical conditions of 90rpm, in dark at 25 ± 2°C and the results in terms of biomass accumulation (on weight basis) and product formation (analysis of betalaines) were recorded. Spent medium was also analyzed periodically.

2.2.1.2. Yield co-efficient for product formation

Since culture conditions and varying volumes affect the product formation, yield may not be always directly proportional to the substrate depletion. In such cases, it is necessary to establish the relationship between biomass and product formation under different volumes and inoculum densities that may lead to variation in yield co-efficient. Therefore, the same may be calculated based on data on the amount of product formed from a known amount of substrate, which may be calculated using the following formula.

$$q_{sp} = \frac{r_S}{C_x}$$

Where, q_{sp} is the product yield co-efficient, C_x is biomass concentration and an r is the rate of substrate utilization.

To find out the reason for reduction in biomass at higher volumes the stress exerted on the biomass due to hydrodynamic pressure was calculated. The fluid flow in the shake flasks can be modeled as a case of forced vortex, wherein the angular velocity remains constant along the radius of the flask. The rotary motion gives rise to hydrodynamic pressure

which acts radially on the submerged tissue $P_{hyd} = \rho \omega^2 r^2/2$ where ρ is density of fluid, ω is the angular velocity and r is the radius. Thus, the net pressure acting on a unit area of the tissue is given by $P = (Po + \rho gh) + (\rho \omega^2 r^2/2)$. For this different working volumes as well as flask sizes were selected in such a way that headspace remained constant at 1:2.5 in all the treatments. The ratio between the inoculum: substrate (medium volume) also remained constant at 1mg ml^{-1}.

2.2.1.3. Pulse-feeding

To further ascertain that the stress created due to hydrodynamics, a separate experiment was conducted keeping fixed substrate volumes for control cultures and step-wise increase in substrate (medium) concentration (Table 2.1) as is normally done for fed-batch cultures.

Table 2.1. The pattern of pulse feeding over a period of four weeks

Control Medium volume (ml)	Pulse feeding medium volume (ml)			
	I Week	II week	III week	IV week
80	80	-	-	-
100	80	+20	-	-
120	80	+20	+20	-
140	80	+20	+20	+20

The experiments were conducted in 250ml Erlenmeyer's flask

2.2.1.4. Evaluation of different methods for online estimation of biomass

2.2.1.4.1. Conductivity

Conductivity of the spent medium was measured at different growth stages in the spent medium of cultures grown in 40 and 80ml medium in 150ml Erlynmeyer flasks by using Wiss-Tech-werkstatten model LF-54 (Weihelm, Germany) at a constant temperature of 25°C and expressed in mS (milli Siemens) units.

2.2.1.4.2. Osmolarity

After measuring conductivity, the spent medium, at different stages of hairy root growth, was used for measuring osmolarity using an automatic cryoscopic osmometer (Osmomat-3; Gonotech, Berlin, Germany). A sodium chloride solution of 300 mOsmol Kg^{-1} was used as standard. Osmolarity was expressed as mOsmol kg^{-1} units. The following

equation was used to estimate the biomass.

$$X = Xt_{max} + K (T_{max} - T)$$

Where, X = dry cell concentration; T_{max} = maximum osmolarity reached; T = osmolarity of the culture medium; Xt_{max} = dry cell concentration at T_{max} and K = empirical constant. This experiment was conducted over a period of 4 weeks, at weekly intervals, wherein the initial inoculum was 20mg.

The conductivity and osmolarity measurements were also done for the experiment with different sugars to monitor the nutrient depletion in the spent medium.

2.2.2. Influence of carbon source on growth and metabolism

2.2.2.1. Hairy root culture in media with different sugars

About 50mg fresh weight of hairy roots was inoculated into 40ml filter sterilized MS liquid medium with different types of sugars (Table 2.5) in 150ml flask with 6 replicates and cultured under condition specified earlier. Equi-carbon level of sugars was maintained based on anhydrous weight of 30g L^{-1} for all sugars and 35g L^{-1} for glycerol. Betalaine synthesis was growth - dependant and that both biomass accumulation and betalaine content were highest on 21st day (Bhagyalakshmi and Ravishankar 1999; Thimmaraju et al. 2003). Therefore, all experiments were conducted for a three-week period. For fresh weight measurement, the fresh roots were gently blotted to dry the surface taking care that colour did not leach out and the weight was recorded.

2.2.2.2. Seedling culture in media with different sugars

Filter-sterilized media with different sugars was prepared and aseptically dispensed to culture tubes containing filter paper (Whatman No. 1) bridges (pre-sterilized by autoclaving) and used to grow one week old aseptically germinated seedlings that were incubated in 16h photoperiod of 40μmol m^{-2} s^{-1} at 25 ± 1°C. Data on morphology and weights were recorded after two weeks using 10 replicates (Table 2.6).

2.2.2.3. Estimation of betalaines: Was carried out as presented in the section 1.2.9 of chapter 1.

2.2.2.4. High performance liquid chromatography (HPLC)

For analysis and quantification of sugars (glucose, fructose or sucrose) in the spent medium, HPLC was done using aminopropyl column (Shimadzu) with RI detector using HPLC-grade acetonitrile - water (7:3) at a flow rate of 1ml min^{-1} (Prabha and Bhagyalakshmi 1998).

2.2.2.5. Measurement of pH

The medium pH was adjusted to 5.8 immediately after the addition of the respective sugar, and the medium was then filter-sterilized. Change in pH of the medium, starting from one hour after adding inoculum and at 5 days intervals till the end of growth phase (21 days) was recorded by using digital pH meter (Control Dynamics, India).

2.2.2.6. Morphological observations

Cultures were routinely observed under stereomicroscope as presented in section 1.2.11 of chapter 1. For detailed observation, scanning electron microphotographs were taken by fixing the root tips from each treatment, at the end of the growth phase, in 2.5% (v/v) glutaraldehyde (prepared in absolute alcohol) for 48h. The treated samples were repeatedly washed in distilled ethanol and dried at room temperature in a desiccator. The dried samples were gold coated under vacuum, scanned using LEO – 435 VP, UK.

2.2.3. Studies on scale up of hairy root culture

Hairy root cultures were grown in a 3L bubble column reactor (for details see Fig 2.1 A) with a working medium volume of 1.75L. Hairy root inoculum was prepared by sub culturing about 100mg of hairy roots of clone LMG-150 in 40ml medium in conical flasks for 10 days under standard conditions mentioned earlier. Totally 10g fresh weight of actively growing hairy root inoculum was transferred aseptically through inoculation port into the anchorage basket of the growth chamber. The bioreactor was maintained in dark at 23 ± 2°C with air supply through a sparger at a rate of 33.4cm^3 s^{-1}. After the running period of 20 days, the biomass and the pigment produced were analyzed. To improve the mass transfer conditions another model of bioreactor with a bubble column reactor attached with an air enrichment tank was developed (Fig 2.1 B)

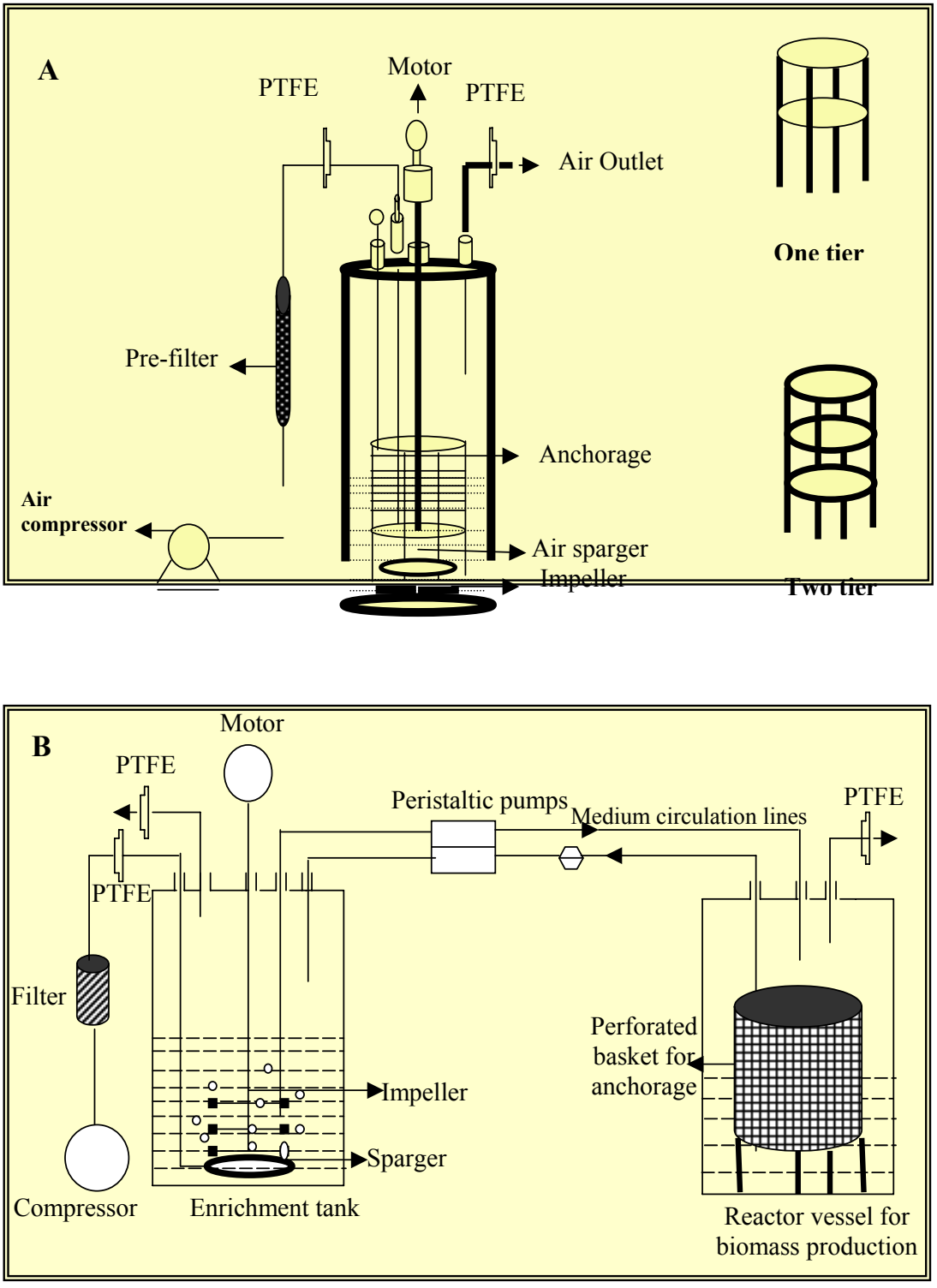


Figure 2.1. Diagrammatic representation of the bubble column bioreactor model with different types of baskets (A) and the same with an air enrichment tank (B) used for the growth of hairy root clone LMG-150. The reactor was of 3L capacity with a working volume of 1.75L. About 10g of actively growing hairy roots were inoculated into the anchorage basket held in growth vessel having MS basal liquid medium with 3% sucrose. The air was supplied at a rate of 33.4cm^3 s^{-1} through the glass sparger of 45cm height and 7cm diameter. The reactor was operated in dark at 23 ± 2°C and after the required running

period the reactor was dismantled and the biomass and spent medium were analyzed for POD.

2.3. RESULTS & DISCUSSION

2.3.1. Growth, pigment production and effect of culture volume

2.3.1.1. Growth curve and biomass maxima

Growth curve is the value obtained due to continuous accumulation of biomass over the initial inoculum during specific time intervals. The results are shown in Fig. 2.2 the maximum biomass achievable under this set of conditions was about 6g where the growth showed a typical sigmoid curve, with maximum growth on the 20th day of culture. The growth cycle of pigmented root culture was characterized by a lag phase of 5 days, a phase of slow increase in growth (pre-exponential phase) during 5-10 days, followed by an exponential growth up to 20th day. The fresh weight of the hairy roots increased by 150-fold over the initial inoculum of 20 ± 4.5mg during the growth cycle and the maximum biomass achievable was about 6g flask^{-1} or 150g FW L^{-1}.

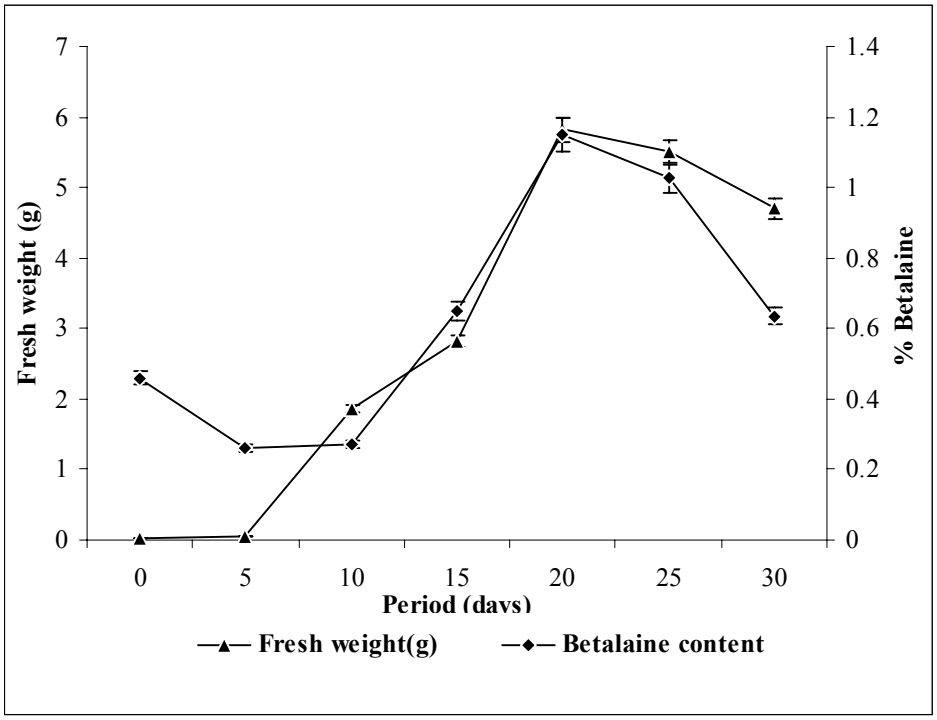
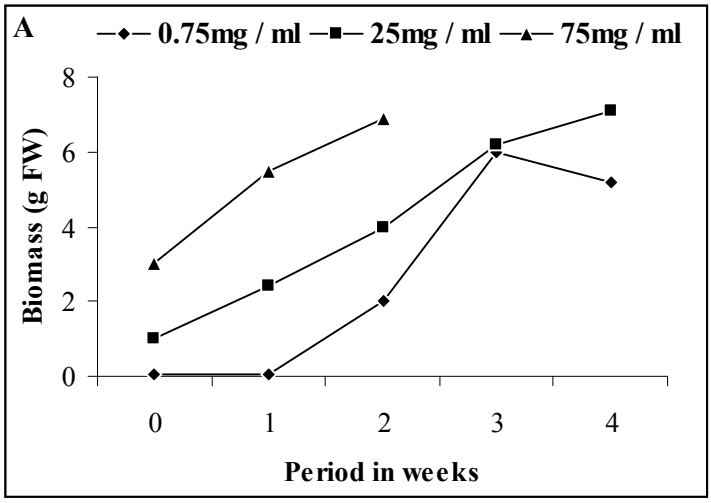


Fig. 2.2. Growth pattern and betalaine content in hairy root clone LMG-150 grown in 40ml MS basal medium kept on a rotary shaker set at 90 rpm in dark at 25 ºC.

The rate of biomass increase, which is an index for all other parameters, basically depends on the initial inoculum. For red beet hairy roots, different initial inoculum densities

directly influenced the pattern of biomass accumulation and more so when specific growth rate was considered (Fig. 2.3A & B). The initial lag phase was evident for low initial inoculum of 0.75mg ml^{-1}, with no lag phase as well as steady log-phase for 25mg ml^{-1} inoculum, though both 0.75 and 25mg ml^{-1} inoculums resulted in the same quantity of biomass by the end of 3rd week. Both these treatments resulted in decline in specific growth rate, which was more apparent when the inoculum size was lowest, indicating culture death and loss of weight between 3rd and 4th occuring mainly due to respiration and catabolic activities. The continuous increase in biomass at 25mg ml^{-1} inoculum (Fig. 2.3A) and decline in specific growth rate (Fig 2.3B) indicates some amount of biomass accumulation. The ratio is much lesser than the already accumulated biomass indicating loss of culture vigor at this stage, which may be linked to lack of nutrients in the medium. A higher level of loss of specific growth rate in the lower inoculum size indicates the loss of nutrients during slow growth, during which most of the nutrients are also spent for catabolic activities.



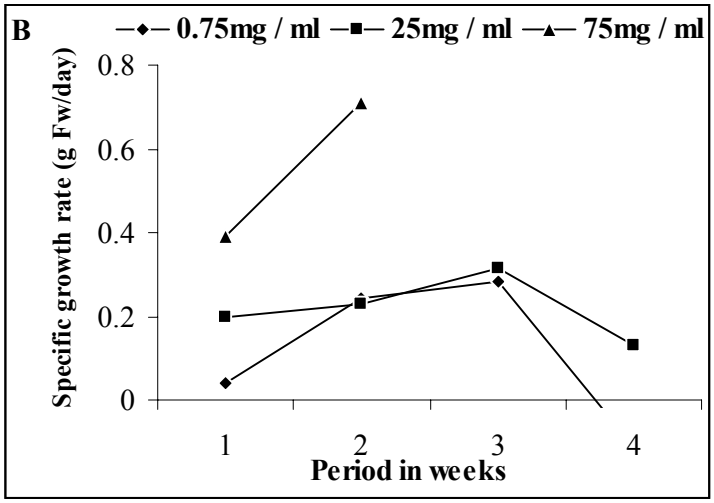


Figure 2.3. Effect of initial inoculum densities on biomass accumulation (A) and specific growth rate (B). Values are the mean of six replicates and the bars represent standard error of mean.

A very high inoculum density of 75mg ml^{-1} showed rapid biomass accumulation as well as specific growth rate (Fig. 2.3B). Though initial inoculum is known to significantly influence productivities, rarely attempts have been made to study the inoculum properties for scale-up. Kondo et al (1989) used 200mg dry weight of carrot hairy root inoculum per each litre of medium and studied their performance in different bioreactors. Hilton and Rhodes (1990), Ramakrishna et al (1994) and Tescione et al (1997) studied growth pattern using various inoculum densities and demonstrated that quality of inoculum plays an important role and generalized that the fast growing roots maintained for 1 to 2 weeks are most suitable. In the present experiments, the hairy roots were grown for 2 weeks, and the optimal inoculum density was found to be between 25-75mg ml^{-1}, which would yield almost the same amount of biomass at the end of 2 weeks as that of 75mg ml^{-1}.

2.3.1.2. Kinetics of substrate depletion

Sucrose consumption is an important aspect in specific growth rate and the results are presented in table 2.2. The sucrose depletion showed a linearity with the increase in biomass and thus inversely proportional to the biomass as well as product formation. Here it should be noted that the calculated yield of biomass also matched with the actual biomass yield. In the present study, the relationship between sucrose depletion and the biomass accumulation indicate that 6g of biomass was produced for 1.2g of sucrose utilized. In the situation of high growth rate, which occurred during rapid log phase (10-20 days), the yield relationship between rates of biomass increase versus the rate of substrate utilization (sugar) can be calculated for higher volumes as well as for stoichiometric control of medium replenishment and biomass accumulation. Thus the data generated in the present study at shake flask level would allow one to set parameters of biomass for scale-up.

2.3.1.3. Effect of higher medium volumes at constant shaker speed

Scale-up, in simple term, means production in larger volumes. When live cultures are involved, this increased volume exerts pressure on the biomass (HR) by its own weight. Thus it is necessary to understand growth and product formation in higher culture volumes. An initial experiment with six replicates was conducted to find out whether beet hairy roots were sensitive to larger culture volumes. For this, different volumes of medium were used in Erlenmeyer flaks of different capacities. Here, care was taken to keep the ratio of medium

and the headspace constant. Similarly, care was also taken that the ratio between initial inoculum densities with the medium volume remained unchanged. The experimental pattern with different culture flask volumes and the medium volume as well as inoculum are compiled in table 2.3.

Table 2.2. Biomass accumulation, pigment content in the clone LMG-150, medium remaining and left-over sucrose in the medium during the course of 20 days cultivation period using 100mg initial inoculum in 40ml MS liquid medium.

Days	Biomass (g)	Betalaine (%)	Volume of spent medium (ml)	Residual sucrose (%)
0	0.1	-	40	100
5	2.0	0.37	37	83.3
10	4.0	0.28	35	50.0
15	5.0	0.9	30	25.0
20	6.0	1.14	25	1.6

Table 2.3. Effect of medium volume and culture density on growth and biomass accumulation

Volume of flasks (ml)	Volume of medium (ml)	Inoculum density (mg)	Final fresh weight (g)	Biomass productivity (gL^{-1})
250	100	100	23.7	240
500	200	200	41.6	208
1000	400	400	50.5	121
2000	800	800	60.0	75

The above results clearly indicate that as the volume increased the biomass yield also increased, but the rate of accumulation i.e., the number of folds of biomass increase, in other words the biomass productivity per litre medium, steadily decreased (Table 2.3). The results presented in table 2.4 shows that there was a steady increase in hydrodynamic pressure acting on the culture which was associated with increase in medium volume though the container size also increased. Though the values presented seem as very marginal increase, the increase of about 1000pa pressure from 250ml to 1000ml container size explains the

sensitivity of hairy root culture to hydrodynamic stress as indicated by drastic decrease in the rate of biomass production.

Table 2.4. Effect of container size and medium volume on the hydrodynamic pressure exerted on the hairy root culture maintained at 90rpm on a gyratory shaker for 30 days*.

Container capacity (ml)	Medium volume (ml)	Height of free surface (m)*	Dia of base (m)	Hyd dyn pressure at the periphery (Kpa)	Hyd dyn pressure at the centre (Kpa)	Hyd dyn pressure at the r/2 (Kpa)	Inoculum wt (mg)	Total biomass (gL^{-1})
250	100	0.025	0.085	101.66	101.57	101.59	100	240
500	200	0.030	0.100	101.75	101.62	101.63	200	208
1000	400	0.035	0.125	101.87	101.68	101.73	400	121
2000	800	0.045	0.160	102.10	101.79	101.94	800	75

* The data is the mean of three independent experiments

2.3.1.4. Effect of medium volume and shaker speed on hydrodynamic stress

Figs 2.4 A & B show the observations made for different culture volumes as a function of shaker speed. While the increase in volume retarded biomass yield at constantly higher shaker speed, the low speed of 90rpm showed higher biomass, which could be correlated with lower hydrodynamic stress (Fig 2.4 B). Though a higher agitation is expected to improve better mass transfer when higher volumes are considered, the beet hairy roots appear to prefer lower shear stress over that of higher mass transfer. Therefore, from these shake flask experiments of the present study it can be concluded that the red beet hairy roots are highly sensitive to hydrodynamic stress.

2.3.1.5. Effect of medium volume on pigment synthesis and other medium parameters

The product formation i.e., the synthesis of betalaine was rapid at lower culture volumes during the growth period with maximum on third week. Whereas, at higher volume the betalaine accumulation was steady reaching a maximum of 1.2% (DW) on fourth week (Fig 2.5). Conductivity measurement has been used as an indirect means of estimation of biomass accumulation on-line as well as stoichiometric replenishment of medium. Conductivity of the medium linearly decreased with increase in biomass as shown in fig 2.6(B) during 4-week growth period. A linear decrease in osmolarity was recorded with an increase in biomass. As all the medium constituents contributed to osmolarity value, its

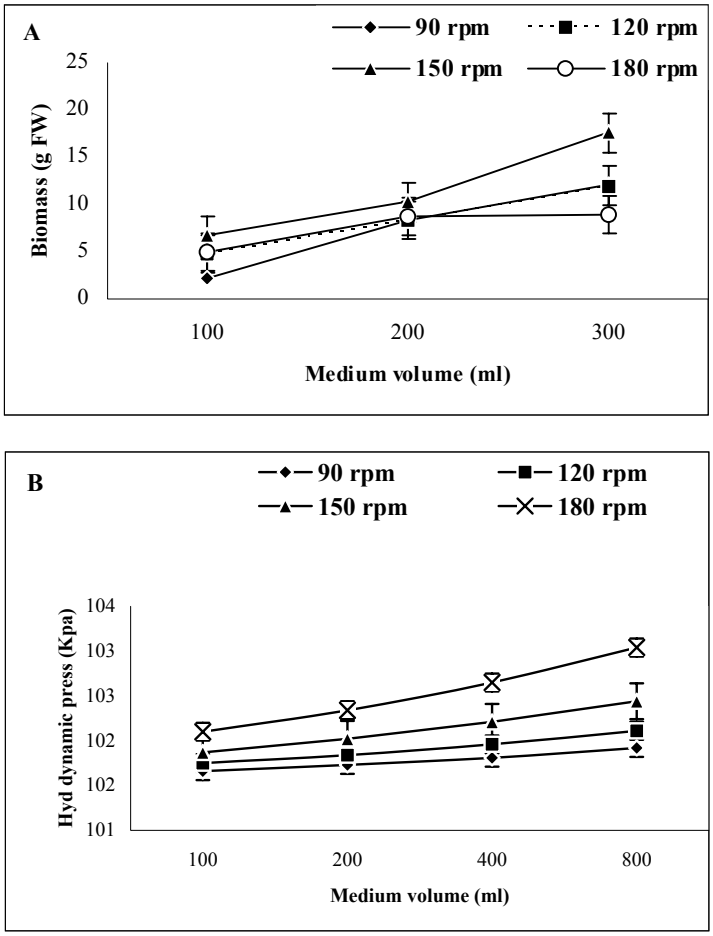


Figure 2.4. Effect of shaker speed and medium volume on biomass production (A) and hydrodynamic stress (B) exerted on hairy root cultures. The data is the mean of ten replicates.

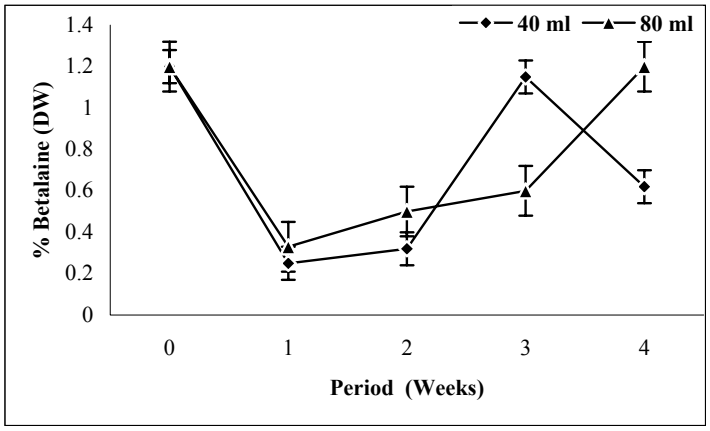


Figure 2.5. Influence of medium volume on pigment synthesis in the culture grown at constant shaker speed of 90 rpm. The data is the mean of ten replicates and the vertical bars indicate standard error of mean.

measurement correlated well with increase in biomass, which is shown in fig 2.6(C) whereas, the other medium parameter, the pH (Fig 2.6A) remained almost unchanged.

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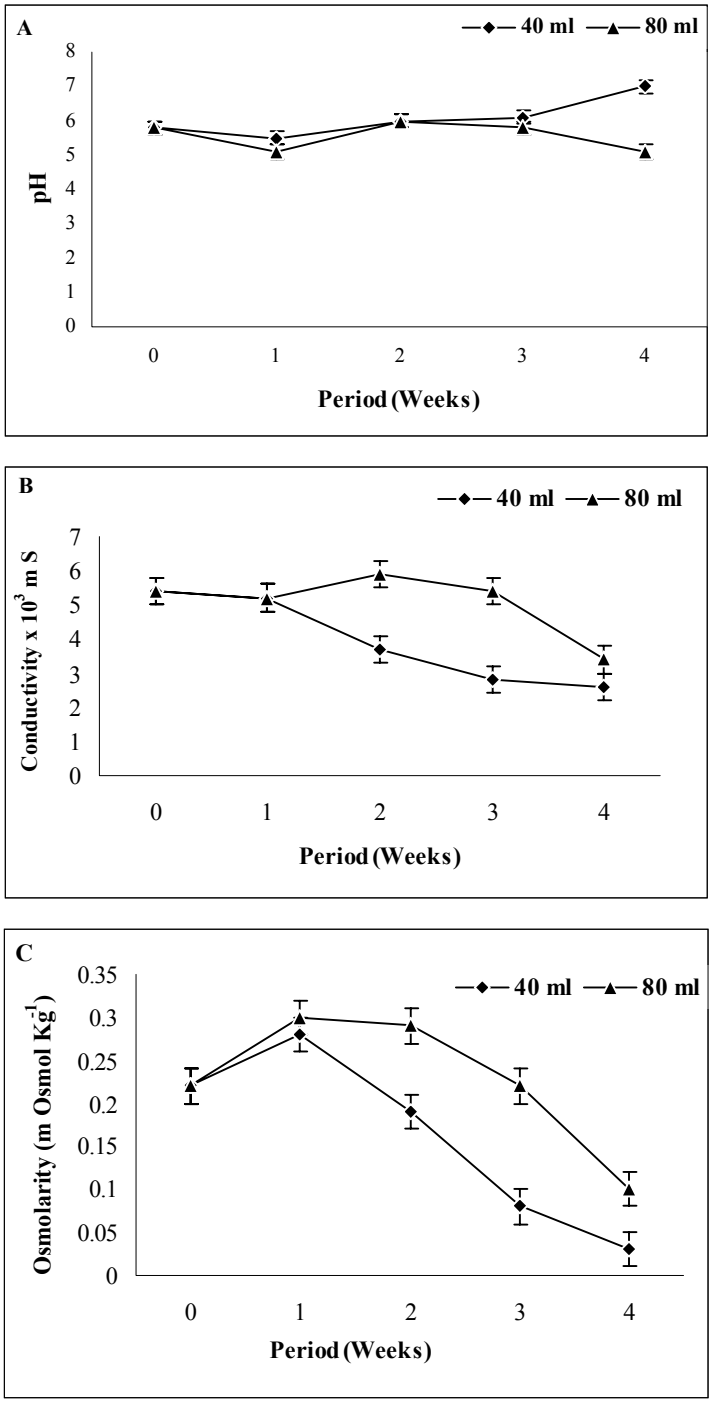


Figure 2.6. pH (A), conductivity (B) and osmolarity (C) of the spent medium over a period of 4 weeks as influenced by the medium volume. The data is the mean of ten replicates and the vertical bars indicate standard error of mean.

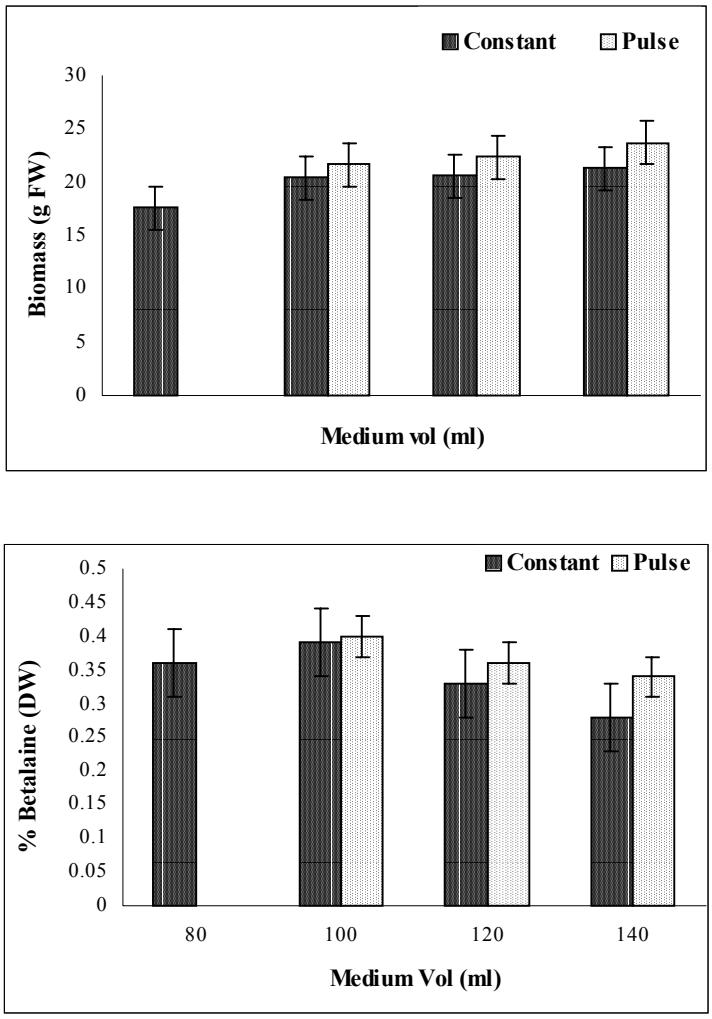


Figure 2.7. The biomass accumulation and betalaine production in control cultures with different constant initial volumes of media and pulse-fed cultures. Pulse-fed cultures produced more biomass and pigment compared to control. The increase in biomass was steady with 10% more in pulse-fed cultures as compared with the respective controls.

To check whether the reduction in growth and pigment synthesis at different culture volumes were due to nutrient limitation or because of hydrodynamic stress, an experiment was done by feeding nutrient medium to the roots in pulses (Table 2.1). The results presented in the figure 2.7 indicate that the increase in biomass and pigment production was steady with about 10% additional biomass and pigment production in pulse fed cultures when compared to respective control cultures.

Shake flasks have been applied extensively to study various parameters both kinetic and stoichiometric for the growth of hairy root cultures. Various parameters such as nutrient

levels (Hilton and Rhodes 1990), carbon source (Inomata et al. 1993) hormone regime (Yoshikawa and Furuya 1987; Robins et al. 1991; Repunte et al. 1993) have been studied for various hairy root systems. Apart from the other parameters, medium osmolarity, medium pH has also been reported to be very important to get an understanding of the system for scale up. The results of various culture parameters studied in the present study such as culture volume, inoculum size shaker speed, and effect of medium volume on growth and pigment synthesis are very important for the scale up studies on red beet hairy roots.

2.3.2. Effect of different sugars on growth performance and betalaines

2.3.2.1. Growth pattern and betalaine content

Most organisms have developed a sensing mechanism and signaling cascade to respond to changes in the availability of sugars (Winter and Huber 2000). In most plant cells sugar sensing is specific for sucrose (Gibson and Graham 1999) while in other cases glucose or fructose can also serve as signaling molecules (Jang and Sheen 1994; Jang and Sheen 1997). In the first experiment of this study, growth of hairy roots and betalaine content of hairy roots was compared on media containing different sugars were tested where the inoculum had been taken from the stock cultures (grown in MS medium with 3% sucrose). A significantly ($p \geq 0.01$) higher biomass was observed on sucrose, followed by maltose, glucose and fructose. The other sugars such as lactose, galactose, xylose and glycerol failed to support the growth of beet hairy roots (Table 2.5). These results clearly point out that though beet hairy roots require sucrose as a carbon source, they also partially utilize maltose (a glucose dimer) and other hexoses, the degree varying with each hexose. Hexose sensing is usually by hexokinase pathway, where hexokinase hydrolyses sucrose to glucose and fructose at membrane or vacuolar level (Pego et al. 1999), thereby indicating that sucrose influx into the cell is a highly programmed activity. In the present study it is also clear from the lack of growth on glucose + fructose that these hexoses are not utilized as a carbon source or as substrates to synthesize the needed sucrose, both by hairy roots and seedlings (Tables 2.5 & 2.6). Other studies indicated the hydrolysis of sucrose to glucose and fructose by invertase at cell surface (Kraemer et al. 2002; Schripsema et al. 1991) before being absorbed into the cell where either glucose or fructose may then preferentially be utilized by the cell (Street, 1969). The levels of total and reducing sugar were assayed at different periods of hairy root growth, both by chemical method and by HPLC to know whether sucrose was hydrolyzed to glucose and fructose, and if so the pattern of uptake of individual hexoses.

Data compiled in Table 2.7 indicates sucrose hydrolysis did not occur and there was negligible presence of reducing sugars (hexoses) in the medium at initial stages with a drop at the later stages by chemical method, whereas by HPLC (data not presented) no glucose or fructose were identified at any stage of hairy root growth in sucrose medium.

When betalaine productions on various sugar-supplemented media was checked, the greatest betalaine accumulation was observed in sucrose-supplemented medium and in glucose & maltose-supplemented medium (Table 2.5). Betalaine synthesis in fructose and glucose supplemented medium was very low, logically due to poor growth. The ratio of betacyanine to betaxanthine differed significantly ($p \geq 0.001$) with a much higher level of betaxanthine in medium with maltose (1**:**4.4) than in sucrose (1**:**3) and glucose (1:12) (Table 2.8). Contrarily, in fructose-supplemented medium betacyanine content in hairy roots was much higher than the betaxanthine (1:0.7) (Fig. 2.8).

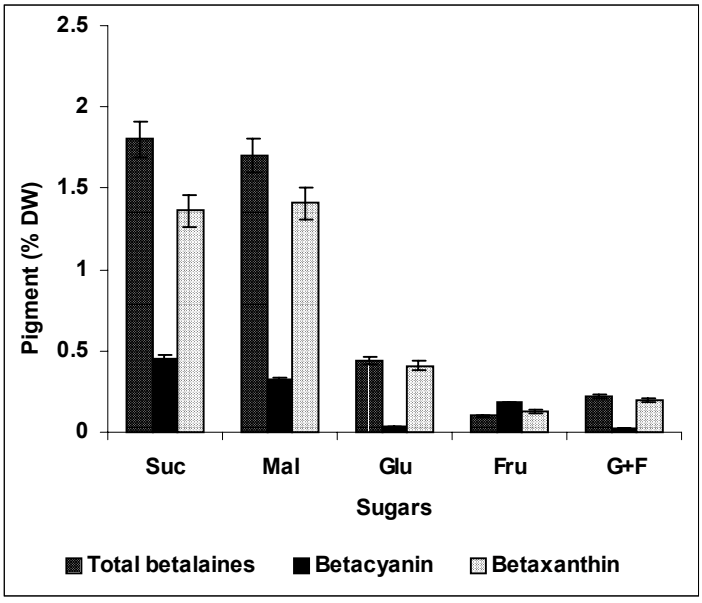


Figure 2.8. Betacyanine, betaxanthine and betalaine contents in hairy root cultures of *Beta vulgaris* grown on Murashige and Skoog's medium supplemented with sucrose, maltose and different hexoses. Each bar represents the mean ± S.E of six replicates.

Though the variations in the ratio of pigment can be related to stressed condition of hairy roots because of un-congenial carbon supply, the involvement of sugars in diverse plant metabolic processes such as nitrogen metabolism, lipid and storage protein accumulation (Gibson and Graham 1999) and their possible involvement in pigment synthesis cannot be overlooked, particularly when these pigments are stored as different glycosides.

Table 2.5. Effect of different sugars on the growth, morphology and betalaine content of red beet hairy roots (20 days after inoculation).

Type of Sugar	Root* Length (cm)	Length of laterals (cm)	Density of laterals (cm^{-1})	Color	Hairiness	Length of the root from tip to branch initiation point	Fresh Biomass FW (g)
Sucrose	7.20± 0.045a	2.90± 0.003 a	9.20± 0.05a	Red	Very high	2.36± 0.003a	7.4± 0.04a
Glucose	3.76± 0.02b	1.26± 0.001b	5.60± 0.03b	Yellowish / white	Very little	0.92± 0.001b	1.80± 0.01c
Fructose	0.30± 0.001d	Nil	0.00	Yellowish	Very little	0.0±0.0d	0.05± 0.0003d
Glucose + Fructose	0.4± 0.002d	Nil	0.00	Yellow	Nil	0.0±0.0d	0.05± 0.0003d
Maltose	1.08± 0.006c	0.56± 0.0007c	1.60± 0.01c	Yellowish	Nil	0.3± 0.0003c	3.45± 0.02b
Galactose	No growth	Nil	Nil	Nil	Nil	Nil	Nil
Xylose	,,	Nil	Nil	Nil	Nil	Nil	Nil
Lactose	,,	Nil	Nil	Nil	Nil	Nil	Nil
Glycerol	,,	Nil	Nil	Nil	Nil	Nil	Nil

Table 2.6. Effects of sucrose, maltose and different hexoses on the growth of one-week-old seedlings of red beet.

Type of sugar	Total length (cm)	Length of the shoot (cm)	Length of the root (cm)	Density of laterals / cm	FW of 5 seedlings (g)
Sucrose	5.02± 0.03a	1.94± 0.01a	3.09± 0.02a	9.6± 0.06a	0.29± 0.00008a
Maltose	4.74± 0.03b	1.78± 0.01a	2.96± 0.02b	5.2± 0.03c	0.28± 0.00008a
Glucose	4.0± 0.02b	1.6± 0.01a	2.2± 0.01c	7.4± 0.05b	0.22± 0.00006a
Fructose	1.8± 0.01c	1.36± 0.009b	0.44± 0.003d	1.4± 0.009d	0.13± 0.0003b
Glucose+ Fructose	0.94± 0.006d	0.94± 0.006c	0.0±0.0d	0.0±0.0d	0.21± 0.0006a

Table 2.7. Pattern of sucrose utilization as depicted by the content of reducing and total sugar in the medium.

Days after inoculation	Reducing sugar %	Total sugar %	Non reducing %
0	0.00	100.0	100.00
1	1.30	84.0	82.7
5	1.30	58.4	57.1
10	1.30	53.6	52.3
15	0.65	25.0	24.35
20	0.65	5.0	4.35

As maltose and glucose also supported growth and pigment synthesis to various extents, though not so well as sucrose, it was of interest to know whether hairy roots may adapt (habituated) to maltose, glucose and fructose upon repeated exposure.

Table 2.8. Growth and pigment formation in hairy root cultures of *Beta vulgaris* when repeatedly subcultured in media with different sugars.

Sugars (3%)	Sub culture No.	Biomass (g. FW)	BC (% DW)	BX (% DW)	BN (% DW)	Ratio of BC: BX
Sucrose	I	6.96±0.05a	0.45	1.36	1.81	1: 3
	II	6.7±0.05a	0.5	1.31	1.8	1: 2.7
	III	6.7±0.05a	0.5	1.2	1.7	1: 3
Maltose	I	3.45±0.02b	0.32	1.41	1.71	1: 4.4
	II	2.9±0.02c	0.33	0.92	1.27	1: 3
	III	0.09±0.0006f	0.045	0.16	0.21	1: 3.6
Glucose	I	1.80±0.01d	0.03	0.41	0.44	1: 12
	II	0.16±0.001e	0.04	0.24	0.28	1: 6.5
	III	No growth	Nil	Nil	Nil	Nil
Fructose	I	0.95±0.007de	0.18	0.13	0.31	1: 0.7
	II	No growth	Nil	Nil	Nil	Nil
	III	No growth	Nil	Nil	Nil	Nil

Table-2.8 summarizes the behavior of hairy roots when sub-cultured in medium with a particular type of sugar. While the growth and BN content in cultures maintained in sucrose supplemented medium remained unchanged for three subcultures, those on maltose, glucose and fructose steadily declined ($p \geq 0.05$) in subsequent cultures. The ratio of betacyanine to betaxanthine remained unchanged in these cultures except in second subculture on glucose-medium where the betaxanthine content was lower ($p \geq 0.05$) than that of the first culture (Table 2.8).

These results suggest that beet hairy roots do not sense the presence of maltose/hexoses except to a very limited extent, probably due to the lack of uptake and phosphorylation step leading to glycolysis. It is surprising that though beet hairy roots are fully heterotrophic, and though glucose is needed as an energy source and abundantly present in the external medium, cells were unable to use it. Thus sugar sensing is an extremely interesting aspect of study in case of red beet hairy roots. Such hexose insensitivity has also been reported in mutants of *Arabidopsis* (Wenzler et al. 1989; Yokoyama et al. 1994).

2.3.2.2. Influence on hairy root phenotype

Sucrose, maltose, glucose, fructose and the combination of glucose and fructose significantly influenced hairy root morphology. The root branching pattern differed significantly between treatments. While sucrose induced very elongated moderately branched luxuriant growth of roots, roots on media with other sugars - glucose, maltose, fructose and glucose + fructose showed variable branching (Table 2.5).

Sugars have been proven implicated in both the number and placement of lateral roots through signal pathways (Malamy and Ryan 2001). Sucrose in particular is known to retard lateral root initiation especially under low nitrogen levels (Malamy and Ryan, 2001). The morphological changes were distinct from an early stage itself. The roots showed compact tip region on sucrose or maltose treatments whereas, glucose treatment resulted in slender roots tips with a dark root-cap-like region. On fructose the roots turned dark and loosely arranged cells. Scanning electron photomicrography also revealed significant differences in the morphology of the tips as well as the root elongation region, with well organized symmetrically arranged cells in the root tips grown in sucrose medium (Fig. 2.9), whereas roots grown in other media appeared more unorganized with disturbed cell arrangements. The cultures on fructose medium showed frequent branching with callusy cells in root elongation region, whereas the roots grown in maltose and glucose media showed loosely arranged cells that appeared to slough off from the root elongation region. When grown in the mixture of glucose and fructose, roots mainly expressed phenotypic changes similar to those observed on fructose medium (Table 2.5). Maltose appeared to suppress hairs when compared to standard sucrose supplemented medium whilst hairs were highly enhanced by glucose where tufts of root hairs appeared to originate from a common point (Fig. 2.10). Fructose treatment showed densely arranged hairs of variable lengths. The hairs were sturdier in glucose medium than on sucrose medium. However, when both glucose and fructose were present in the medium, there was only initiation of hairs without further elongation (Fig. 2.10).

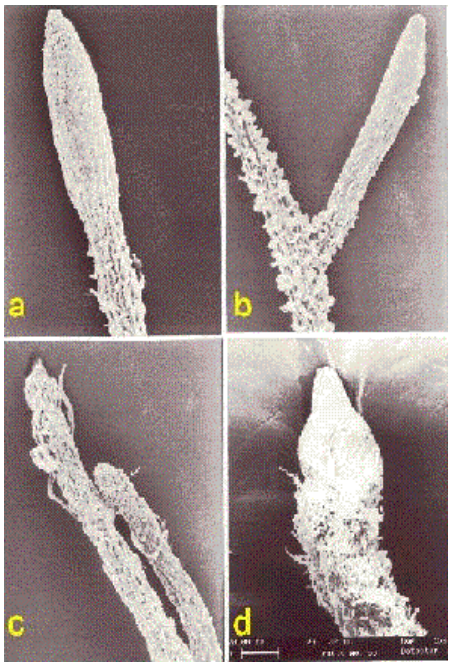


Figure 2.9. Scanning electron photomicrographs of hairy root tips of *Beta vulgaris* grown in Murashige and Skoog's liquid medium with different sugars such as sucrose (a), maltose (b), glucose (c) and fructose (d). Note the callusy nature in fructose (d) treatment and the highly porous nature of tips in glucose (c) medium.

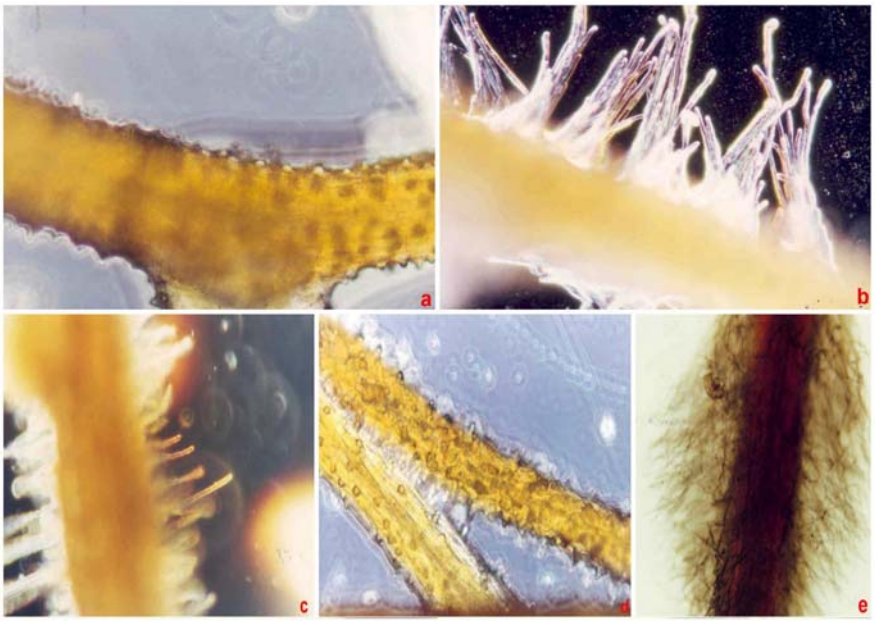


Figure 2.10. Phase contrast microscopic pictures showing highly repressed root hairs in case of maltose treatment (a), suppressed root hair elongation in case of glucose + fructose (d), tufts of root hairs arising together from a common point in case of glucose (b), sparsely arranged variable length root hairs in case of fructose (c) associated with a complete absence of pigment betacyanine, and profuse and long root hairs with high level of red pigment synthesis (betaxanthine + betacyanine) in case of sucrose (e) treatment.

On maltose medium, which supported moderate biomass and a level of betalaine almost equivalent to that on sucrose medium, the root hairs were arrested at the formation level without any further elongation. This effect was more pronounced than that on glucose + fructose (Fig. 2.10).

2.3.2.3. Influence on medium status

In order to establish whether different sugars influence the physical status of the medium, the parameters such as conductivity, osmolarity and pH were recorded. The medium conductivity is usually influenced by nitrates, calcium chloride, major / minor other elements, whereas osmolarity is largely influenced by sugars and similar exudates leached into the medium by the cells / hairy roots. The conductivity of the medium was initially constant (5.4×10^3) in all the media except for a negligible higher value of 5.5×10^3 in the case of maltose medium (Fig. 2.11). At the end of culture period there was a reduction in the conductivity of all the media with a lowest value (46% reduction) in the sucrose medium. There was 27% reduction in maltose medium and about 20% reduction in the conductivity of the other treatments (Fig. 2.11). These results clearly indicate that minerals are taken up significantly from the sucrose-medium, and to a very limited extent in the others. The similar reduction of conductivity in fructose-medium without a concomitant increase in the biomass is something we are not able to relate with any constructive activities, but the increase in medium osmolarity in fructose-medium at the end of growth phase suggests the exudation of certain neutral compounds into the medium at the expense of minerals.

There are various reports indicating the hydrolysis of sucrose to glucose and fructose (Kraemer et al. 2002; Schripsema et al. 1991) and maltose to glucose (Last and Brettell 1990) before being absorbed into the cell by invertase at the cell surface (Street, 1969) and glucose is then preferentially utilized by the cells until the supply is exhausted when the only remaining hexose, is subsequently taken up from the medium. In such instances where sucrose is hydrolyzed in the medium, the combined effect of hexoses (glucose + fructose) is known to impart a lower osmolarity to the medium than the equimolar level of sucrose (Lazzeri et al. 1988), but no such difference in osmolarity was observed when glucose alone was used in the place of maltose (Last and Brettell 1990). The lower osmolarity was observed in sucrose medium right from the beginning till the end. Different initial osmolarity values were observed for different sugars in the medium (Fig. 2.11) where the highest of 0.27 mOsmol Kg^{-1} was recorded for fructose as well as for the glucose-fructose combination. At the end of culture period drastic reduction in osmolarity by 95% was observed only in sucrose medium indicating its continuous uptake by hairy roots. Among the rest, there was a

narrow reduction in maltose and Glu + Fruc, medium whereas the rest showed increased osmolarity, which was highest in fructose-supplemented medium probably due to partial exudation of cellular substances, which are only neutral but not ionic, as there was no concomitant increase in conductivity in these treatments. Thus the higher osmolarity values, observed at the end of culture period in other sugars, are invariably due to non-utilization of nutrients and hence leading to poor growth.

There was an insignificant fall in medium pH upon adding the inoculum and therefore, a little change in all media except in sucrose medium in which the initial drop gradually changed reaching a high pH of 6.2 at the end of growth phase. Aseptic adjustment of pH to 5.8 throughout the growth period suggested that these minor fluctuations in pH neither affect sucrose sensing nor its further metabolism, which is applicable to the rest of the hexoses as well.

To screen whether the observations are well confined to hairy roots, the experiments were repeated using the red beet seedlings. Though the overall response on each sugar was comparable to that of hairy roots (Table 2.6), there were also significant differences. For seedlings, fructose was generally root inhibitory, which was more pronounced in the presence of glucose, though glucose alone did not cause similar retardation of root growth, especially lateral roots on maltose, with the seedling weight nearly same as that on sucrose suggests that there is good level of metabolic activity in seedlings. If these growth patterns are compared with the hairy root growth (Table 2.5), root lengths were more pronounced on glucose rather than on maltose, suggesting that there are certain differences between the genetically transformed hairy roots and the non-transformed seedling roots as far as sugar utilization patterns are concerned.

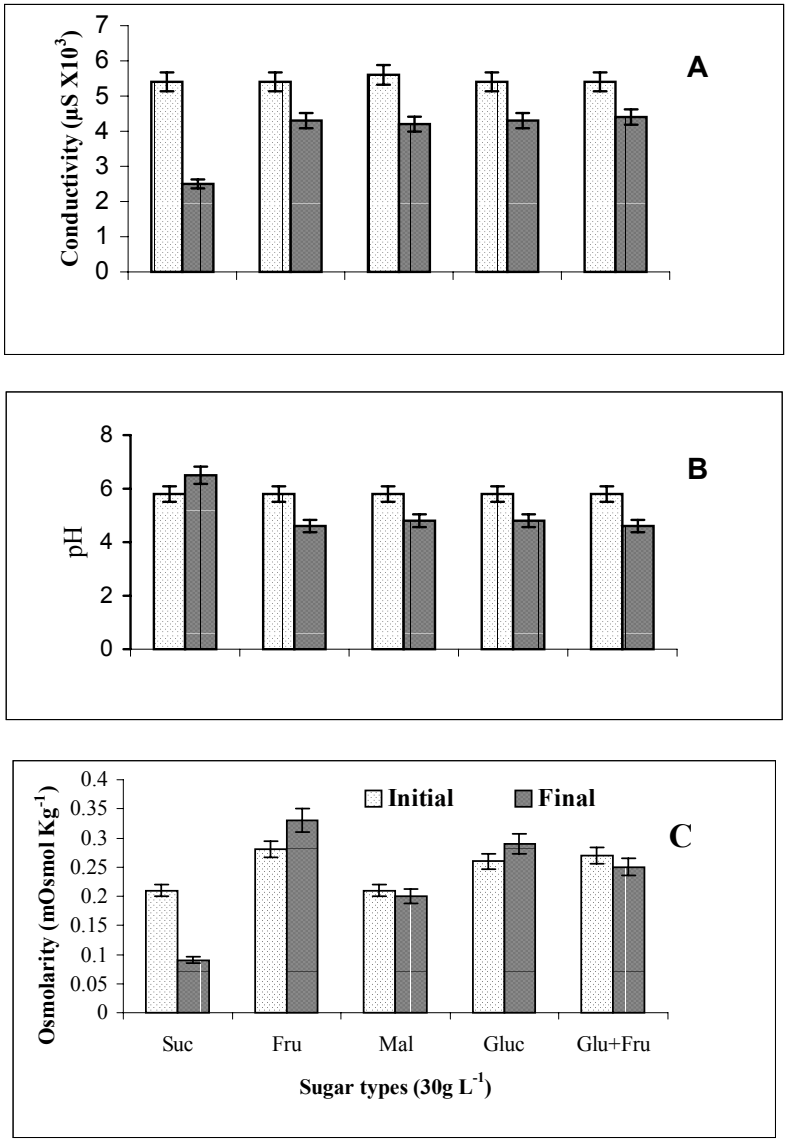


Figure 2.11. Influence of type of sugar on the conductivity (A), pH (B) and osmolarity (C) of the medium used, for growth of hairy roots of *Beta vulgaris*. The vertical bars indicate standard deviation obtained from six replicates of three different experiments. Initial values were taken prior to the addition of inoculum and the final values were recorded at the end of growth phase, i.e., 21st day. Each bar represents the mean ± S.E of six replicates.

2.3.2.4. Reversibility of hexose effects

Repeated culturing on particular sugars especially the non-sucrose treatments, resulted in gradual death of hairy roots (Table 2.8). The possibility of reversing the adverse effect caused by the hexoses and rejuvenation of sucrose sensing was tested. For this experiment we selected the roots of third subculture on medium with maltose, glucose, fructose and glucose + fructose. Table 2.9 summarizes re-growth pattern as well as betalaine content two weeks after transferring back to sucrose-supplemented medium. Roots grown on fructose medium, were dark, lignified and appeared dead, but upon transfer to sucrose medium were rejuvenated and grew many elongated roots with normal branching and dense root hairs (Fig. 2.12). This was also the case with other individual hexoses with various levels of biomass (Table 2.9). In contrast cultures from glucose and fructose that had scanty hairs and stunted lateral branching ($p \leq 0.01$) continued to show the abnormality even after two weeks of incubation on sucrose (Fig. 2.12). The roots grown on maltose and glucose recovered quickly and by the end of second week (Table 2.9) indicating that firstly, maltose imparts lesser repressive effects than hexoses and secondly, the sucrose signal in the presence of maltose lasts longer than in conjunction with other hexoses. These observations clearly indicate that the extent of rejuvenation directly correlates with extent of repression caused by unfavorable hexoses. The fact that, the hexoses were able to keep the roots alive for quite a long period indirectly suggest that the quantum of sensing is not further translated probably due to lack of phosphorylation through hexokinase step as has been well documented in other systems (Graham et al. 1994; Jang and Sheen 1997; Pego et al. 1999) or simply the hexoses send repressive signals (?), which need to be established in subsequent studies.

Figure 2.13 shows further growth of hairy roots (after 3 weeks on sucrose medium), which had been grown on different hexoses for a month. While most of the roots reverted back to normal growth, the highly branched nature of glucose-treated roots (Fig. 2.13) would still be traceable to phenotypic effects caused by glucose. However, hairiness and pigment synthesis (Table 2.10) recovered to an extent comparable to control cultures ($p \leq 0.001$).

Table.2.9. Effect di-hexoses and hexoses on growth, morphology and betalaine content of hairy roots and the reversal effects after transferring back to sucrose supplemented medium each grown for 2 weeks on respective sugar medium.

Type of Sugar	Root Length (cm)	Length of the laterals (cm)	Density of laterals/ cm	Length of the root from tip to branch initiation point	Hairiness	Color	Fresh Biomass FW (g)	Betalaine content % DW
Effect of different sugars								
Sucrose	7.2± 0.08a	2.36± 0.005a	9.20± 0.1c	2.90± 0.006b	Dense	Yellowish Red	7.4± 0.08a	1.81
Maltose	1.08± 0.01c	0.3± 0.0006d	1.60± 0.01	0.56± 0.001d	Nil	Yellow	1.0± 0.01e	1.71
Glucose	3.76± 0.04b	0.92± 0.002c	5.60± 0.06e	1.26± 0.002c	Sparse	Yellowish white	0.16± 0.001f	0.44
Fructose	0.30± 0.003d	0.0± 0.0e	0.0± 0.0e	Nil	Sparse	Yellowish	0.05± 0.0005g	0.31
Glucose + Fructose	0.4± 0.04d	0.0± 0.0e	0.0± 0.0e	Nil	Nil	Yellow	0.05± 0.0005g	0.22
Reversal effect upon transferring to sucrose								
Maltose	7.16±0. 08a	0.70± 0.001cd	8.20± 0.09d	3.2± 0.007a	Dense	Red	4.8± 0.05c	0.24
Glucose	7.00±0. 07a	0.96± 0.002c	14.20± 0.16a	2.28± 0.005b	Dense	Purplish Red	2.8± 0.03d	1.88
Fructose	3.72±0. 04b	1.40± 0.003b	11.20± 0.12b	1.60± 0.003c	Dense	Yellowish red	6.5± 0.07b	2.19
Glucose + Fructose	6.70±0. 07a	0.76± 0.001cd	11.20± 0.12b	3.24± 0.007a	Dense	Red	4.8± 0.05c	4.24

Table 2.10. Effect of sucrose, maltose and different hexoses on growth, morphology and betalaine content of hairy roots and the reversal effects after transferring back to sucrose supplemented medium each grown for 3 weeks on respective sugar medium.

Type of Sugar	**Root Length (cm)**	**Length of laterals (cm)**	**Density of laterals / cm)**	**Length of the root from tip to branch initiation point**	**Hairiness**	**Color**	**Fresh Biomass FW (g)**
Effect of different sugars							
Sucrose	3.2± 0.01b	1.5± 0.001a	6.8± 0.029bc	1.02± 0.001cd	Dense	Yellow	3.4± 0.01a
Maltose	1.08± 0.004c	0.3± 0.0003e	1.60± 0.006f	0.56± 0.0006 f	Nil	Yellow	0.50± 0.002d
Glucose	3.76± 0.01b	0.92± 0.001b	5.60± 0.02c	1.26± 0.001c	sparse	Yellow	0.16± 0.0006e
Fructose	0.30± 0.001e	0.0±0.0ef	0.0±0.0f	Nil	sparse	Dark	0.02± 0.00008f
Glucose + Fructose	0.4± 0.001de	0.0±0.0ef	0.0±0.0f	Nil	Nil	Dark	0.02± 0.00008f
Reversal effect upon transferring to sucrose							
Maltose	4.4± 0.01a	0.44± 0.0004e	7.6± 0.03b	2.96± 0.003b	Dense	Purple	2.0± 0.008c
Glucose	4.90± 0.02a	0.92± 0.001b	4.60± 0.01d	3.50± 0.003a	Dense	Purple	1.9± 0.008c
Fructose	3.04± 0.01b	0.82± 0.0009b	9.20± 0.03a	1.60± 0.001c	Sparse	Yellowish white	2.5± 0.01b
Glucose + Fructose	3.54± 0.01ab	0.28± 0.0003ef	5.2± 0.02c	2.16± 0.002b	Dense	White	1.9± 0.008c

Figure 2.12. Hairy roots 2 weeks after transfer to sucrose from maltose (a), glucose (b), fructose (c) and glucose + fructose (d) showing variable branching and pigment synthesis.

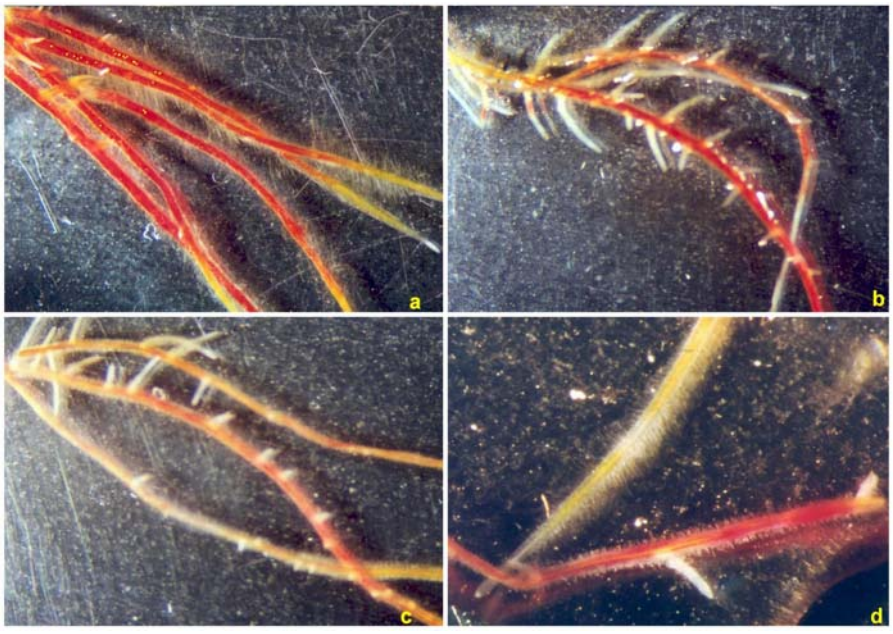


Figure 2.13. Hairy roots 3 weeks after transfer to sucrose from maltose (a), glucose (b), fructose (c) and glucose + fructose (d) showing variable branching and almost normal growth and pigment synthesis as observed in sucrose control.

2.3.2.5. Signaling role of sucrose with hexoses

It is clear that beet hairy roots grow well on sucrose and to a limited extent on maltose and other hexoses only when sub-cultured from sucrose-containing medium. Glucose and fructose being the breakdown products of sucrose, and glucose being the product of maltose are extensively involved in various metabolic activities of plant cells, and such a breakdown may occur at membrane level or cytosolic level. The present study clearly establishes that beet hairy roots not only fail to sense these hexoses, but that the growth is repressed when exposed to hexoses. The failure of beet hairy roots to sense hexoses as the external carbon source has also been reported in other studies (Smeekens, 2000; Wenzler et al. 1989). Now the question arises that if a part of the external carbohydrate is supplied as sucrose along with its hydrolyzed products (glucose and fructose) whether sucrose acts as a signal for the uptake of the hexoses, and if so, to what extent. To obtain an answer to this we supplied medium with different levels of sucrose with the remaining part as glucose, fructose or glucose + fructose to ultimately obtain 30g l^{-1} of carbohydrate. Since glycerol is an intermediary compound in various cellular functions, glycerol was also used at various levels. To know the nutritive role of sucrose the respective level of sucrose, without any hexose or glycerol counterpart, was used as control (Fig. 2.14). Glycerol was not utilized alone, but was taken up in the presence of a low level of sucrose (1g l^{-1}). However, a steady decline in glycerol uptake in the presence of higher levels of sucrose was apparent. Thus we now know that though glycerol is a far off intermediate in the glycolytic pathway it is still sensed and utilized for growth indicating that sucrose helps to trigger the initial steps responsible for the uptake of the glycerol molecule. This glycerol uptake was useful only for growth to a limited extent but not for pigment synthesis (Fig. 2.13), whereas the higher levels of glycerol appeared to nullify the effect of sucrose in facilitating uptake. The higher level of growth in the presence of 1gL^{-1} sucrose + 29gL^{-1} glucose that is nearly 10-fold higher than the respective control (1 sucrose), clearly indicates the signaling role of sucrose, as negligible growth was observed in the presence of glucose alone.

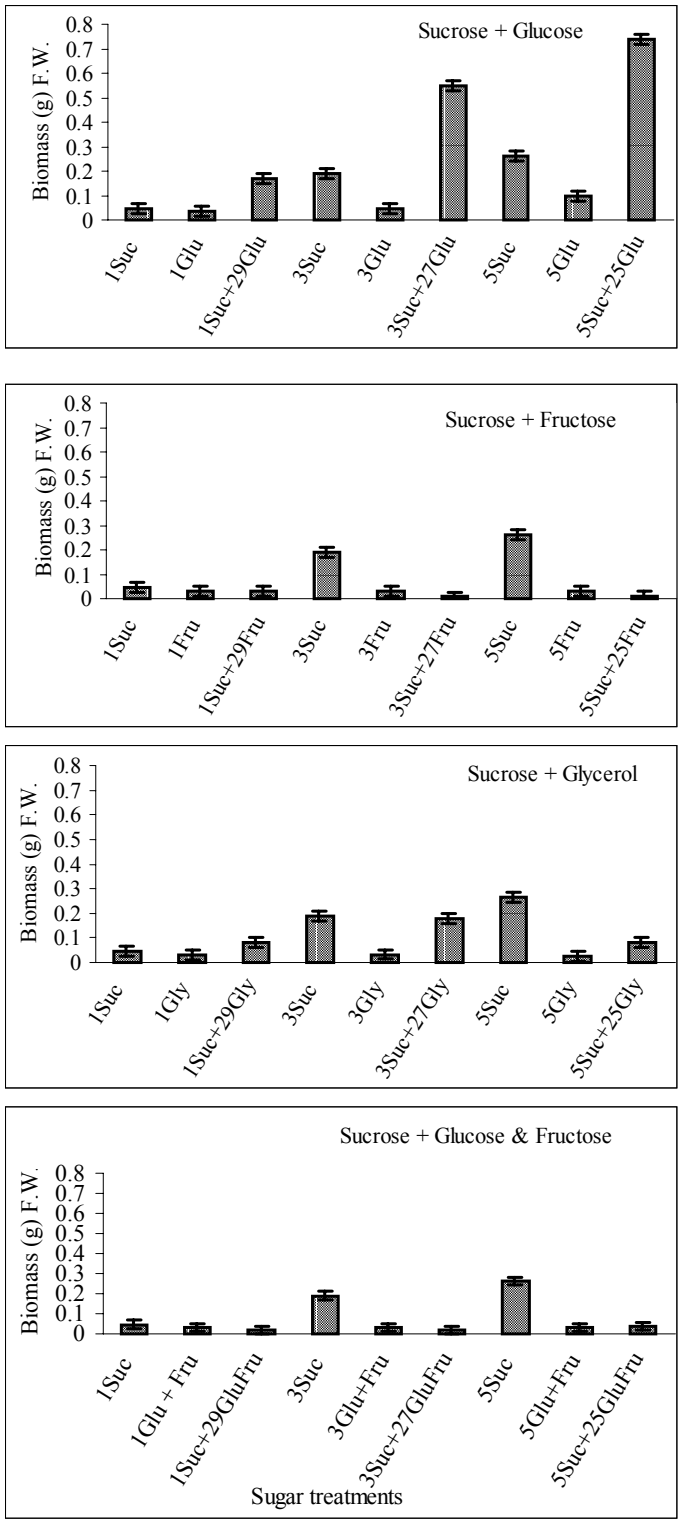


Figure 2.14. Complementary effects of different levels of sucrose on its hexoses/glycerol acting as a signal and / or a nutrient. Suc: Sucrose, Gluc: Glucose, Gly: Glycerol, Fru: Fructose, Glu + Fru = 1:1. Pre-fix numbers represent g l^{-1} of respective carbohydrate in medium. Each bar represents the mean ± S.E of six replicates.

Higher levels of sucrose along with glucose were utilized in a dose-dependent manner for both hairy root growth and pigment synthesis, especially for betaxanthine whereas for the

induction of betacyanine, higher levels of sucrose were essential. Such participations of sugar sensing pathways for secondary metabolite synthesis have been observed in a few other systems (Gibson, 2000; Mita et al., 1997; Muller et al., 2000). Fructose on the other hand was inhibitory and appears to impart a strong growth repressive effect irrespective of the level of sucrose present (within the limits of the levels tried in the present study). Even in the presence of glucose (i.e., glucose + fructose), the positive effects of both glucose and sucrose were strongly repressed by fructose. Such a strong inhibitory effect of fructose has not been reported in higher plant system.

2.3.3. Studies on scale up of hairy root culture

The hairy root cultures are heterotrophic shear sensitive systems based on the studies on the results of various culture conditions in shake flasks the studies were continued further using bioreactors. The results presented in the table 2.11 shows the biomass accumulated and betalaine studies using different types of anchorages such as one tier and two tier baskets.

Table 2.11. Growth and pigment production in bioreactor

Reactor	Biomass (FW g L^{-1})	Betalaine content (mg L^{-1})
1.Bubble column reactor		
One tier basket	30	100
Two tier basket	50	115
2. With air enrichment tank	60	125

The bubble column reactor with two-tier basket where air was continuously bubbled produced about 1.66-fold higher biomass compared to one tier basket (Fig 2.1A). Since the hairy root cultures are highly sensitive to bubble impact and shear, a modified bubble column was designed where an additional air enrichment tank was connected. The medium was in enrichment tank constantly bubbled (33.4cm^3 s^{-1}) with air to increase the dissolved oxygen level. The air enriched medium was circulated to and fro in to the reactor vessel where the culture was grown in a two-tier basket. When the beet hairy root culture was grown in this set up, a two-fold increase in biomass production was achieved with 1.25-fold higher betalaine production. The increased betalaine and biomass production in the modified reactor set up may be because of very low hydrodynamic and oxygen stress. The effect of hydrodynamic stress has already been explained with shake flask experiment. In the modified bubble column reactor one may expect greater oxygen diffusion to roots from the liquid because the roots come constantly in contact with the oxygen enriched medium without air bubbles causing a shear stress on the roots. Improved growth and metabolite formation was observed with improved mass transfer and oxygen supply to root cultures as also observed in other studies (Kondo et al. 1989; Yu and Doran 1994; Ramakrishnan and Curtis 1995).

In total, the present chapter considered the studies on various culture parameters such as effect of medium volume, shaker speed, and inoculum size on growth and pigment synthesis in the best clone LMG-150. Apart from the culture conditions, the chapter also dealt with the importance of carbon source and the role of sugars as nutrients and as signaling agents affecting various growth and metabolic attribute. Finally, the results of the studies on various parameters were applied for developing a bioreactor system with improved mass transfer and minimum shear on the hairy root cultures leading to improved growth and pigment production.

Chapter highlights

- **Importance of various kinetic parameters on the growth and pigment production was established.**
- **The nutritive and signaling role of sucrose in the growth of cultured hairy roots was established.**
- **An improved bioreactor model for the growth of hairy roots and betalaine production was developed.**

CHAPTER 3

STUDIES ON PEROXIDASE ENZYME

CONTENTS

SUMMARY

*T*he genetically transformed roots of red beet have been shown, to produce very high levels of peroxidase (POD; EC 1.11.1.7) accounting for $1.21x10^6$U L^{-1}. Each hairy root clone differed significantly from the others in growth, hormone dependency and POD production where LMG-150 produced highest biomass (140g FW L^{-1}) as well as POD (ranging from 8000-9000U g^{-1} FW and $1.18x10^6$U L^{-1} with a specific activity of 600U mg^{-1} protein) on hormone-free medium, both in shake-flask as well as in bioreactor, with a further enhancement to $1.21x10^6$U L^{-1} upon the addition of extra calcium chloride (5 mM). PAGE with active staining showed 5 distinct bands of R_m 0.06, 0.16, 0.25, 0.38 and 0.46 in the biomass and bands at R_m 0.06, 0.16, 0.25 and one extra band of R_m 0.575 in the spent medium where isozymes of R_m 0.38 and 0.46 were totally absent. The pH optima and other properties were grossly comparable with the standard horseradish peroxidase (HRP) with better thermal stability than HRP. In an effort to elicit POD activity further, the cultures were contacted with biotic elicitors such as dry cell powders of microbial cultures (0.1– 0.5% w/v) and the respective culture filtrates (1-5% v/v). Similarly, abiotic elicitors, particularly metal ions (2-8 folds of that present in the nutrient medium), plant hormone Thidiazuron (at 0.25-1 ppm) and other bio-molecules such as Glutathione (at 0.5mM-10mM) and Methyl jasmonate (at 20-100μM) were used. It was observed that dry cell powder of *Candida versatalis* significantly elicited the enzyme activity (3.52-fold higher than the control) followed by glutathione (3.44-fold) and *Rhizophus oligosporus* (3.09-fold). Among abiotic ones, thidiazuron, Mg and Ca salts elicited 2.49, 3.03 and 2.8 fold activities respectively. While most of the biotic elicitors were effective when added on 15^{th} day of culture, the abiotic elicitors were effective when added on 20^{th} day. Combination of highly effective elicitors indicated that glutathione (1mM) and dry cell powder of *R. oligosporus* caused 4-fold enhancement in enzyme activity, accounting for $10.9x10^6$U L^{-1}. Therefore, the present source appears to offer a cheaper and additional alternative for the commercial production of POD. Apart from this the chapter also deals with the partial purification and characterization of POD from hairy root cultures

Publications

Thimmaraju R, Vinod Kumar, Bhagyalakshmi N and Ravishankar GA (2005). Peroxidase production from hairy root cultures of red beet (*Beta vulgaris*). Electronic Journal of Biotechnology 8(2): 185-196.

Thimmaraju R, Bhagyalakshmi N and Ravishankar GA (2005). Elicitation of peroxidase enzyme activity in genetically transformed root cultures of *Beta vulgaris* L. Electronic Journal of Biotechnology (Communicated).

3.1. INTRODUCTION

Peroxidase (E. C. 1. 11. 1.7) (POD) is an enzyme known to play a very crucial role in scavenging free radicals (peroxides) within the plant system (Regalado et al. 2004) in addition to its involvement in various metabolic activities (Section G1.6.1; Chapter G). Outside the plant system this enzyme has several commercial applications, the major ones being its use as an important component in chemical diagnostics and laboratory experiments (Regalado et al. 2004) (Section G1.6.2; Chapter G). Because of broader catalytic activity, a wide range of chemicals can be modified using POD and hence has varied applications in waste water treatment to remove phenolics, synthesis of various aromatic compounds and removal of peroxides from foodstuffs, beverages and industrial wastes (Torres et al. 1997; Ayala et al. 2000). An extensive array of use though can be found for this enzyme, a major bottleneck lies in its current high cost.

3.1.1. Production

Presently, horseradish has been the source of high quality POD for biochemical / clinical applications whereas certain agricultural wastes / byproducts have been suggested for the production of commercial-grade POD. However, these sources of POD are limited by difficulties in purification (Uozumi et al. 1992; Srinivas et al. 1999). A few plant cell/suspension cultures have been reported to produce considerable levels of POD (Veitch, 2004). For example, cell cultures of horseradish, radish, suspension cultures of carrot, peanut, tobacco, spinach, *Vaccinium* sp. and cowpea (Melo et al. 1995; Veitch, 2004) produced appreciable levels of POD enzyme. However, these systems were of limited applications due to the slow growth rate of cell cultures, inconsistent product yield and genetic instability.

Hairy root cultures of carrot produced a higher level of POD of about $19.2Ug^{-1}$ FW than the suspension cultures (Uozumi et al. 1992). Horseradish hairy root cultures have also been reported to produce considerably high levels of PODs (Uozumi et al. 1992; Flocco et al. 1998).

3.1.2. Elicitation

Enhancement of secondary metabolites and their related precursors, including the associated enzymes such as POD, were achieved by using different strategies such as selection of clone, changing growth conditions, particularly with reference to hormones and nutrients. Another interesting strategy is the exposure of cultures to changed environment such as treatment with elicitors. Elicitors are compounds of biological or non-biological

origin, which upon contact with plant cells, trigger defense related compounds through over-expression of relevant enzymes (Eilert et al. 1984; Robins et al. 1985; Eilert et al. 1986; Flores and Curtis 1992; Bhagyalakshmi and Bopanna 1998; Gomez-Vasquez et al. 2004). The secondary products and the respective enzymes, particularly those of phenylpropanoid pathway (Fig. 3.1) get significantly enhanced under the influence of elicitors.

Peroxidase (POD) is one such enzyme that is associated with the plant defense pathway (Fig 3.1) and gets elicited when challenged with elicitors (Gomez-Vasquez et al. 2004; Perera and Jones 2004). For example, treatment with different abiotic elicitors such as $AgNO_3$ and $CuSO_4$ caused about 100% increase in POD production in transformed root cultures of horseradish. Further, the metal ions caused secretion leading to an overall productivity of about 12-fold higher POD enzyme (Flocco et al. 1998). The heterotrophic hairy roots of *Ipomoea aquatica* were also found to produce POD at levels 250U g^{-1} FW. When such roots were made autotrophic, the POD activity within the tissue nearly doubled which the authors related to the requirement of POD for scavenging the high release of toxic oxidants, the peroxides (Kino-Oka et al. 2001). Nevertheless failure of one elicitor does not necessarily mean that the metabolic pathway cannot be triggered. A combination of inappropriate medium and elicitor as well as unsuitable concentration of the latter can result in ineffective elicitation. For example, phenylpropanoid (PP) pathway was not induced in all cultures of *Vanilla planifolia* by yeast extract (Funk and Brodelius 1990), whereas the same elicitor was used by the same group of workers to induce phytoalexin in cultures of *Glycine max*. The PP pathway could however be triggered in *V. planifolia* by using chitosan as an elicitor (Funk and Brodelius 1990). Thus, successful application of elicitation requires extensive screening. Most of the earlier studies on elicitation of peroxidase enzyme involved *in vivo* plants (Perera and Jones 2004; Gomez-Vazquez et al. 2004) in response to pathogenic organisms. However, a few studies focused on the elicitation of POD activity in *in vitro* cultures using mainly one elicitor (Uozumi et al. 1992; Agostini et al. 1997; Flocco et al. 1998; Flocco and Guilietti 2003; Xu et al. 2004) but the present study is the first report on elicitation of POD activity in red beet hairy roots.

3.1.3. Purification and characterization of plant PODs

Though characterization of POD from hairy roots is essentially same as that from any plant or animal source, there have been difficulties. Early studies on POD involved purification and partial characterization of tomato extensin POD (Brownleader et al. 1995). The tomato extensin POD was estimated by assaying for the high molecular weight extensin molecules, which are cross-linked when incubated in the presence of H_2O_2. A significant

degree of purification was achieved by using CM-cellulose chromatography, which resolved the active POD into two peaks (I and II). Though both the enzymes of peak II and I performed the oxidation of guaiacol, only peak I was found to be an extensin cross linker (Brownleader et al. 1995). The RZ value of peak II was 3.2 which was about 2.3 fold higher than the RZ value of peak I. The peak I was further purified by using Mono S FPLC where four main protein peaks were resolved with NaCl gradient. While all of them possessed POD activity when assayed with guaiacol, the extension cross-linking was demonstrated only for EPIII (RZ 1.5) and EPIV (RZ 1.8). All the proteins were able to display a single band on SDS-PAGE. Further, the molecular mass estimated for EPIII and EPIV revealed a MW of about 37 and 34 KD respectively, which was further correlated with the MW determined by gel filtration. Hence it was concluded that EPIII and EPIV as monomeric proteins. (Brownleader et al. 1995). Both EPIII and EPIV showed a pI of 9.0 indicating that they are basic PODs, which were found to be consistent with a cation binding column assay. As in the case of many PODs, the N-terminals were found blocked when subjected for amino acid sequencing. Both the isoforms showed similar amino acid composition but both of them lacked cystein, which was quite unusual when compared to a number of published cationic PODs.

Barley cationic PODs were purified by $(NH_4)_2SO_4$ precipitation coupled with ion exchange chromatography using a cation exchanger where, among the isoforms resolved, the pI was between 7.3-9.3 and MW of 53-55 KD except for the isoform with pI 9.3 which had MW of 33 KD (Kristensen et al. 1999). Similarly purification of PODs from a majority of plant sources involved precipitation with $(NH_4)_2SO_4$ followed by ion exchange chromatography with further partial characterization by SDS-PAGE and iso-electric focusing (Nair and Showalter 1996; Wititsuwannakul et al. 1996; Christensen et al. 1998; Aruna and Lali 2001). However, Srinivas et al (1999) used a different strategy which involved aqueous two phase extraction coupled with gel filtration for purification of a POD extracted from the leaves of *Ipomea palmata*. This method used PEG/ammonium sulphate/NaCl (24/7.5/2.0%, w/v) to obtain a purification factor of 2.18, volume reduction of 57.5% and finally about 49-fold purification using a Sephadex G-100 column with a recovery of about 75.3%.

Since this enzyme has various commercial applications, the present chapter focuses on production, elicitation, partial purification and characterization of the enzyme from red beet hairy roots.

3.2. MATERIALS AND METHODS

3.2.1. Growth and POD production in different clones of hairy roots

Once the hairy root clones were confirmed for their transformed nature (chapter1), they were characterized for their growth performance, POD production and their responses to auxins and abiotic elicitors. For testing growth performance, about 50mg of root tips of 10 hairy root clones were subcultured in 50ml Erlenmeyer's flasks containing 15ml of MS basal liquid medium with 3% sucrose and grown on a rotary shaker as described earlier. The biomass accumulation was monitored at an interval of 5 days for a total period of 25 days. Fresh weight increase was recorded after surface drying the roots by keeping between folds of blotter sheets. The POD activity in each clone was estimated on 20^{th} day of the growth period. Further, the POD activity in the clone LMG-150 was monitored at an interval of 5 days for a total period of 30 days. Similarly, the non-transformed beetroot parts and seedling parts (grown both *in vivo* and *in vitro*) were also tested for the enzyme.

3.2.2. Extraction of POD, assay and estimation of protein

POD was extracted by homogenizing 50mg fresh tissue of hairy roots, in 1ml of sodium phosphate buffer (pH 6). After homogenization the homogenate was centrifuged at 12,000rpm twice and the supernatant was assayed for POD activity by following the method of Agostini et al (1997). Briefly, 1ml assay mixture was prepared which consisted of 100µl of 1% H_2O_2, 100µl of 0.25% ortho-di-anisidine hydrochloride, 10µl of enzyme extract and 790µl of respective buffer. The change in OD at 460ηm per minute (dA min^{-1}) at 27ºC was recorded using kinetic program in UV- visible spectrophotometer (Shimadzu UV-160A). The results were expressed as units per litre medium i.e., activities in biomass + medium for one litre of medium input (productivity). Activity was quantified on the basis of standard curve of horseradish POD enzyme obtained from ICN-biochemicals. To quantify the extra cellular POD in the medium, the spent medium was centrifuged twice at 12,000rpm at 4ºC to remove cell debris and the supernatant was assayed for enzyme activity. Estimation of total protein content in the crude extracts was done by adding reducing agents such as NaCl and cysteine to the extract, followed by precipitation of total protein using trichloro acetic acid (TCA), re-dissolving in 2N NaOH to determine total protein concentration by following the method of Lowry et al (1951). The protein content data was used to check the specific activity of POD whenever required.

3.2.3. Influence of auxins

Ten mg each of different hairy root clones were inoculated into 50 ml conical flasks containing 15ml of MS liquid medium with 0.5 and 1.0ppm of either indole acetic acid (IAA) or naphthalene acetic acid (NAA) and incubated on an orbital shaker kept at 90rpm maintained at 25ºC. The total biomass (fresh weight in g L^{-1}) and POD activity were analyzed at the end, i.e., on 20th day and POD productivity level was expressed as units of enzyme produced per litre of medium.

3.2.4. Influence of salts

For horseradish hairy roots the additional ion-supplementation considerably enhanced the release of POD into the medium (Uozumi et al. 1992). Therefore, to check for similar influence in the present system the cultures of LMG-150 were exposed to MS liquid medium containing additional levels of different ions in salt forms such as NaCl, KCl, $MgCl_2$, $CaCl_2$, Na_2SO_4, Na_2CO_3, KNO_3, $FeCl_3$ and EDTA at various concentrations such as 5, 10 and 15mM. Hairy roots were cultured in 50ml Erlenmeyer's flask containing 15ml of MS medium incorporated with different ionic salts keeping rest of the conditions as mentioned earlier. Biomass and POD levels were estimated at the end of 20th day and the POD productivity (activity U L^{-1}) and the POD released into the medium (also measured in terms of activity) was calculated.

3.2.5. Intracellular and extra-cellular POD

To further characterize the enzyme, the POD zymogram was prepared by polyacrylamide gel electrophoresis (7.2%) (PAGE) carried out at 120V for 4h using 12 × 14 × 0.3cm gel without SDS using standard protocol. The gel was stained for POD activity with a 100ml solution of sodium phosphate buffer (0.2M, pH 6.0) containing 10ml of 0.25% ortho-di-anisidine hydrochloride and 10ml of 1% hydrogen peroxide (Agostini et al. 1997) and immediately photographed.

3.2.6. Production of POD in bioreactor

Hairy root cultures were grown in a 3L bubble column reactor (Chapter 2; Fig 2.1A) with a working medium volume of 1.75L. Hairy root inoculum was prepared by sub culturing about 100mg of hairy roots of clone LMG-150 in 40ml medium in conical flasks for 10 days under standard conditions mentioned earlier. Totally 10g fresh weight of actively growing hairy root inoculum was transferred aseptically through inoculation port into the anchorage basket of the growth chamber. The bioreactor was maintained in dark at 23 ± 2ºC

with air supply through a sparger at a rate of 33.4cm^3s^{-1}. Hairy root cultivation was terminated after 10 days and the POD in biomass and the spent medium was estimated.

3.2.7. Elicitation of POD activity

3.2.7.1. Biotic elicitors

3.2.7.1.1. Crude elicitors

Based on earlier reports and the availability of cultures, culture filtrate (CF) and dry cell powder (DCP) of various fungi, yeast and bacteria were used (Food Microbiology Department CFTRI, Mysore, kindly supplied all the microbial cultures). Fungi used were *Aspergillus parasiticus, Aspergillus niger, Penicillium notatum, and Rhizophus oligosporus.* Yeast species used was *Candida versatalis.* Among the bacteria *Lactobacillus helveticus* was used.

3.2.7.1.2. Purified biotic elicitors

The compounds and their levels were selected based on an earlier study (Suresh et al. 2004). Thus methyl jasmonate (Mej) was used at 20, 40, 60, 80, 100μM whereas glutathione (GSH) was used at different levels such as 0.5-10mM.

3.2.7.1.3. Maintenance of microbial cultures

Fungi and Yeast: The fungal and yeast cultures were maintained as slants in potato dextrose agar (PDA) medium containing hot water extract from 200g potato, 20g of dextrose and pH adjusted to 5.5 with 10% tartaric acid and 20g of agar was added and the final volume was made up to one litre using distilled water. Autoclaved medium was used in culture tubes as slants. Liquid medium prepared similarly without agar was used to grow fungal mycelia for elicitor preparation.

Bacteria: Bacterial culture was maintained on agar slants with solid medium containing proteose peptone (10g L^{-1}), yeast extract (5g L^{-1}), beef extract (10g L^{-1}), dextrose (20g L^{-1}), tween-80 (1g L^{-1}), ammonium citrate (2g L^{-1}), sodium acetate (5g L^{-1}), magnesium sulphate (0.1g L^{-1}), manganese sulphate (0.05g L^{-1}) and di-potassium phosphate (2g L^{-1}). The pH was adjusted to 6.5 before addition of agar. In case of liquid medium, agar was deleted.

3.2.7.1.4. Elicitor preparation

The cultures maintained on agar slants, were transferred to 100ml of the respective liquid medium in 250ml flasks and incubated at room temperature. The bacterial and yeast cultures were kept on a shaker (90rpm), while the fungal cultures were allowed to stand without shaking. The cultures were harvested after they reached their stationary phase (i. e.,

3 weeks for fungal cultures, 72h for yeast culture and 48h for bacterial culture). The flasks were autoclaved and the fungal mycelial mat which floated at the surface of the medium was carefully removed and washed a few times with sterile distilled water and allowed to dry in a hot air oven at 40°C, crushed into powder using mortar and pestle and used as DCP. The remaining medium i.e., CF was centrifuged to remove suspended particles, filtered through Whatman No. 1 filter paper and the clear solution so obtained was stored at 4°C for further use. Similarly, for yeast and bacterial cultures, the culture broth was centrifuged at high speed for 1h and the cell sediment was air-dried and used as DCP and the respective spent medium for use as CF was stored at 4°C until further use.

3.2.7.2. Abiotic elicitors

Different abiotic elicitors used were metal ions such as calcium and magnesium, and an hormonal elicitor thidiazuron (TDZ), Metal ions were used at various levels such as 2, 4 and 8 folds of their respective concentrations in the normal MS medium. TDZ was used at 0.25ppm, 0.50ppm and 1.0ppm in MS liquid medium.

3.2.7.3. Addition of elicitors

Fifty milligrams of hairy roots were grown in 15ml Murashige and Skoog's (1962) liquid medium (MS) in an Erlenmeyer flask, incubated in dark on a rotary shaker with 90rpm at 25 ± 1°C for 15, 18 and 20 days. The mycelial / cell powders prepared as above were weighed and added to MS basal medium and the pH was adjusted to 5.8 before autoclaving at 15psi for 20min. The culture filtrates of respective fungi, bacteria and yeast were also used (v/v) similarly to prepare the elicitation medium. The so-prepared elicitation medium was used to treat hairy root cultures at three different time periods such as 15^{th}, 18^{th} and 20^{th} day of the total culture period of 25 days by replacing the spent medium of respective culture stages. Control cultures received fresh MS medium without elicitor addition. The enzyme production was estimated by extracting on 25^{th} day of the culture period. Abiotic elicitors such as calcium, magnesium and TDZ were weighed and added to MS medium and the pH adjusted before autoclaving as mentioned above. Other two abiotic elicitors such as GSH and Mej were used by preparing appropriate concentration of stock solution were directly added to autoclaved medium after filter sterilization using 0.45µ Sartorius® filters. Culture conditions for further monitoring of growth and POD activity were as explained earlier (Section 3.2.1; Page 90).

3.2.7.4. Combined effect of biotic elicitors and GSH

Possible combined effects of best complex microbial elicitor and purified biotic elicitor GSH was studied by selecting effective fungal elicitors such as 0.25% DCP of *R. oligosporus*, 5% CF of *A parasiticus* and 0.1% DCP of yeast *C versatalis* with different levels of GSH such as 0.5-2mM. The elicitor medium was prepared as explained earlier for the respective fungal and yeast elicitors and filter sterilized (0.45μ Sartorius) GSH was added at various levels and used for treating hairy roots grown for 15 days. The enzyme was extracted on 25th day, assayed, quantified and expressed as productivity in terms of UL^{-1} medium used.

3.2.7.5. Partial purification and characterization of hairy root POD (HR-POD)

3.2.7.5.1. Ammonium sulphate fractionation

The crude enzyme extract prepared as given in the section 3.2.2 was subjected for ammonium sulphate precipitation by progressively increasing the degree of saturation form 0-20, 20-40, 40-60, 60-80, 80-100. Solid ammonium sulphate was added to the extract with constant stirring at 4°C and kept overnight to complete precipitation. The mixture was centrifuged at 8000Xg for 20min at 4°C and the fraction with maximum specific activity was dialyzed in a dialysis tubes of 12000kD cut-off range (Sigma –Aldrich, USA).

3.2.7.5.2. Anion exchange chromatography (AEC)

About 10g of ion exchange resin DEAE-cellulose (Sigma-Aldrich) soaked in double distilled water for 48 hours was activated by following standard protocol. Briefly, the matrix was washed with 0.1M HCl for 10min and the pH of the matrix was brought back to neutral by repeated washing with excess of double distilled water. Again the matrix was treated with 0.1M NaOH for 10min and washed several times to bring the pH to neutral. The activated matrix was equilibrated with pH 6.0 sodium phosphate buffer (0.2M) and packed onto a column of bed volume 25ml.

The packed resin was equilibrated with sodium phosphate buffer (0.2M pH 6.0) by washing overnight at a flow rate of about 12ml h^{-1}. The column was loaded with 80% dialyzed ammonium sulphate fraction of HR-POD. The unbound enzyme was collected at about 2ml per fraction by washing the loaded column with approximately 3 bed volumes with buffer. The bound fractions were eluted by a linear gradient of 0 to 0.5M-sodium chloride prepared in 0.2M sodium phosphate buffer (pH 6.0), collecting fractions of 2ml each. The OD at 280ηm and activity of POD in each fraction was monitored and plotted. The fractions corresponding to the peak with maximum activity were pooled and concentrated by lyophilization and used for SDS-PAGE analysis.

3.2.7.6. Partial characterization

3.2.7.6.1. Optimum pH for activity and stability

The pH optima for the activity of the HR-POD was found out by assaying for enzyme activity at different pH. The assay was carried out by taking buffers of different pH such as pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 separately in an assay mixture of 1ml containing 10µl of the 60-80% dialyzed fraction of the enzyme, 100µl 0.25% orthodianisidine hydrochloride, 100µl of 1% H_2O_2 and 790µl of buffers of different pH. The experiment was repeated using standard horseradish peroxidase.

For the assay of optimum pH for stability, 100µl of the 60-80% dialyzed fraction and AEC non retained fraction of the enzyme were taken separately and mixed with 100µl each of different buffers such as pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 separately. The mixture was allowed to stay at room temperature (24 ± 1ºC) for overnight (ca 12h). The activity was measured as explained in the section 3.2.2 at pH 6.0. The experiment was repeated using standard horseradish peroxidase procured from ICN-Biochemicals.

3.2.7.6.2. Determination of K_m and kinetic mechanism

Initial experiment was carried out to determine the K_m and V_{max} values for H_2O_2 by assaying the activity at different concentrations of H_2O_2 ranging from 0.01 to 0.2mM at a constant orthodianisidine hydrochloride concentration (2mM). Based on this experiment a constant concentration of 0.5mM H_2O_2 was selected for determining the K_m and V_{max} values for other substrates such as ABTS (2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (1 - 10mM), orthodianisidine hydrochloride (0.1 – 2mM) and guaiacol (30 - 50mM) at various concentration ranges indicated in the parenthesis. The K_m and V_{max} values were determined by using the Lineweaver-Burk reciprocal plot graphic method (Segel 1993) for the two substrate ping-pong mechanism followed by peroxidase. Assuming initial rates (v_0), a general equation for this mechanism in the forward direction was given by Whitaker (1994) is as below

$$1/v_0 = 1/V_{max} + K_a / A_0 V_{max} + K_b / B_0 V_{max} \text{----------(1)}$$

Where, V_{max} = maximum velocity, $K_a = K_m$ for substrate A (hydrogen peroxide), A_0 = concentration of substrate A, $K_b = K_m$ for substrate B (H donor), and B_0 = concentration of substrate B, from a plot of equation (1), enzyme systems following sequential mechanism (random or ordered) give lines that intersect to the left of y-axis, whereas, the systems that follow a ping-pong mechanism give parallel lines (Whitaker, 1994). When A_0 is constant, equation (1) will yield a slope intercept given by

$$\text{Slope} = K_b / V_{max} \text{----------(2)}$$

Therefore, the constants K_m and V_{max} can be determined from equation (2).

3.2.7.6.3. Peroxidase inhibition

The potassium periodate, and sodium azide inhibition of the AEC partial purified peroxidase was determined by using a constant orthodianisidine hydrochloride concentration (0.5mM), in individual experiments in which the concentrations of potassium periodate and sodium azide ranged from 0.1 to 0.5mM at three different concentrations of H_2O_2 (0.5, 0.6 and 0.9mM). The required aliquots of potassium periodate and sodium azide was added to a 1ml reaction mixture containing 0.5mM orthodianisidine hydrochloride and individual concentrations of H_2O_2. The blank consisted of all the reagents except potassium periodate (or sodium azide) and H_2O_2. The K_i was determined from the intercepts of a plot of 1/v (reciprocal of initial velocity) versus potassium periodate (or sodium azide) concentrations.

3.2.7.6.4. Thermal inactivation of POD

Heat inactivation of the crude and AEC-derived enzyme was carried out by measuring the residual activity after incubating 100µl of the enzyme (the extraction of which has been explained earlier) at different temperatures in a shaking water bath. Aliquots (10µl) taken at different time intervals were assayed immediately and residual activity was expressed as per cent decrease of activity.

3.3. RESULTS AND DISCUSSION

3.3.1. Biomass accumulation and POD activity in hairy root clones

Among the ten hairy root clones, there was a clear-cut difference from one clone to another in terms of biomass accumulation and the profile of POD activity (Fig 3.2A & B). While the root clone LMG-150 showed high biomass productivity of 140g FW L^{-1} on hormone-free medium, the other clones produced very low biomass. The clone LMG-150 was unable to grow in the presence of any level of IAA whereas growth was fair in the presence of NAA though the latter also suppressed biomass when compared with control (Fig 3.2A). IAA significantly supported biomass formation in the second clone of A 2/83 whereas NAA supported better biomass than in control in the first clone of A4, followed by third clone of the same and the third clone of A20/83. In general, both the auxins IAA and NAA appeared to suppress the biomass accumulation in clone LMG-150, whereas for most of the other clones either IAA or NAA was beneficiary either marginally or significantly (Fig 3.2A).

Unlike the biomass, the productivity (U L^{-1}) of POD (pH 6.0) in the best clone LMG-150 was almost similar with or without auxin treatment accounting for about 1×10^6U of POD activity per litre medium (Fig 3.2B). Similarly, in most of the other clones, the POD productivity was enhanced significantly by the auxins and in some of them the productivities were on par with that of the best clone. The clones A 20/83(2), A 20/83(3), A4(1), A4(2) and A4(3) performed better than their respective controls when supplemented with 1ppm of NAA producing about 1×10^6U of POD activity per litre. Whereas the clones A 2/83(2) and A 2/83(3) produced higher POD in the presence of 0.5ppm IAA accounting for about 0.8×10^6 and 0.6×10^6U L^{-1} respectively (Fig 3.2B). These differences may be attributed to the secondary variation in the T-DNA insertion, copy number, size and location of integration of T-DNA of Ri -plasmid into the plant genome (Doran, 2002) as also noted in horseradish hairy roots (Flocco et al. 1998).

Thus, the transformed nature of red beet hairy roots harbouring genes for root morphology i.e., *rol* genes (Lemcke and Schmulling 1998; Thimmaraju and Bhagyalakshmi 2005) is probably one reason for the over-expression of POD, as observed in seedling roots rather than in the mature tubers. The higher expression of POD in the skin of red beer tuber may be attributed to defense related activities or due to elicitation by surface borne micro-organisms, since several soil-borne microbes are known to elicit phenyl propanoid pathway, enhancing the intermediary enzymes as well (Radman et al. 2003).

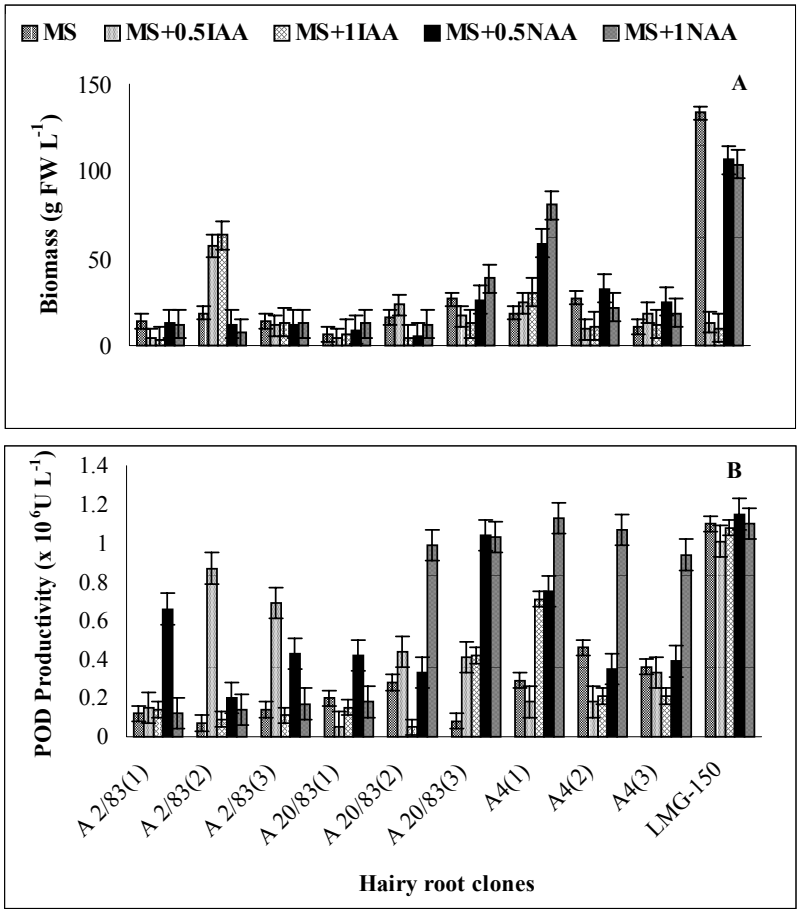


Figure 3.2. Growth (A) and peroxidase activity (B) in different hairy root clones grown in hormone free MS liquid medium and in MS with either IAA or NAA at either 0.5ppm or 1.0ppm observed on 20th day of culture period. The data is the average of 5 replicates of two independent experiments and the bars indicate SE of mean values.

The fact that auxins are involved in growth and morphogenesis of various organs, especially the roots, is well established. The exogenous application of two auxins in the present study did not cause any significant improvement of biomass accumulation in most of the clones, except in clones A 2/83(2) and A4(1) where there were significant improvements in biomass after the supplementation of IAA and NAA. Such hormone dependency for growth has also been observed in hairy root cultures of *Cichorium intybus* (Bais et al. 2001) and horseradish hairy roots (Uozumi Kobayashi 1997). Contrarily, there was a very high variability when POD production was concerned. In many cases the POD activity increased in response to specific concentrations of auxins. Such responses may be attributed to the hormonal support or stress created on the hairy root clones in addition to the well-known fact of PODs significant implication in auxin metabolism (Liu et al. 1996). In cases where increased growth was observed, such as in clones A 2/83(2) and A4(1), the oxidants

produced during various metabolic activities would demand for the synthesis of this enzyme for scavenging $O^{\cdot}_2$ (pro-oxidants) and H_2O_2 (peroxides) (Kino-Oka et al. 2001). In addition, the POD being necessary for the cell during the stress-related metabolism, the presence of auxins may switch on the stress signal for the production of POD causing sudden increase of the same in most of the other clones where biomass was otherwise suppressed. Interestingly, the best clone LMG-150 did not respond significantly for auxin where POD levels remained almost constant even after treatment (Fig 3.2B) indicating that this clone neither utilizes exogenous auxin nor that the addition of hormones impart any stressed physiological conditions. The non-responsiveness of this clone may be attributed to the lack of auxin-uptake metabolism from the cell exterior, particularly at levels that are tried in this study. However, it may be recalled that these levels were able to induce rooting and/or callus in untransformed cultures (Narayan et al. 2004). Since the different clones induced by the same strain of *A. rhizogenes* such as three clones each from A2/83, A20/83 and A4 responded differently to different auxins, the present study clearly indicates that red beet hairy roots form an excellent model system to unravel the influence of T-DNA on auxin metabolism.

3.3.2. POD production in LMG-150

In root clone LMG-150, the activity of POD extracted at pH 6.0 was highest on 20th day producing about 10000U g^{-1} FW followed by 9000U g^{-1} FW on 15th day, and of the total about 30% of the POD in each case was secreted into the medium (Fig 3.3A & B). However, the enzyme extracted each at acidic (pH 4.0), basic (pH 9.0) and neutral (pH 7.0) showed significantly lesser POD activity. In the medium on 15th day, about 45% of the total activity was that of acidic and nearly 20% were those from pH 6.0 and neutral. However, the basic POD was released at high levels accounting for 28% of the total found in the medium (Fig 3.3B). The total protein was highest on 15th day and remained consistently at 15mg g^{-1} FW biomass (Fig 3.3C). The clone A 20/83(2) expressed higher level of POD i.e., nearly 12000U g^{-1} FW on 20th day in MS liquid medium (data not shown). Since, the biomass was lower in this clone than LMG-150 accounting for lower productivity per litre medium, the latter clone LMG-150 was considered for detailed study. All the other clones produced either less or negligible total POD activity compared to the clone LMG-150. The best clone LMG-150 established in the present study produced POD of about 9000U g^{-1} FW, which was significantly higher than the POD level reported for horseradish hairy roots (Flocco et al. 1998). The kinetic studies showed that the POD activity was high during mid-exponential growth phase and declined further as the biomass reached its peak on 25th day as also observed by Flocco et al (1998). This is probably due to high metabolic activity during

which the pro-oxidants are formed sending signals for the release of POD to quench the oxidant molecules, as has been suggested for *Ipomoea aquatica* (Kino-Oka et al. 2001). The low productivities of PODs of other pH may also be due to the same reason that most of the metabolic activities occur at pH ranging from 5.5 - 6.0, which is well known.

Similarly, to check whether the high level of POD activity observed in hairy roots was due to transformed nature of root or not, the non-transformed tissues were also screened for POD activity. Table-3.1 shows that among the seedling parts whether grown *in vitro* on MS medium or in the soil, the roots expressed almost similar activity of POD (9000U g^{-1} FW) whereas the cotyledonary leaf that was used for inducing hairy roots in the present study showed much lesser level of about 3591U g^{-1} FW of POD activity. In the mature red beet tuber (grown in the field), peel showed maximum activity of 6116U g^{-1} FW, which again was much lesser than that found in hairy roots (Table 3.1). In non-transformed seedling tissues and tubers it is the seedling root that expressed maximum POD activity (irrespective of *in vivo* or *in vitro* conditions of seedling growth) and not the tuber indicating that it is the rooty morphology that probably plays a role in signaling POD expression. Peel of a tuber invariably has a higher defensive role than the interior tissue and hence higher POD activity may be expected.

Table 3.1. Peroxidase activity (U g^{-1} FW) in different parts of 20-day-old red beet seedling and mature tuber at pH 6.0

Plant part	Total POD activity U g^{-1} FW
20 day old seedling	
Leaf blade	**3591.5**
Petiole	**2623.4**
Root	**9700.3**
Mature tuber	
Peel	**6116.0**
Pulp	**1492.5**
Pulp + Peel	**4398.0**

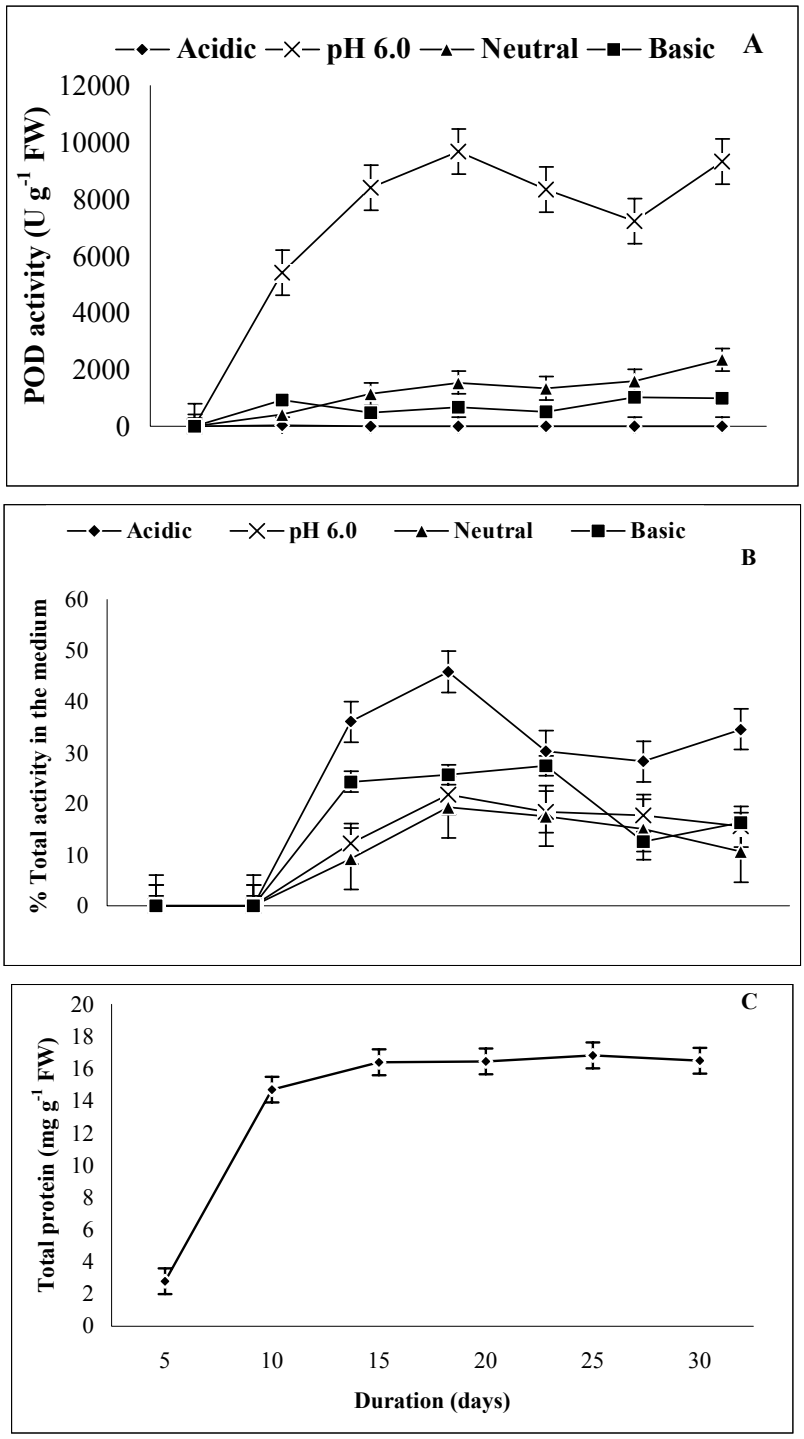


Figure 3.3. Total activities of different peroxidases (A), and the pattern of release of different peroxidases into the medium (B) and total protein content (mg g^{-1} FW) (C) in the clone LMG-150 cultured for 30 days. POD activity was monitored at an interval of 5 days for a total period of 30 days. Different POD fractions were extracted separately using separate buffers and the supernatant was taken from both medium and the extract from biomass for POD and total protein analysis. The data is the average of 5 replicates of two separate experiments. The bar indicates SE of mean.

3.3.3. Influence of salts on total turnover and secretion of POD

Among the different salts used, in addition to those already present in the medium, $FeCl_3$ as well as EDTA completely suppressed growth and POD activity levels even in the best clone LMG-150 (data not presented). Among the other metal ions used $CaCl_2$ at 5mM concentration produced slightly higher POD activity of about 1.21×10^6U L^{-1}. Further higher concentrations appeared to suppress the enzyme activity in a dose dependent manner. KCl produced slightly higher POD activity (Fig 3.4A) than the control accounting for about $1.11\times10^6UL^{-1}$ with a suppression of activity at higher treatment levels. Most of the other ions either produced same amount of POD or appeared to suppress the enzyme at higher treatment levels, except for KNO_3 where higher the level better was the POD activity (Fig 3.4A).

Maximum release of POD measured in terms of activity was observed in the presence of 5mM KNO_3 (nearly 50%) that was followed by only NaCl (43%) (Fig 3.4B), whereas treatments with other metal ions at three different concentrations did not cause any significant effect on POD secretion.

Data presented in Fig 3.4A showed that the cations Mg^{2+}, Ca^{2+} and K^+ from their respective salts caused slight increase in total turn-over of POD while the other anions used such as SO_4^{2-}, CO_3^- and Cl^- appeared to suppress the same. NO_3^- ions also caused a suppression of POD at low concentrations while enhancing the same at higher concentration. Na_2-EDTA and $FeCl_3$ completely suppressed the growth of the hairy roots, as has also been observed in other hairy root systems (Uozumi et al. 1992).

Among the different cations Na^+ and Mg^{2+} ions improved the secretion POD into the medium thus acting as effluxing agents. The involvement of these ions in membrane permeabilization acting as ion channels has been well established (Karpen and Ruiz, 2002). The other cations such as K^+ & Ca^{2+} did not cause any significant increase in POD secretion though these ions are also involved in the membrane activities and reported to cause secretion of POD in horseradish hairy roots (Uozumi et al. 1992). Supplementation of medium with anions such as SO_4^{2-}, CO_3^{2-} & NO_3^- increased the extra-cellular POD activity with NO_3^- causing highest secretion of about 50% of the total POD (Fig 3.4B). This was in contrast to the earlier reports where NO_3^- had very little effect on secretion of POD (Uozumi et al. 1992). Interestingly white rot fungus *Phanerochaete flavido-alba* has also been observed to respond positively to carbon limitation and excess of nitrogen leading to the leaching of lignin POD (Hamman et al. 1997).

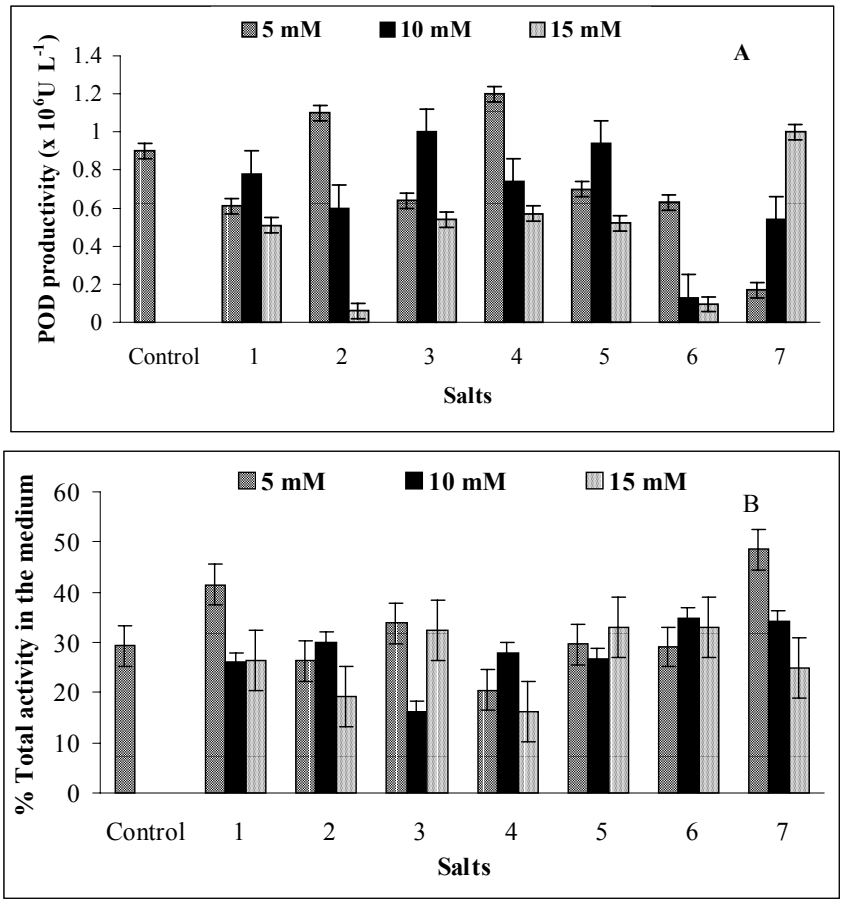


Figure 3.4. Effect of different salts at various concentrations on the total turn-over of activity (A) and secretion (B) of peroxidase enzyme in hairy root clone LMG-150 estimated on 20th day of the culture period (X-axis: 1: NaCl, 2: KCl, 3: Mg Cl_2, 4: $CaCl_2$, 5: Na_2SO_4, 6: Na_2CO_3, 7: KNO_3).

3.3.4. Intracellular and extra-cellular POD in LMG-150

Figure 3.5A&B shows the active staining of of intra- and extra-cellular PODs separated on 7.2% polyacrylamide gel where POD from the hairy roots of clone LMG-150 showed 5 distinct bands each corresponding to relative mobility (R_m) of 0.06 (designated as F), 0.16 (E), 0.25 (D), 0.38 (C), 0.46 (B). Of these isoforms, D, E and F were found in the spent medium. The bands B and C were not present in the medium. Whereas the band with R_m value of 0.575 (A) was found only in the medium, which was absent in the biomass and this may be the isozyme that was completely secreted in to the medium. Therefore, the present study shows that red beet hairy roots produced totally about 6 POD isoforms of which three were secreted partially into the medium and only one was completely secreted into the medium. All the isoforms present in the hairy root biomass, except for two, were also found in the extra cellular medium but only one isoform of R_m 0.575 was found only in the medium but not in the biomass. This pattern indicates that four of the six isozymes were

of secretary-type-proteins but the degree of secretion varied allowing a good scope for improving the secretion. The involvement of specific signals in assisting the leaching of a specific type of POD isozyme forms an interesting study for the future. Further, there was a clear distinction between the different isoforms differing by R_m value of at least 0.15, which implies that the isoforms are amenable for easy separation and further purification (Fig 3.5).

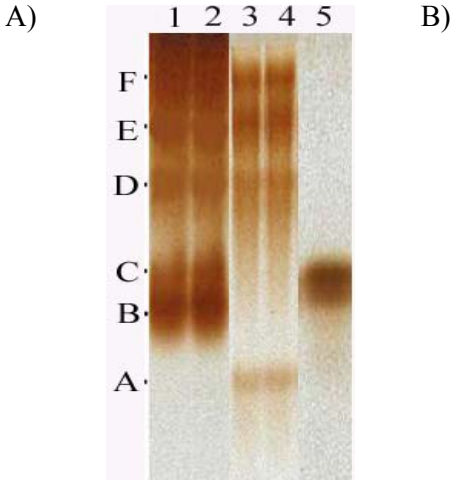


Figure 3.5. Zymograms of (A) peroxidase isozymes from roots (lanes 1 & 2) and from spent medium (lanes 3 & 5) developed by activity staining of the crude enzyme separated on 7.2% native polyacrylamide gel and partially purified fraction (lane 5). (B) SDS-PAGE analysis of crude (lanes 1-3) and purified fraction of (lane 4) of POD from hairy root clone LMG-150. The electrophoresis was carried out by loading about 20µl each of crude enzyme preparation from both biomass and spent medium in a 7.2 % polyacrylamide gel (12 × 14 × 0.3 cm) (PAGE) and 10% SDS-PAGE carried out at 120V for 4h. The lane M = Protein molecular weight markers.

3.3.5. POD production in bioreactor

The best clone LMG-150 was grown in a bubble column reactor for 10 days. As shown in table 3.2, even here this clone showed almost the same amount of total POD activity as observed in the shake flask, except that there was slight reduction in the activity of extra cellular POD. A total POD of about 9000U g^{-1} FW was produced in the bubble column reactor (Table 3.2).

Though various metabolites are suppressed in a bioreactor when compared with the respective shake flask culture (Doran, 2002), the selected root clone of red beet i.e., LMG-150 performed well even in the bioreactor cultivation producing comparable levels of POD. Though there was a suppression of biomass in the bioreactor, leading to the lower overall productivity, such suppression was not very significant. Interestingly, the release of enzyme

into the cell exterior was very limited in bioreactor, except for the one at acidic pH (Table 3.2). Contrarily there are other organized systems grown in bubble column bioreactor such as the somatic embryos of sandalwood, which released very high levels of PODs when grown in bioreactor (Pal et al. 2003). These observations indicate that the red beet hairy root system has several metabolic activities that need to be unraveled and hence appear as an interesting system for future studies.

Table 3.2. Activities of different peroxidase fractions in hairy root clone LMG-150 grown in bubble column reactor (U g^{-1} FW) and in the spent medium (percent of the total activity).

Peroxidase fraction	**Total units of POD activity per g^{-1} FW**	**% of the total activity found in the medium**
Acidic (pH 4.0)	13.8	13.4
pH 6.0	9932.0	5.9
Neutral (pH 7.0)	3495.1	0.4
Basic (pH 9.0)	4754.3	4.9

3.3.6. Elicitation of POD activity

The results of the present study show (Table 3.3) that among the fungal elicitors, majority of the treatments resulted in elicited levels of enzyme activities. The highest were those with DCPs of both *R. oligosporus* (added on 15th day) and *P. notatum* (added on 20th day) causing enhancements to the tune of 2-2.5-fold higher activity than the control. High levels (5% v/v) of culture filtrates of *A. parasiticus* and *P. notatum* (2.5 – 5% v/v) enhanced POD activity by nearly 3-fold. However, low level of (1%) culture filtrate of *R oligosporus* was needed to cause similar levels of elicitation in a short period of 5 days.

Table 3.3. Elicitation of peroxidase activity ($\times 10^6$U L^{-1}) using dry cell powders (DCPs) and culture filtrates (CFs) of fungi. Each value is the mean ± standard error of six replicates.

Dried cell powders (DCPs)		Time of elicitor addition (day)		
	Treatment % (w/v)	**15**	**18**	**20**
Control*		2.75 ± 0.05	2.85 ± 0.03	2.80 ± 0.04
A. parasiticus	0.10	4.15 ± 0.12^{ef}	2.30 ± 0.21^{e}	7.40 ± 0.32^{b}
	0.25	3.80 ± 0.23 ^{ef}	2.55 ± 0.12^{e}	5.15 ± 0.21^{de}
	0.50	5.95 ± 0.22^{cd}	6.55 ± 0.41^{a}	3.95 ± 0.10^{ef}
A. niger	0.10	2.40 ± 0.23^{ef}	6.53 ± 0.38^{a}	2.53 ± 0.12^{g}
	0.25	7.51 ± 0.10 ^{b}	4.90 ± 0.15^{bc}	5.55 ± 0.31^{d}
	0.50	7.05 ± 0.23^{bc}	4.79 ± 0.12^{bc}	6.60 ± 0.23^{c}
P. notatum	0.10	7.66 ± 0.41^{b}	6.25 ± 0.31^{ab}	8.45 ± 0.41^{a}
	0.25	4.20 ± 0.21^{ef}	3.40 ± 0.23^{d}	5.67 ± 0.21^{d}
	0.50	4.15 ± 0.10^{ef}	2.18 ± 0.05^{ef}	5.13 ± 0.23^{de}
R. oligosporus	0.10	5.55 ± 0.23^{cd}	1.90 ± 0.25	4.30 ± 0.12^{e}
	0.25	8.51 ± 0.22^{a}	3.40 ± 0.03^{d}	5.55 ± 0.34^{d}
	0.50	1.70 ± 0.04^{g}	2.35 ± 0.08^{e}	2.25 ± 0.23^{g}
Culture filtrates (CFs)				
	Treatment % (v/v)	**15**	**18**	**20**
A. parasiticus	1.0	5.20 ± 0.21^{bc}	6.58 ± 0.23^{c}	3.77 ± 0.16^{e}
	2.5	5.63 ± 0.22^{b}	6.93 ± 0.32^{bc}	6.63 ± 0.24^{cb}
	5.0	6.58 ± 0.34^{a}	8.47 ± 0.21^{a}	5.88 ± 0.35^{c}
A. niger	1.0	3.74 ± 0.12^{cd}	6.02 ± 0.05^{cd}	6.46 ± 0.31^{cb}
	2.5	4.20 ± 0.24^{cd}	5.20 ± 0.21^{de}	6.93 ± 0.21^{b}
	5.0	6.08 ± 0.32^{ab}	3.21 ± 0.34^{fg}	7.50 ± 0.20^{ab}
P. notatum	1.0	3.30 ± 0.10^{de}	7.76 ± 0.42^{ab}	5.96 ± 0.32^{c}
	2.5	4.48 ± 0.15^{c}	6.00 ± 0.12^{cd}	8.02 ± 0.25^{a}
	5.0	4.29 ± 0.23^{c}	5.99 ± 0.11^{cd}	8.19 ± 0.21^{a}
R. oligosporus	1.0	3.63 ± 0.14^{d}	4.84 ± 0.15^{de}	8.19 ± 0.27 ^{a}
	2.5	5.60 ± 0.51^{b}	6.65 ± 0.10^{c}	6.52 ± 0.11^{cb}
	5.0	4.11 ± 0.21^{cd}	4.17 ± 0.09^{ef}	7.28 ± 0.05^{ab}

Table 3.4. Influence of Yeast (*C. versatalis*) and Bacterial (*L. helveticus*) elicitors on peroxidase activity ($\times 10^6$U L^{-1}). Each value is the mean ± SE of six replicates.

Dried cell powders (DCPs)		**Time of elicitor addition (day)**		
	Treatment % (w/v)	**15**	**18**	**20**
Control*		2.75 ± 0.05	2.85 ± 0.03	2.80 ± 0.04
C. versatalis	0.10	9.68 ± 0.33^{a}	9.63 ± 0.28^{a}	5.43 ± 0.12^{a}
	0.25	8.76 ± 0.16^{ab}	8.25 ± 0.15^{bc}	5.03 ± 0.11^{ab}
	0.50	7.32 ± 0.33^{c}	7.83 ± 0.22^{bc}	4.92 ± 0.03^{ab}
L. helveticus	0.10	6.45 ± 0.12^{d}	8.07 ± 0.21^{bc}	3.56 ± 0.32^{c}
	0.25	5.35 ± 0.24^{e}	7.40 ± 0.18^{c}	3.51 ± 0.41^{c}
	0.50	4.35 ± 0.02^{f}	6.95 ± 0.41^{cd}	3.47 ± 0.19^{c}
Culture filtrates (CFs)				
	Treatment % (v/v)	**15**	**18**	**20**
C. versatalis	1.0	1.25 ± 0.11^{a}	0.72 ± 0.33^{ab}	1.18 ± 0.15^{a}
	2.5	0.77 ± 0.32^{ab}	1.41 ± 0.12^{a}	0.49 ± 0.20^{ab}
	5.0	0.36 ± 0.04^{ab}	0.58 ± 0.25^{ab}	0.62 ± 0.34^{ab}
L. helveticus	1.0	0.53 ± 0.02^{ab}	0.76 ± 0.05^{ab}	0.67 ± 0.11^{ab}
	2.5	1.19 ± 0.14^{a}	0.85 ± 0.11^{ab}	0.40 ± 0.01^{ab}
	5.0	0.68 ± 0.32^{ab}	0.20 ± 0.34^{b}	0.56 ± 0.30^{ab}

The dry cell powders of yeast elicitor *C. versatalis* elicited the activity of POD up to 3.5-fold at very low concentration whereas the culture filtrates suppressed the turn over of enzyme activity (Table 3.4). *L helveticus* elicited nearly 3-fold activity in a contact period of 8 days only at lower concentration. Higher levels of yeast DCP was however inhibitory in a dose-dependent manner (Table 3.4). The results clearly indicate that, similar to most of the earlier studies where fungal elicitors have been found feasible for elicitation, in the present work also most of the biotic elicitors were found to cause elicitation of POD. Important observation made from the present screening study was that the concentration and time of elicitor contact were very critical factors for efficient elicitation of POD as observed in the elicitation of secondary metabolites in other species (Funk et al.1987; Bonhoff and Griesbach 1988; Buitelaar et al. 1992). The fact that CF of *C. versatalis* (Table 3.4) did not cause any elicitation of POD at all the levels used, instead there was a concentration-dependent suppression of POD, indicates that the receptors for elicitation in this case are responsive for high molecular weight cell wall polysaccharides that are present in DCP rather than in CF.

In horseradish hairy roots significant enhancement of POD (100%) occurred upon treatment of cultures with certain metal ions such as $AgNO_3$, $CuSO_4$ and fungal extracts such as *Verticillium* sp., *Monodyctis cataneae* and *Aspergillus niger* (Uozumi et al. 1992).

Table 3.5. Elicitation of POD activity ($\times 10^6$U L^{-1}) purified biotic (GSH & Mej) and abiotic elicitors (Mg, Ca & TDZ). The values are mean ± SE of six replicates.

Treatment	Time of elicitor addition (day)		
	15	18	20
Control*	2.75 ± 0.05	2.85 ± 0.03	2.80 ± 0.04
Glutathione (GSH)			
2 mM	9.46 ± 0.32^{a}	5.65 ± 0.41^{b}	5.73 ± 0.32^{cd}
4 mM	4.81 ± 0.23^{ef}	6.08 ± 0.07^{ab}	5.84 ± 0.21^{cd}
6 mM	4.44 ± 0.22^{f}	5.63 ± 0.21^{b}	7.18 ± 0.10^{cb}
8 mM	4.22 ± 0.18^{gf}	6.59 ± 0.38^{a}	6.24 ± 0.41^{cd}
10 mM	4.73 ± 0.09^{ef}	6.29 ± 0.12^{a}	6.63 ± 0.12^{c}
Methyl jasmonate (Mej)			
20 μM	1.39 ± 0.10^{i}	2.33 ± 0.38^{ef}	1.38 ± 0.23^{h}
40 μM	1.83 ± 0.41^{ih}	1.66 ± 0.15^{f}	1.52 ± 0.12^{h}
60 μM	1.59 ± 0.05^{i}	1.64 ± 0.03^{f}	1.43 ± 0.12^{h}
80 μM	1.15 ± 0.23^{ji}	1.71 ± 0.21^{ef}	0.11 ± 0.05^{ih}
100 μM	1.17 ± 0.08^{ji}	1.68 ± 0.08^{ef}	1.39 ± 0.06^{h}
Magnesium (Mg)			
2 fold	6.10 ± 0.23^{ed}	5.01 ± 0.31^{cb}	6.05 ± 0.12^{cd}
4 fold	6.20 ± 0.10^{ed}	5.04 ± 0.05^{cb}	7.20 ± 0.31^{cb}
6 fold	7.40 ± 0.23^{c}	5.51 ± 0.21^{b}	8.49 ± 0.23^{a}
Calcium (Ca)			
2 fold	7.15 ± 0.41^{cd}	6.38 ± 0.05^{a}	6.18 ± 0.41^{cd}
4 fold	6.67 ± 0.21^{d}	6.39 ± 0.12^{a}	7.87 ± 0.21^{ab}
6 fold	5.87 ± 0.10^{ed}	5.26 ± 0.08^{cb}	7.68 ± 0.23^{b}
Thidiazuron (TDZ)			
0.25 ppm	5.54 ± 0.23^{e}	3.75 ± 0.23^{cd}	6.74 ± 0.12^{c}
0.50 ppm	5.54 ± 0.22^{e}	3.61 ± 0.10^{d}	3.53 ± 0.34^{f}
1.00 ppm	5.04 ± 0.04^{ef}	6.85 ± 0.23^{a}	3.63 ± 0.23^{f}

Heterotrophic hairy root cultures of *Ipomea aquatica* were found to produce twice the amount of POD when they were made photo-autotrophic in which case the requirement of POD for scavenging the released toxic oxidants, the peroxides, has been suggested (Uozumi et al. 1992). Nevertheless, the hairy roots used in the present study have been observed to produce much higher levels of POD than that reported so far under non-elicited conditions and further enhancement was accomplished upon elicitation. The fact that the enzyme POD has several isoforms that are active at several physiological pH (Thimmaraju et al. 2005) indicates that each isoform might respond to elicitation differently for a particular elicitor.

Among the pure compounds (Table 3.5), GSH used at 5 concentrations at three different treatment time caused highest elicitation at lowest concentration of 2mM producing about 3.44-fold higher accumulation of POD activity than the respective control where a contact period of 10 days was required (elicitor addition on 15th day). Mej, a well-known secondary signaling molecule, when used at 5 different concentrations caused high suppression of POD activity by about 50% when compared to control cultures (Table 3.5).

While the effect of magnesium was based on concentration, calcium caused a fluctuating effect. In the present study, a production of highest POD activity of 3.01-fold higher over the control cultures (Table 3.5) was observed with the highest concentration of Magnesium in a shortest contact period of 5 days. Calcium also caused elicitation in a similar fashion, in a concentration dependent manner. Elicitation effects of these metal ions could be attributed to their reported role as both nutrient (Agostini et al. 1997) and secondary signaling molecules in the responses that follow elicitation (Pitta-Alvarez et al. 2000). Since these ions are readily available as salts, they are more practical and cost-effective unlike biotic elicitors that need several processing steps. It is well-known that most of the defense responses by a plant cell to microbial attacks are mediated either through alterations in membrane properties followed by signal transduction during which calcium has been proven participant molecule triggering a cascade of biochemical reactions within the cell, including efflux and influx of other ions (Blatt et al. 1990). Similarly Mej and GSH have been implicated in signaling functions mediating a cascade of defense responses (Graham and Graham 1999; Vranova et al. 2002). Mej in particular has been reported to elicit secondary metabolites (where POD is implicated) in suspension cultures of various plant species (Gundlach et al. 1992). Though Mej failed to elicit POD in the present study, another secondary metabolite of the phenylpropanoid pathway, the betalain, was enhanced (Suresh et al. 2004). TDZ caused highest elicitation of POD activity at 1ppm whereas a low level of 0.25ppm also caused almost a similar elicitation when added at a later stage i.e., on 20th day. Conclusively, the

present study has established that among the abiotic elicitors it is only GSH that significantly enhanced POD activity and more so in the presence of DCP of *R oligosporus* (as explained below).

3.3.7. Combined effect of GSH and crude biotic elicitors

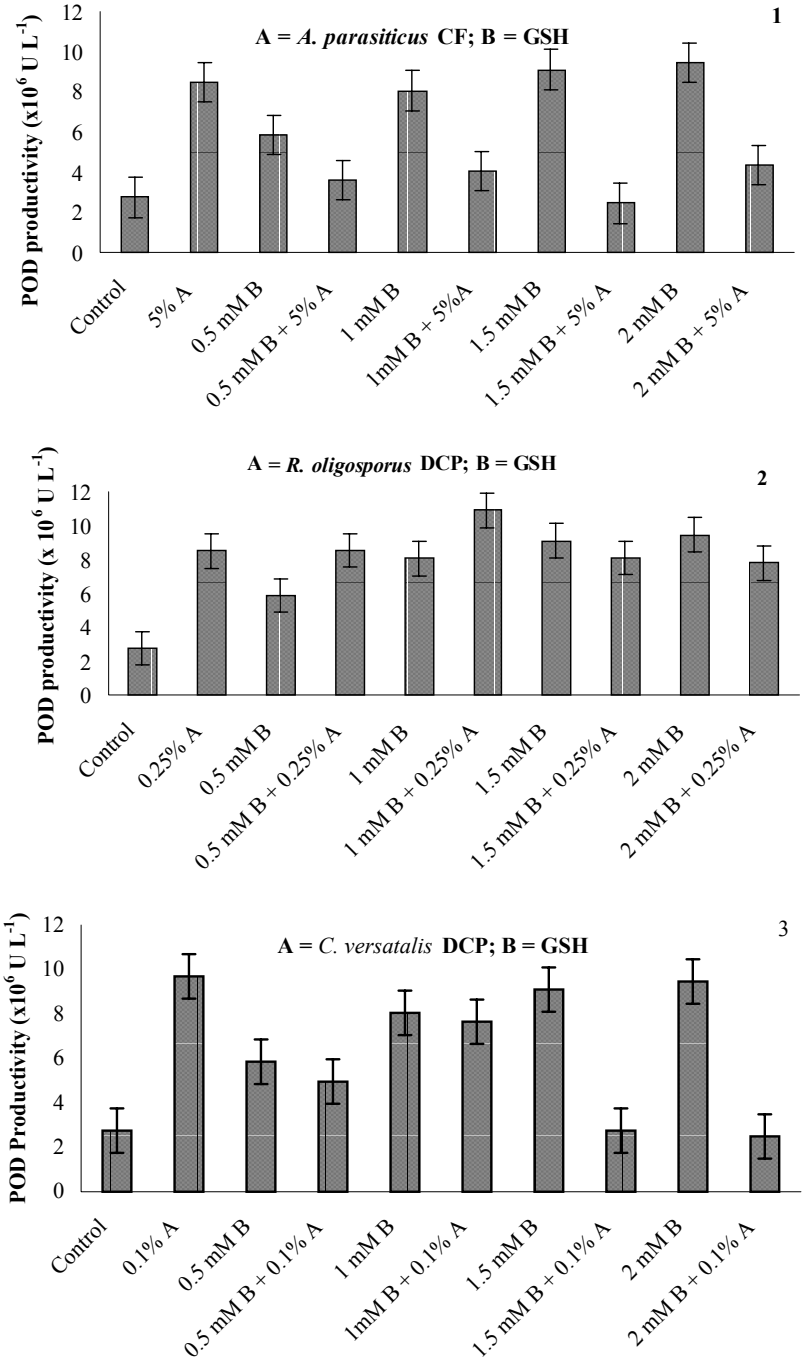


Figure 3.6. Combined effect of glutathione (GSH) and 5% CF of *A. parasiticus* (1), 0.25% DCP of *R. oligosporus* (2) and 0.1% DCP of *Candida versatalis* (3). The data on POD level was recorded on 25th day as an average of five replicates of two independent experiments.

Among various combinations of the three elicitors such as GSH, CF of *A. parasiticus,* DCPs of *R. oligosporus,* and *C. versatalis,* only the combination of 1mM GSH with 0.25% DCP of *R. oligosporus* caused highest elicitation of about 4-fold with a total POD activity of $10.9x10^6U\ L^{-1}$ when compared to the individual components (Fig 3.6). Other combinations either suppressed or caused very little elicitation of POD activity when compared to the effect of individual components (Fig 3.6).

Although many workers have studied elicitation in various systems and recorded the suppression of biomass in elicitor treatment, no attempt was made to check the possibility of effectively using the elicitor at the late exponential growth phase. For pigment we observed that the addition of elicitor at late exponential phase could enhance the overall productivity (Savitha et al. 2005). The activity of POD was generally high at early exponential phase (Thimmaraju et al. 2005) and therefore, maximum elicitation also occurred at treatments on 15^{th} and 18^{th} day rather than on 20^{th} day. Therefore, by judiciously selecting and timing the addition of elicitor, there is a possibility of enhancing both POD and betalain in the same process, in which case the process of online recovery of pigment developed earlier by us (Thimmaraju et al. 2004, see chapter 4) could further be extended for the online recovery of POD as proposed by Agostini et al (1997) for turnip hairy roots.

3.3.8. Partial purification

3.3.8.1. Ammonium sulphate fraction

Progressive fractionation of POD was carried out by ammonium sulphate precipitation from 0-20, 20-40, 40-60, 60-80 and 80-100%. The fraction obtained with 60 – 80% showed maximum activity and minimum total protein content. This fraction was dialyzed for 48h using 12000kD cut off range dialysis bag against 0.2M sodium phosphate buffer (pH 6.0). The dialyzed fraction was used for further purification by anion exchange chromatography.

3.3.8.2. Anion exchange chromatography

The dialyzed 60-80% ammonium sulphate fraction when loaded on to a DEAE cellulose column that had been activated, pre washed and equilibrated with 0.2M sodium phosphate buffer (pH 6.0). From the ion exchange chromatography experiment it was found out that the most of the activity was recovered in the non-retained fraction from the column. Two peaks one single major and one minor peak were recovered when eluted with 0.2M sodium phosphate buffer (pH 6.0) (Fig 3.7).

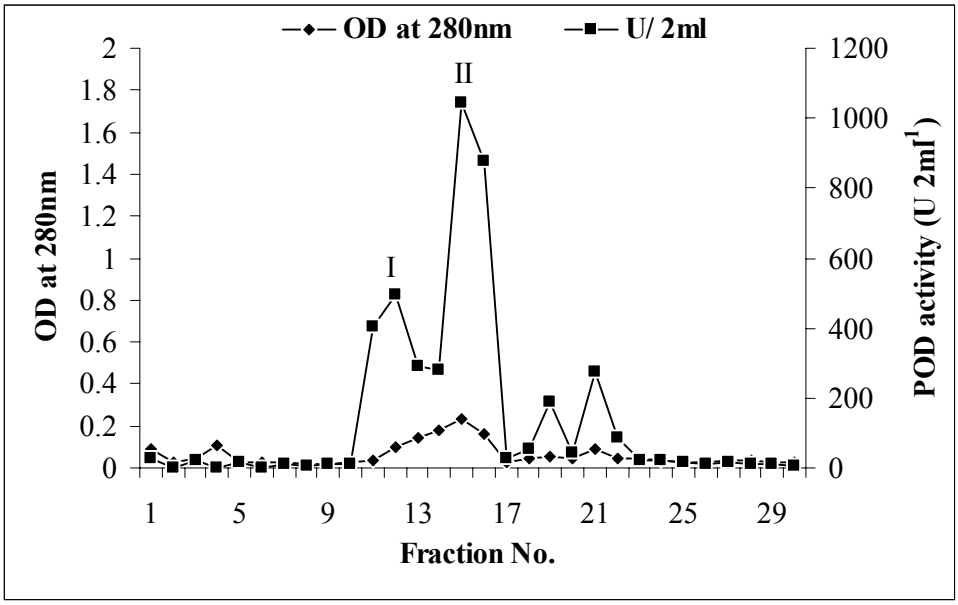


Figure 3.7 Elution profile of the non-retained fraction from DEAE cellulose column eluted with 0.2M sodium phosphate buffer (pH 6.0), collected as 2ml fractions.

Negligible any activity was recovered when the column was subjected for elution with 0-0.5M NaCl gradient in 0.2M sodium phosphate buffer (pH 6.0). The major peak from AEC column was recovered dialyzed against double distilled water for about 48h. The enzyme activity and protein content were estimated and the results expressed in terms of specific activity showed about 15-fold increase in specific activity when compared to crude resulting in about 15-fold purification (table 3.6). The fraction was concentrated by lyophilization and used for further characterization.

Table 3.6. Different methods and levels of purification of POD

Sample	Protein (mg)	Activity (units)	Specific activity (U mg^{-1} protein)	Fold (s) of activity
Crude extract	168.0	1.17 x 10^6 U	700.0	1.0
Amm. Sulphate precipitation	123.5	2.16 x 10^6 U	1750.0	2.5
AEC* (DEAE Cellulose)	9.5	0.99 x 10^6 U	10500.0	15.0

3.3.9. Partial characterization

3.3.9.1. Molecular weight and purity

The molecular weight and the purity was analyzed by SDS-PAGE (Fig 3.5;Section 3.3.4). After comassie brilliant staining the peak-I showed some impurities, whereas the peak-II showed a single band (Fig 3.5) and hence this fraction was used for further studies due to its higher degree of purity.

The molecular weight was calculated as 45kD (Fig 3.5;Section 3.3.4; Page 104) this is similar to that of HRP (40-46kD) (Paul and Stigbrand, 1970). Molecular weights of most of the peroxidases vary from 30 to 60 kD (Srivastava and van Huystee, 1977).

3.3.9.2. Substrate specificity

Generally peroxidases are specific for H_2O_2 as a substrate, but can also use a number of H donors such as orthodianisidine hydrochloride. Hence the experiments were carried out to test the specificity of red beet HR-POD. The purified (AEC) fraction of red beet HR POD was assayed at various concentrations of different H donors and H_2O_2. The equation (1) and (2) were used to determine the K_m and V_{max} values from the Lineweaver-Burk plots for various POD substrates such as H_2O_2, orthodianisidine hydrochloride, ABTS and guaiacol. The figure 3.8A is the Lineweaver – Burk (LB) plot for H_2O_2 at 2mM othodianisidine, which shows lowest K_m value of 0.1 indicating the specificity of red beet HR POD to H_2O_2. Among the H donors studied the enzyme appeared to have highest affinity in the order orthodianisidine>ABTS>guaiacol i.e., the enzyme showed more affinity towards orthodianisidine as indicated by the lowest K_m value among the H donors (Fig 3.8B & 3.9A & B).

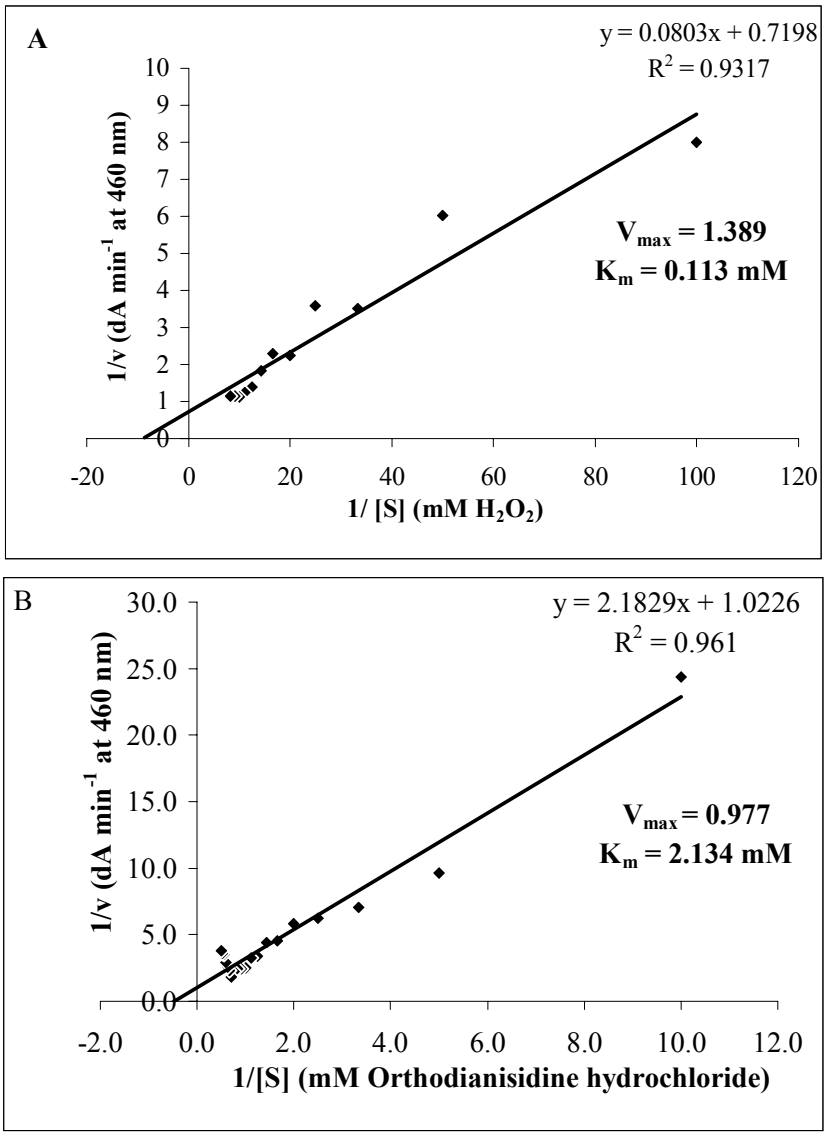


Figure 3.8. Lineweaver – Burk plot for POD activity at various concentrations of H_2O_2 at 2 mM orthodianisidine hydrochloride (A) and orthodianisidine hydrochloride at 0.5 mM H_2O_2 (B). The assay was carried out at 25ºC using 0.2M sodium phosphate buffer (pH6.0).

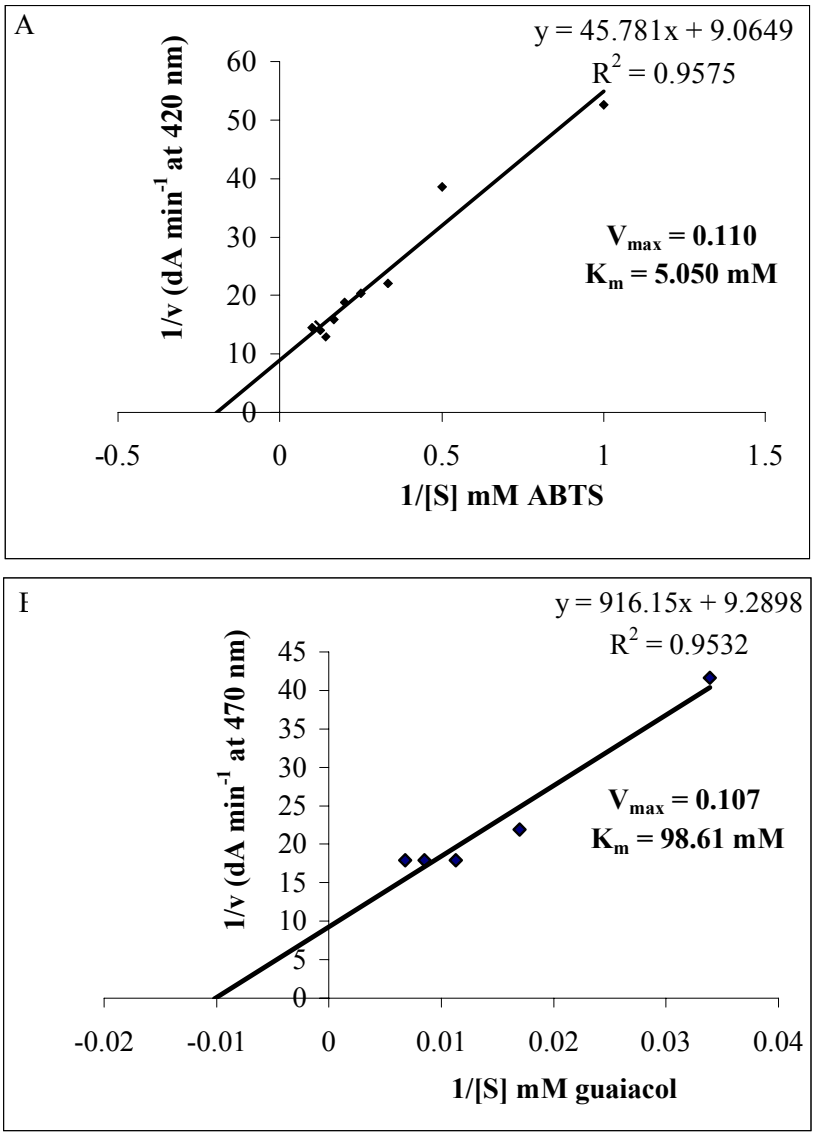


Figure 3.9. Lineweaver – Burk plot for POD activity at various concentrations of ABTS at 0.5 mM H_2O_2 (A) and guaiacol at 0.5 mM H_2O_2 (B). The assay was carried out at 25ºC using 0.2M sodium phosphate buffer (pH 6.0).

3.3.9.3. pH optima for activity and stability

When studied for pH optima using orthodianisidine hydrochloride as H donor, the red beet HR crude POD showed maximum activity at pH 5-6 whereas the commercial HRP activity was highest at pH 4-5. The enzyme was stable over a wide range of pH from 4 to 9 and showed highest stability at pH 7 and 9 (Fig 3.10A).

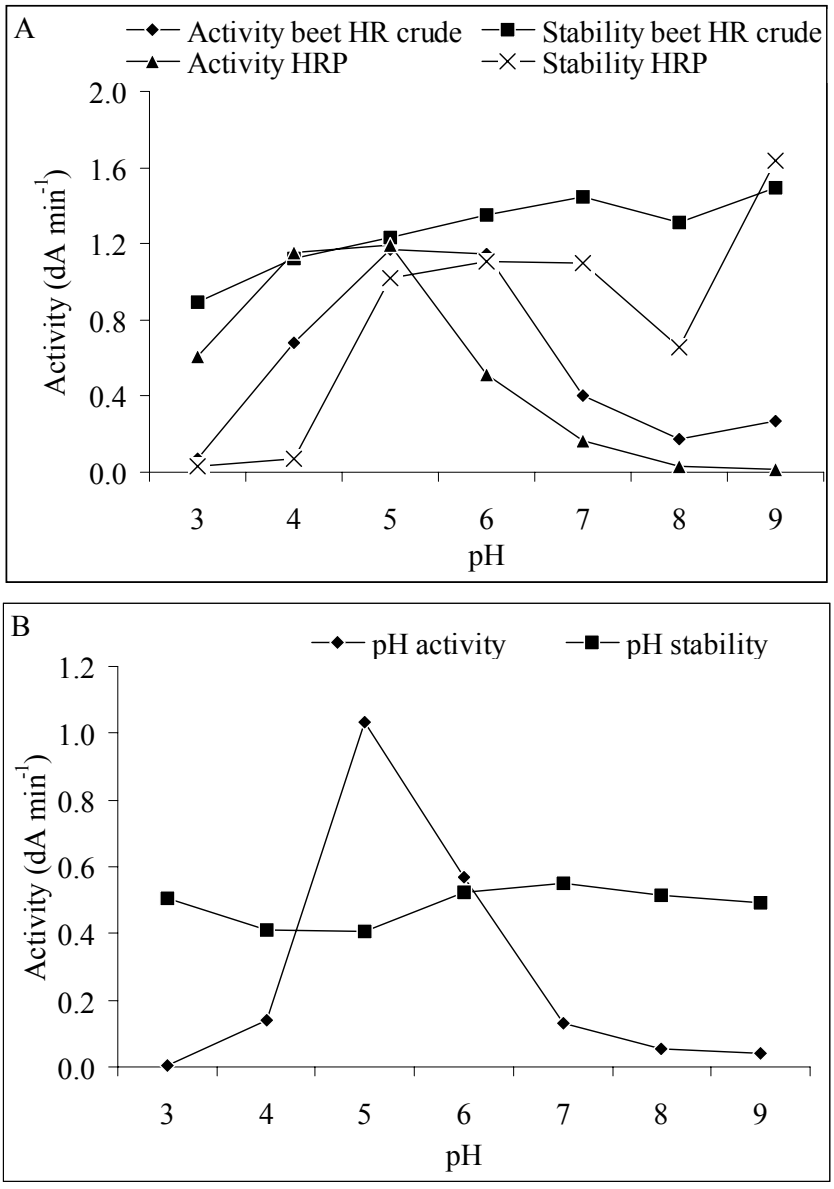


Figure 3.10. Effect of pH on the enzymatic activity and stability (incubated for 12 h) of crude beet HR POD, horseradish POD (A) and AEC fraction of beet HR POD (B). The buffers used were pH 3-5 sodium citrate (0.1M), pH 6-8 sodium phosphate buffer (0.2M); pH 9 Tris-HCl (0.2M).

The pH optima of purified fraction indicated that the enzyme shows highest activity at pH 5 where as stable over a wide range from pH 3-9 with highest stability from 6-8 (3.10B). The

commercial HRP when studied for activity and stability, showed highest activity at slightly more acidic pH (4-5) compared to crude and purified beet HR POD (Fig 3.10B).

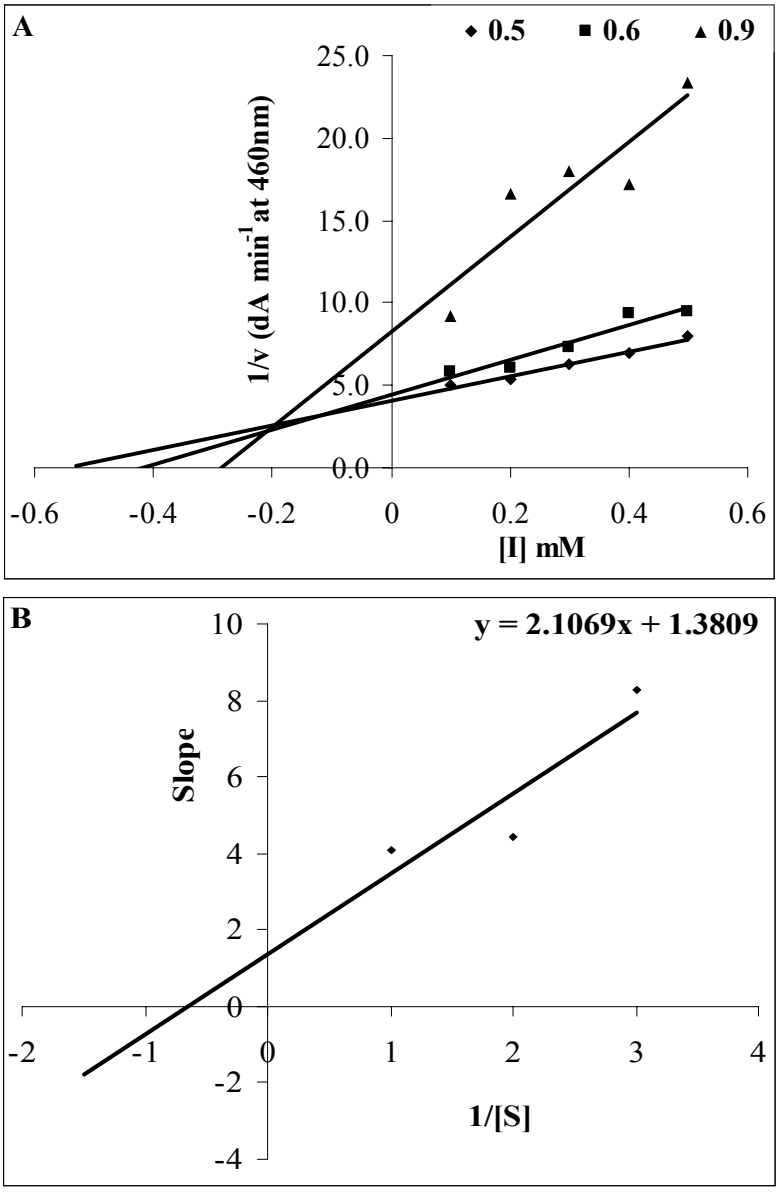


Figure 3.11. Dixon plots for POD inhibition, the experiments were conducted by assaying for POD activity at different inhibitor concentration (0 – 0.6mM) potassium periodate (A) and slope re-plot (B). From the plot it can be inferred that the periodate acts in a competitive manner in inhibiting the beet HR POD. The values are the average of three independent experiments.

The commercial enzyme was stable at a narrow range of pH 4-6 and showed highest stability at pH 9 similar to crude beet HR POD (Fig 3.10A). However the pH optima for red beet HR POD activity was very similar to that of strawberry fruit (pH 6), tomato (5.3 – 5.5), soybean (5.5) and turnip (5.5) (Sessa and Anderson, 1981; Heidrich et al. 1983; Civello et al. 1995; Duarte-Vazquez et al. 2001). Therefore, the red beet HR POD can very well find applications that involve these pH ranges. Activity of HR POD being similar to commercial HRP with an added advantage of higher stability of beet HR POD over a wide range of pH when compared to commercial HRP.

3.3.9.4. Inhibition of HR-POD

Potent inhibitors of hemoprotein catalyzed reactions such as periodate (Fig 3.11A & B) and sodium azide (Duarte-Vazquez et al. 2001) were studied for beet HR POD inhibition and the data was analyzed by using Dixon Plots (Segel, 1993). The plots indicate that the periodate acts in a competitive manner (Ki = 0.2mM) whereas the sodium azide causes the POD inhibition in an uncompetitive manner (Fig 3.12).

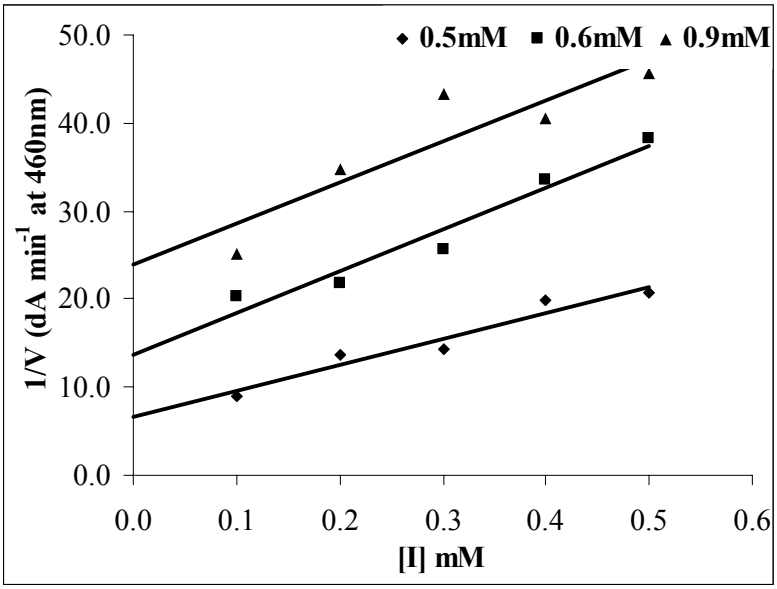


Figure 3.12. Dixon plots for sodium azide inhibition of POD, the experiments were conducted by assaying for POD activity at different inhibitor concentration (0– 0.6mM). From the plot it can be inferred that the sodium azide acts in an uncompetitive manner in inhibiting the beet HR POD. The values are the average of three independent experiments.

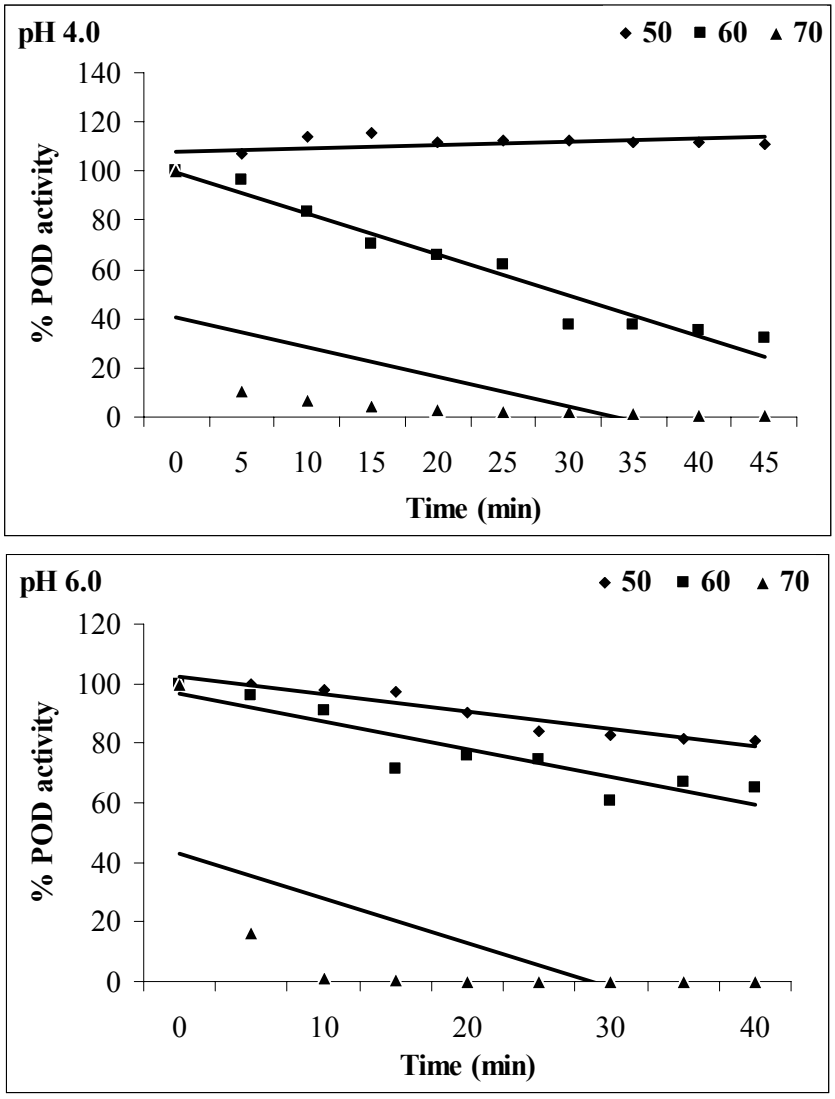


Figure 3.13. Thermo-stability of crude peroxidase enzyme fractions at three different temperatures (50, 60 and 70^{o}C) recorded for 40min time period. About 500µl of the crude enzyme extract prepared at different pH (4.0 & 6.0) was incubated at three different temperatures and the rate of loss of activity was monitored by taking 10µl from each of the treatments and measuring the activity i.e., dA min^{-1} at 460ηm. The data is the average of 5 replicates of two independent experiments.

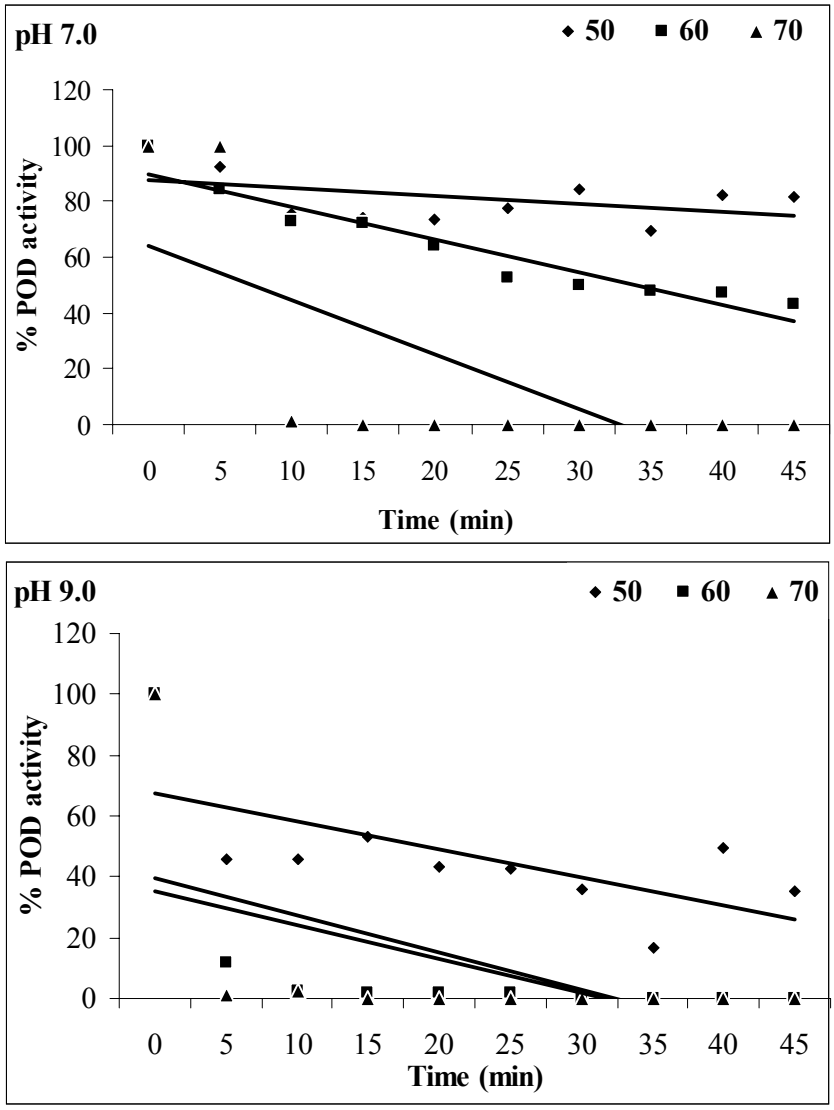


Figure 3.14. Thermo-stability of crude peroxidase enzyme fractions at three different temperatures (50, 60 and 70 °C) recorded for 40min time period. About 500µl of the crude enzyme extract prepared at pH (7.0 & 9.0) was incubated at three different temperatures and the rate of loss of activity was monitored by taking 10µl from each of the treatments and measuring the activity i.e., dA min^{-1} at 460ηm. The data is the average of 5 replicates of two independent experiments.

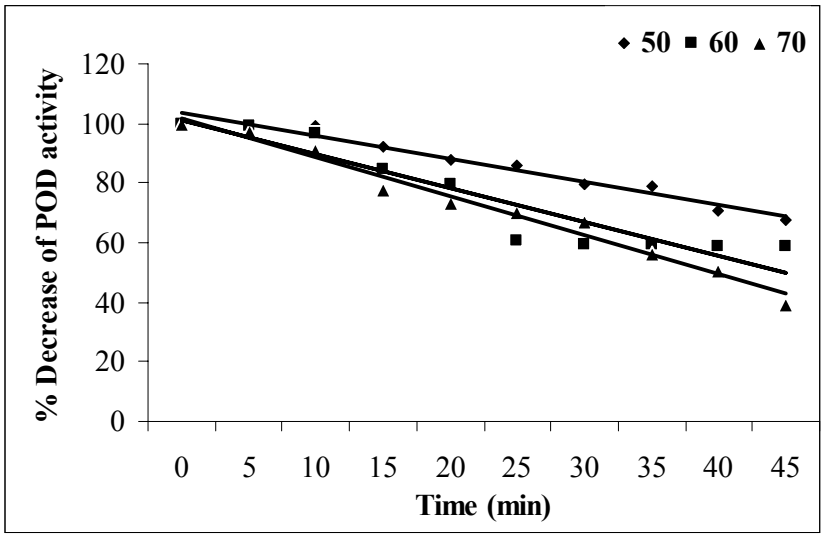


Figure 3.15. Thermo-stability of purified peroxidase enzyme at three different temperatures (50, 60 and 70°C) recorded for 45min time period. About 500µl of the crude enzyme extract prepared at pH 6.0 was incubated at three different temperatures and the rate of loss of activity was monitored by taking 10µl from each of the treatments and measuring the activity i.e., dA min^{-1} at 460ηm. The data is the average of 5 replicates of two independent experiments.

3.3.9.5. Thermo-stability of crude POD

Figure 3.13, shows the percent increase or decrease of POD activity as a function of three different temperatures during a total period of 45min. The thermal inactivation of PODs extracted at acidic and neutral pH was negligible up to 50ºC with almost 95% of the activity being retained even after 40 min. However, the POD of basic pH was very sensitive to temperature with 50% loss at 50ºC in 40 min, with the total loss of activity at 60ºC (Fig 3.14). At the latter temperature (60ºC) the acidic and neutral PODs retained more than 70% of the activity up to 40min whereas these enzymes were completely inactivated at 70ºC. However, the purified enzyme was more stable (Fig 3.15) compared to its crude state as it retained more than 70% activity at 70°C even after 20min while the commercial HRP had lost most of its activity at 70°C within 11min (Duarte-Vazquez et al. 2001).

In conclusion the present chapter has dealt in detail on the characterization of hairy root clones for the production of POD in terms of total units of activity and its elicitation to further enhance the productivities. Finally the enzyme was purified to electrophoretic homogeneity where the molecular weight of the purified isozyme has been detected to be 45 kD. The enzyme was also partially characterized for various parameters such as pH optima for activity, stability and substrate specificity, apart from studying the enzyme inhibition for

two important and well-known POD inhibitors. In most of these studies the enzyme showed the properties either on par or better than the commercial HRP. Therefore the red beet HR-POD may be a good alternative for commercial HRP.

Chapter highlights

- **The hairy root clones were screened and a clone having high activity of peroxidase was selected.**
- **A best elicitor combination was obtained after screening various biotic and abiotic elicitors for enhancement of peroxidase.**
- **The enzyme was purified to electrophoretic homogeneity and partially characterized.**

CHAPTER 4

DOWNSTREAM PROCESSING

CONTENTS

SUMMARY

The chapter mainly considers various down stream processing aspects such as in situ release of pigments by non-destructive methods, followed by adsorption and recovery by desorption. An attempt has also been made for the application of aqueous two- phase extraction (ATPE) technology for the simultaneous recovery of the betalaine and POD. A prototype reactor model for continuous production and online recovery of betalain and POD has been worked out. Various physical, chemical and biological agents were studied in detail for the permeabilization and the release of pigment where, the chemical agent CTAB was found ideal to recover the max quantity of the pigment. Some studies on the involvement of calcium channel in the pigment release through chemical agents were also made. The free and wall bound lipid fractions of food grade microbes were also tried for the recovery of betalaines with or without oxygen stress. It was found that the free lipid surfactants of the biological agents were most ideal for the permeabilization of hairy root cultures. Among the different adsorbents screened for *in situ* recovery of released pigments, alumina: silica (1:1) appeared ideal showing *in situ* adsorption of 97% in a unit time of 30min accounting for *in situ* recovery of 71.39% of the total betalaine effluxed. *Ex situ* recovery of betalaine was done using various combinations of alumina-silica and processed sand and different column geometries which indicated that alumina with processed sand at 2:1 ratio (weight/weight) and a minimum column material of 2cm height and 2cm diameter was good enough to cause 97% adsorption from a solution containing 1.6mg ml^{-1} of pigment. Desorption and recovery of pigments *ex situ* from columns were affected by various elution mixtures, where, a gradient elution with ascending levels of HCl-ethanol in water resulted in 100% recovery of adsorbed pigments in a significantly lesser volume of eluent in a short period of 1h. The chapter also deals with other parameters such as flow rates, elution time and separation of betacyanine and betaxanthine. A strategy for simultaneous recovery of pigment and POD was worked out using ATPE (Aqueous two phase extraction) and finally a bioreactor model for integrating all these unit operations was also suggested for online production and recovery.

Publications

Thimmaraju R, Bhagyalakshmi N, Narayan MS and Ravishankar GA (2003). Kinetics of pigment release from hairy root cultures of *Beta vulgaris* under the influence of pH, sonication, temperature and oxygen stress. Process Biochemistry 38: 1067-1074.

Thimmaraju R, Bhagyalakshmi N, Narayan MS and Ravishankar GA (2003). Food-grade chemical and biological agents permeabilize red beet hairy roots assisting the release of betalaines. Biotechnology Progress 19: 1274 -1282.

Thimmaraju R, Bhagyalakshmi N and Ravishankar GA (2004). *In situ* and *ex situ* adsorption and recovery of betalaines from hairy root cultures of *Beta vulgaris*. Biotechnology Progress 20: 777-785.

4.1. INTRODUCTION AND REVIEW OF LITERATURE

4.1.1. Permeabilization

4.1.1.1. Physical agents

Product recovery and further concentration are of crucial importance as down-stream processing often accounts for 50-90% of the total production costs (Dwyer, 1984; Sahai, 1994). The sensitivity of the plant cell cultures to alterations in culture environment may often result in changes in membrane properties, rendering it amenable for permeabilization. Such a property may be exploited to extract metabolites such as red beet pigments. For safe application of beet colors in food products it becomes essential to use separation methods such as physical agents and food-grade chemicals for causing the efflux of pigments into the cell exterior. Initial trials were focused on the method of introducing a partition to the cell culture medium where part of the metabolite released into the medium accumulated in the immiscible solvent or the solid resin allowing the repeated recovery of the metabolite (Deno et al. 1987; Byun et al. 1990; Shimomura et al. 1991; Byun and Pedersen 1994; Sim and Chang 1997; Peterson, 1999) where often upto 5-fold increase in productivity have been reported (Sim and Chang 1997). Productivity of the shikonin was enhanced by introducing partition into the medium in the form of adsorbents where, the release of color and adsorption were spontaneous (Shimomura et al. 1991). However, beet hairy roots of the present study where betalaines are stored in the vacuoles (Wink, 1997), do not release betalaines to the cell exterior in the presence of adsorption partition (of Amberlite XAD series). Since biological membranes comprise mainly phospholipids, several trials on phosphate limitation in microbes and cultured plant cells caused pores in the membranes allowing the release of certain metabolites (Felix, 1982; Berlin et al. 1988). However, in beet hairy roots, phosphate depletion or its lowering in the medium failed to release any betalaine into the medium (Thimmaraju et al. 2003a). Thus there is a need to induce the reverse sequestering of vacuolar secondary metabolites to the cell exterior using a well-programmed non-lethal permeabilizing method for the advantage of repeated use of the biomass over an extended period. A few earlier trials, made in this direction in other systems, resulted in different degrees of success (Parr et al. 1984; Berlin et al. 1988; Brodelius, 1988; Dilorio et al. 1993; Pedersen et al. 1998), with most of the treatments leading to loss of culture viability. Lowering the medium pH has been implicated with altered cell membrane permeability leading to influx-efflux of solutes and bio-molecules. Certain earlier studies on the recovery of pigments from red beet hairy roots involve treatment with low pH such as pH 2.0

(Mukundan et al. 1998) where a 10min exposure to pH 2.0 followed by return to standard medium of pH 5.5 released about 50% of the pigment in 24h in latter medium. However, the low pH treatment appeared to kill the mature pigment-laden cells, though the extreme tips were not affected and hence were able to grow further. The low pH (of 2.0) involved rapid degradation of betalaines (Nilsson, 1970), and the release by alteration of medium pH (Saenz-Carbonell et al. 1993) leads to erroneous quantification of betalaines as the absorbance of betalaines gets altered due to change in pH. With this background it was necessary to re-establish the efflux of betalaines under the influence of pH.

Among the other physical parameters, treatment by ultrasound of *Beta vulgaris* cell suspension stimulated release of about 5-10% of the total pigment with no adverse effect on viability (Kilby et al. 1990; 1991). By increasing ionic strength of the medium the cells were permeabilized to release certain cytosolic contents including secondary metabolites with no loss of viability (Tanaka et al. 1985). Red beet hairy root cultures of late exponential phase, under the influence of oxygen stress, released over 20% of the pigments into the medium (Tanaka et al. 1985) where the hairy root biomass were used repeatedly for three cycles leading to the enhancement of overall production of betalaines from unit biomass. In all these permeabilizing studies, either the product recovery was very poor or the viability of the hairy roots was lost or the process involved 2-3 changes of nutrient medium (Mukundan et al. 1998b). Therefore, in the present study, all the physical factors were considered to have a comparative picture of beet hairy root permeabilization, as the same has not been documented so far.

4.1.1.2. Chemical and biological agents

To facilitate leaching of rosmarinic acid accumulated in the vacuoles of *Coleus blumeii*, Dimethylsulfoxide (DMSO) was used (Park and Martinez 1992; 1994; Martinez and Park 1993) where 0.1% DMSO was continuously fed to the cultures to acclimatize the cells to the treatment, as DMSO treatment adversely affected the viability of the cells after permeabilization (Parr et al. 1984; Park and Martinez 1992; Reuffer, 1985). Such pre-treated cells of *C. blumeii,* upon exposure to a higher level of 0.5% DMSO, released nearly 65% of the total rosmarinic acid into the medium without loss of viability (Park and Martinez 1992; 1994). Nevertheless, the released rosmarinic acid was quickly destroyed by active peroxidases, which also leached into the medium (Peterson, 1999). In our experiments, DMSO failed to release any pigment into the medium from beet hairy roots (data not presented).

Apart from methods involving effects of chemical and physical agents, there are also biological agents such as live microbial cells, which are known to chew up the cell walls/membranes of higher plant cells, mainly by releasing hydrolytic enzymes, thus allowing the cytosolic contents to leach into the medium or may alter cell surface activity by producing bio-surfactants (Singh et al. 1994) and hence appear attractive to develop strategies for product recovery in both batch as well as continuous cultures. Most of the natural pigments are stored in the cells as glycosides, and decoupling the pigment from its respective glycoside is known to improve the hue value of the pigment (Von-Elbe, 1979). As the microbes used for permeabilization can also act on the glycoside attached to the pigment (Von-Elbe, 1979) the use of biological agents may bring about colour intensification effect, in addition to cell permeabilization followed by pigment efflux. The food-grade microbes such as species of *Lactobacillus* synthesize and release organic acids (particularly lactic acid), which is proven to be a nutraceutical and an anti-microbial in food formulations (Barefoot and Netless 1993). Several food grade microorganisms also act as surfactants, causing changes in the surface activities of higher plant cells. Therefore, it is necessary to study such properties by individually analyzing each component of the microorganism. Therefore, the present chapter summarizes the data obtained on pigment effluxing using biological factors such as food-grade microorganisms, the cell fractions of one microorganism and discusses the other advantages of such system for the food application of released pigment.

4.1.2. Product recovery

Repeated on-line product recovery is an important step for higher productivity in a commercial bioreactor. When once the pigments are outside the cell, they are prone to degradation (Von-Elbe, 1979), therefore, ought to be recovered quickly. Employing either a lipophilic second phase (Becker et al. 1984) or a polar second phase has been reported to be beneficial for the accumulation and extraction of secondary metabolites (Sim and Chang 1997). Brodelius and Nilsson (1983) showed that some solvents were useful for extracting products from immobilized plant cells without affecting cell viability. Berlin et al (1984) used adsorbents to trap volatile plant cell products from cultures of *Thuja occidentalis*. *In situ* extraction of shikonin with hydrocarbons was performed for suspension cultures of *Lithospermum erythrorhizon* (Deno et al. 1987) whereas polymeric adsorbents were used to recover products from *Cinchona ledgeriana* (Rhodes et al. 1986; Robins and Rhodes 1986) and *Catharanthus roseus* (Payne et al. 1988; Payne and Schuller 1988). In certain bioreactor prototypes, extra-cellular product released into the medium was separated by passing the spent medium through a resin column (Amberlite XAD-7) attached as an external loop

(Peterson, 1999) or by directly adding the resin (XAD-2; XAD-7) into the medium (Sim and Chang 1997; Peterson, 1999).

In these experiments, *in situ* product removal enhanced total metabolite production and the products that were selectively released from cells were either held by a second phase composed of solvents or held by the adsorbents. For example, a simultaneous *in situ* extraction and elicitation was feasible for suspension cultures of *Escholtzia californica* for the production of benzophenadrine alkaloids (Byun et al. 1990). Scopalamine, another alkaloid from *Duboisia leichhardtii*, was recovered from the medium using amberlite XAD-2 column and the compound was later eluted with a mixture of methanol and ammonium hydroxide (Muranaka et al. 1992) leading to the recovery of 97% of the released compound. The repeated recovery thus improved scopolamine production by five times when compared to that without *in situ* adsorption. Shikonin, a pigment derived from shikimic acid in the cell cultures of *Lithospermum erythrorhizon,* was enhanced by immobilization coupled with *in situ* extraction (Kim and Chang 1990), which was later adopted for pigment enhancement in hairy roots of the same species (Shimomura et al. 1991; Sim and Chang 1993). All these reports clearly indicate that selection of a suitable adsorbent depends on the characteristics of the target compound which is specific to each culture system. Product removal by adsorption is mainly accomplished by two ways; the first, by *in situ* adsorption where an adsorbent material may directly be added to cultures (where the cells/hairy roots automatically release the product to cell exterior or the cells are permeabilized to cause product release) and the second, by *ex situ* method where the product is passed through an adsorption surface. Most studies use the adsorption isotherms of an adsorbent in a defined temperature range for the design of fixed bed applications (Chern and Chein 2001). However in the present study only one temperature, i.e., 25ºC and pH 5.5 was used for two important reasons; the first is that betalaines are fairly stable at pH 5.5 and 25ºC and the second is that the mentioned pH and temperature do not lead to erroneous quantification due to shift in absorbance of pigment molecules (Thimmaraju et al. 2003a). In case of desorption / elution with aqueous 2% HCl, the pH was adjusted to 5.5 immediately after elution.

Therefore, the objective of the present chapter was to develop a strategy for *in situ* extraction of vacuolar beet pigment by combining physical, chemical and biological effluxing agents, *in situ* recovery by adsorption for which a prototype bioreactor useful for economizing the production of betalaines and POD was worked out apart from attempting for a strategy for simultaneous recovery of pigment and POD by using ATPE.

4.2. MATERIALS AND METHODS

4.2.1. Permeabilization and release of betalaines

4.2.1.1. Physical agents

4.2.1.1.1. Treatment with different pH

Hairy root clone grown for 20 days in 40ml MS medium were subjected to media pH of 2.0, 3.0 and 4.0. For this the spent medium of 20^{th} day culture was replaced aseptically with fresh MS medium of pH 2.0, 3.0 and 4.0 (filter sterilized). Control cultures were also given medium (of pH 5.8) change to rule out the osmotic effects. The pigment released after the treatment was monitored every half an hour by taking the pigment leachate (1ml medium aseptically) and centrifuging and measuring the increase in absorbance at 540 and 480ηm. Further, to study the kinetics of pigment release at pH 2.0 as a function of nutrient ions, a separate experiment was performed using three sets of treatments wherein one set of hairy roots were exposed to acidified water (pH 2.0) and the second to MS of pH 2.0. Pigment release was monitored at different time intervals and the data recorded was also used to assess the stability of released pigment by periodically recording the OD of the earliest removed leachate. The third set of hairy roots were treated with water of pH 2.0 for different periods and transferred to MS liquid medium of pH 5.8 (with a prior wash with MS liquid medium to get rid of already leached pigment) and the release of pigment was monitored in MS medium of pH 5.8 for the next 24h.

For testing the viability after pigment recovery, 50-100mg of the treated culture was transferred to both liquid and agar gelled MS medium and the formation of fresh root tips was observed for two weeks. To test the effect of calcium on the viability of treated culture, after pigment recovery the cultures were given a change of fresh MS medium with (20mM) and without calcium enrichment where growth of biomass and pigment accumulation was observed.

Since the acidic pH is known to affect the absorbance spectrum of most of the pigments, an experiment was conducted to quantify the extent of variation in absorbance from one pH to other. For this, total pigments were extracted using water of pH 5.5 from normal beetroot as well as from hairy roots and concentrated by flash evaporation to obtain $1mgml^{-1}$ of betalaines. 6µl of this stock was added to 3ml of acidified water of different pH ranging from 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 9.0 in a cuvette. The cuvette was inverted with the cap 10 times to mix the color uniformly and absorbance spectrum was recorded individually for each pH. The difference in absorbance, reflected as changed spectrum (Fig. 4.1A & 4.1B) of

betalaine was used as a correction factor to quantify the released pigments at different pH using the value of pH 5.0 as standard. The hue value of betalaines at different pH was also recorded (Fig. 4.2A & B) as an additional evidence for shift in hue value at different pH. The experiment had 5 replicates and the whole experiment was repeated once more.

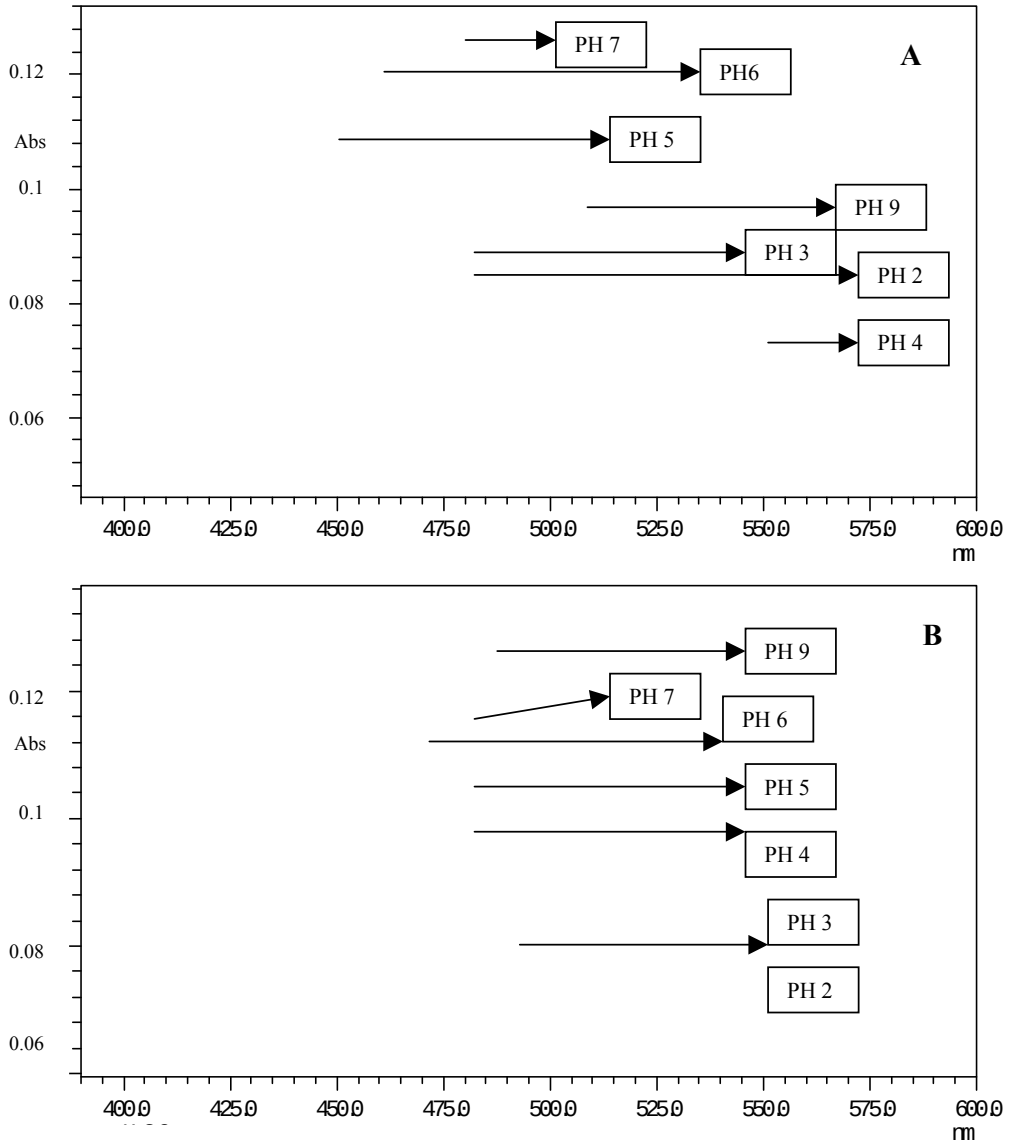


Figure 4.1. Changes in spectral values of betalaines from normal beetroot (A) and hairy roots (B) at different pH.

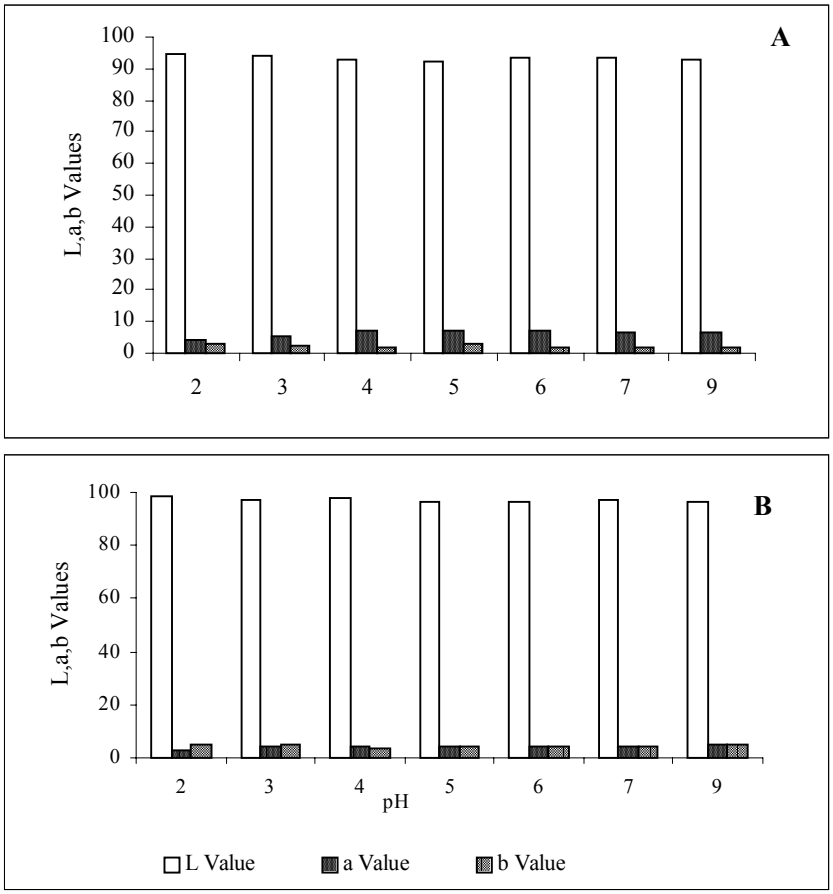


Figure 4.2. Changes in Hunter's color property in terms of L, a & b values of betalaines from normal beetroot (A) and hairy roots (B) at different pH.

4.2.1.1.2. Permeabilization using sonication

Hairy root cultures grown for 20 days in 40ml liquid MS medium were transferred to a 50ml beaker along with spent medium and subjected to a continuous ultrasonic sound of 0.02MHz generated by using a sonicator (Model: W-375 with ¾'' High gain "Q'' horn tip, Heat systems – Ultrasonics, Inc. USA) for different time periods such as 15, 30, 60, and 120 seconds by following the method of Kilby et al. (1990; 1991). Entire biomass along with the leachate was transferred back to original conical flask and incubated for 1h. Control cultures were also treated identically, (transferring to beaker in laminar air flow and transferring back to culture flasks) to sonicated cultures but without sonication to rule out the effect of leaching that occurs due to handling. The medium with effluxed color (which also had a lot of suspended cell debris) was decanted aseptically, centrifuged and quantified spectrophotometrically as explained earlier. The pigment remaining in the biomass was also

quantified as described previously. The sonicated biomass, was given a change of fresh medium, with or without exposure to calcium enriched medium, re-cultured and used for further sonication treatment as well as to check the viability as explained earlier. The experiment had 5 replicates and repeated once more.

4.2.1.1.3. Temperature effect

To study the effect of temperature on the efflux of pigment, 20-day-old cultures with spent medium were incubated at different temperatures of 40, 45 and 50ºC in a water bath shaker for 30 and 60min and the release of the pigment was monitored. The treated hairy roots were checked for viability as explained earlier with or without $CaCl_2$ treatment. The experiment had 5 replicates and repeated once more.

4.2.1.1.4. Oxygen stress

Oxygen stress was imposed by keeping 18-day-old hairy root cultures grown in 150ml Erlenmeyer flask under static condition both in the presence of light (2000Lux) and under dark condition. The release of the pigments was monitored, by reading absorbance at 480ηm & 540ηm, the percent release was calculated and the viability and re growth was checked as described earlier.

4.2.1.1.5. Osmotic stress

Six different types of stress media with MS salts & 3% sucrose containing different levels of sorbitol from 0.5 - 5mM were used to study the effect of osmotic stress on the release of betalaine. Stress was induced by way of total replacement of the spent MS media in the flasks containing 20-day-old cultures with 25ml of the stress media. The cultures were monitored for the release of pigment for 24h under standard conditions.

4.2.1.2. Chemical agents

4.2.1.2.1. Treatment with different chemical agents

Stock solutions (of 10mg ml^{-1}) of each of the chemical agents such as DMSO (Dimethyl sulfoxide), Triton X-100, CTAB (Cetyltrimethylammoniumbromide) and Tween-80 (All from Sigma, Inc., USA) were prepared, filter sterilized (Sartorius 0.45µm) and used for treating the hairy roots. Initially the concentrations of 0.05, 0.1, 0.15, 0.2% each of four chemicals were used to study the pigment release pattern. Since CTAB caused pigment efflux immediately after addition, further studies with CTAB involved lower concentrations ranging from 0.0005, 0.001, 0.002, 0.01, 0.05, 0.1, 0.15, 0.2%. In general, hairy roots grown for 18 days in 40ml MS medium were subjected to treatments either by way of replacing the

spent medium aseptically with fresh MS medium containing different permeabilizing agents or by directly adding the permeabilizing agent to the spent medium. After treatment, the cultures were maintained as either static (oxygen-stressed) or as agitated by incubating on rotary shaker. Release of pigment was monitored for different periods and quantified as explained above.

4.2.1.3. Biotic agents

4.2.1.3.1. Microbial cultures

Pure cultures of Lactobacillus helveticus**,** Saccharomyces cereviseae and Candida versatalis were obtained from Food Microbiology Department, CFTRI, Mysore.

4.2.1.3.2. Treatment with *Lactobacillus helveticus*

A loop full of *L. helveticus* culture was *activated* on 10ml of freshly prepared MRS broth containing 10g L^{-1} proteose peptone, 5g L^{-1} yeast extract, 10g L^{-1} beef extract, 20g L^{-1} dextrose, 1g L^{-1} Tween-80, 2g L^{-1} ammonium acetate, 5g L^{-1} sodium acetate, 0.1g L^{-1} magnesium sulphate, 0.05g L^{-1} manganese sulphate, 2g L^{-1} di-potassium phosphate with pH adjusted to 6.5 at 25ºC (all the chemicals were supplied by Hi-Media Chemicals, India), allowed to grow overnight on a gyratory shaker of 90rpm at 25ºC. A culture density of 0.5 OD at 660ηm was selected and used to treat hairy root cultures grown for 18-20 days in 40ml MS medium, by way of adding 1ml, 2ml, 3ml, 4ml and 5ml of *L. helveticus* culture, each with six replications of two sets where one set was incubated on gyratory shaker of 90rpm (agitated conditions) and the other was kept static. The treated cultures were periodically observed for any leaching of pigment to the medium, which was noticed 8h after treatment. Therefore, from 8h and onwards, 1ml of the leachate was aseptically pipetted out, centrifuged and the OD at 540 and 480ηm was recorded at 12, 24 and 48h of treatment and expressed as the percent pigment released.

4.2.1.3.3. Treatments with *Candida versatalis* and *Saccharomyces cereviseae*

Culture taken from stock of each species with an inoculation loop was inoculated to 10ml of potato dextrose broth containing 20g L^{-1} potato, 20g L^{-1} glucose and pH adjusted to 5.5 at 25ºC allowed to grow overnight on a gyratory shaker of 90rpm at 25ºC. A culture density of 0.5 OD at 660ηm was selected and used to treat hairy root cultures as done for *L. helveticus*. The treated cultures were periodically observed for any leaching of pigment to the medium, which was noticed 24h after treatment in case of *S. cereviseae* and hence the OD at 540 and 480ηm was recorded at 24, 48 and 72h after subjecting to treatment where as

in case of *C. versatalis,* pigment started leaching 8h after treatment as in the case of *L. helveticus* hence the OD at 540 and 480ηm was recorded at 12, 24 and 48h and the pigment was quantified as explained earlier.

4.2.1.3.4. Treatment with killed dry cells and cellular fractions of *L. helveticus*

In addition to the treatments described above, cell fractions of *L. helveticus,* were also used as dry cell powders obtained (from 10g fresh weight) after growing on respective plated medium followed by drying at 45ºC until a constant weight (about 1g), was obtained. The dry cell powders, collected from several replicates, were autoclaved and used either directly or the components were further fractionated (Sadasivam and Manickam, 1992). The total insoluble carbohydrate from such cells was recovered by washing firstly with ethanol followed by profuse washing with petroleum ether and dried till the smell of solvent was absent. Free lipid fraction, from fresh live culture was separated using petroleum ether (40 – 60ºC) from liquid obtained from the supernatant of 1L culture broth of 0.5 OD at 660ηm and cell-bound lipid fraction was obtained from extracting the residue with petroleum ether. The lipid fractions, obtained after evaporating the ether, was re-suspended in 20ml MS medium and used as 1ml aliquot for shake flask cultures and the pigment release was monitored under static as well as agitated conditions.

4.2.1.4. Repeated effluxing of betalaines using CTAB

Repeated effluxing-using CTAB (0.02%) for increasing the productivity was done with and without an additional level of calcium chloride (up to 3folds of that present in MS liquid medium). The cultures were grown in 50ml Erlenmeyer flask containing 15ml MS liquid medium. Twenty-day-old cultures were subjected for effluxing first time and the same cultures were effluxed subsequently, at the end of every week followed by the replacement with 15ml fresh medium with or without calcium. Similarly medium replenished, un-effluxed cultures served as control. The set of cultures with un-effluxed with no medium replenishment served as the overall control. Total pigment yield after subjecting for each additional treatment was calculated and expressed as fold increase of pigment yield over control.

4.2.1.5. Pigment release in the bioreactor

Hairy root cultures were grown in a 3L bubble column reactor with a working volume of 1.75L. Twenty-day old culture was used to study the pigment release pattern by adding filter sterilized (Sartorius 0.45μm) CTAB to achieve a final concentration of 0.002%

(w/v) or 10ml of free lipid fraction obtained as above from culture broth of *L. helveticus*. 5ml of medium sample was periodically drawn and analyzed to monitor the pattern of release as in other experiments.

4.2.1.6. Test for viability

The hairy roots, after treating with different chemical permeabilizing agents and killed whole cell / fractions of cells of biological permeabilizing agents, were checked for their viability by plating a few hairy root tips on agar gelled MS medium as well as by way of replacing the spent medium with the fresh medium. The viability was recorded as positive if there was any emergence of fresh root tips in plated cultures and for re-growth of fresh roots in the liquid medium / bioreactor.

4.2.1.7. Effect of calcium channel modulators on the release of betalaines

Based on the earlier reports a pilot study was conducted to find out effective concentrations of calcium channel modulators and the best concentrations were used for further study. The calcium channel modulators were administered to 20 day old cultures by replacing spent medium with fresh medium containing calcium channel modulators such as EGTA, a calcium chelator (3mM), ionophore A23187 (0.5μM) a calcium channel enhancer, chlorpromazine (10μM) a calmodulin inhibitor and verapamil hydrochloride (10μM) calcium channel blocker. All the calcium channel modulating chemicals were obtained from Sigma-Aldrich, USA. The treated cultures were further subjected for effluxing using various effluxing agents such as CTAB (0.005%), Triton-X-100 (0.005%) and free lipid surfactant from Lactobacillus helveticus (concentration as given in section 4.2.1.3.4.). The lower concentrations of CTAB and Triton-X-100 were selected to achieve a regulated pigment efflux for easy monitoring. The hairy root cultures treated with calcium channel modulators were subjected effluxing at three time periods i.e., after 24h, 48 and 72h of treatment and the pigment released was quantified after 6h of effluxing. The values were expressed as percent increase or decrease of pigment release in each treatment over the respective control.

4.2.2. Adsorption and recovery of betalaines

4.2.2.1. Selection of adsorbents

Two sets of adsorbents, one set for *in situ* (Table 4.2, 4.3) and the second set for *ex situ* (in the column, Table 4.4) were selected assuming that the adsorbents do not bring about any chemical change to betalaines; the temperature and the pH in the adsorbent bags as well as in the column remains constant throughout the operating time and the adsorption mass

transfer is mainly by surface actions / convection (Snyder, 1968). Among the adsorbents used silica gel-G (Silica), aluminium oxide active acidic (alumina), corn starch, maltodextrin, and dextrin white all were from Hi-media chemicals, India; wheat starch, sand - acid purified 40-200mesh (processed sand) were from S. d. fine chemicals, India; β-cyclodextrin (cyclodextrin) is from Sigma, USA and XAD-2 & XAD-4 were from Fluka, Germany. *In situ* experiments were done, by using the adsorbents with and without methanol pre-treatment (done as reported by Sim and Chang 1997) and activating further by keeping at 100ºC for 1h. However, the *ex situ* adsorption experiments were done by using activated adsorbents as above without methanol pretreatment.

4.2.2.2. *In situ* adsorption

Before adding adsorbent, the pigments were effluxed using 0.002% cetyltrimethylammonium bromide (CTAB), as reported earlier (Thimmaraju et al. 2003b). For experimental purpose, the different equilibrium concentrations of pigment were achieved by evaporating pigment-medium mixture in vacuum using a flash evaporator. For *in situ* adsorption, 3g of different adsorbents (Table-4.2), with or without methanol pre-treatment (Sim and Chang 1997) were filled in small bags of 1 sq. inch made up of nylon material having 20μm pore size. The bags, after filling adsorbent, were sealed by stitching the open end carefully (without spilling the adsorbent) and inserted directly into 150ml Erlenmeyer's flask containing pigment (of a known equilibrium concentration) leached from cultured hairy root. Ten sets of flasks along with the bags of adsorbents were incubated on a rotary shaker at 90rpm in dark at 25 ± 2ºC. Each set was analyzed for betalaine content and the quantity adsorbed at the end of 1h was calculated as follows (Payne and Schuler, 1988).

$$q = \frac{(C_i - C)\, V}{A} \qquad (1),$$

where, C and C_i are the measured (un-adsorbed) and initial betalain concentrations, respectively (mg ml^{-1}) ; V is the liquid volume (ml); A is the amount of adsorbent in the nylon bag (g) ; and q is the pigment loaded onto the adsorbent (mg g^{-1} adsorbent). To find out adsorption isotherm constant, the amount of betalaines adsorbed per unit mass of adsorbent (x/m) was plotted against different equilibrium concentrations of betalaines at pH 5.5 at 25ºC and used for further calculations.

4.2.2.2.1. Studies on time-course of adsorption

Once the suitable adsorbent selection was done, the time course study of adsorption was done using nylon bags with 2g adsorbent and introduced into a known volume of pigment extract of particular equilibrium concentration. The adsorbent bags were incubated on a gyratory shaker as mentioned earlier and the pigments remaining in the medium was recorded after every 10min for a total period of 70 min for obtaining a gross picture of the adsorption process. To check whether the periodic analysis of loss of pigment in the medium directly correlated with the amount of pigment adsorbed, the un-adsorbed pigment and the medium in the interstitial spaces of the bag as well as the adsorbent was removed by placing the bags on a Buchner funnel and applying vacuum. The medium thus recovered with un-adsorbed pigment was pooled with the remaining medium and the pigment quantification was done as mentioned earlier. Since this data correlated well with the data of periodic pigment quantification in the medium, the routine experiments were done by analyzing loss of pigment in the remaining medium and considered as the pigment adsorbed by each adsorbent.

4.2.2.2.2. Desorption studies

It is well known that the adsorption phenomenon involves pH as one of the main functional parameters. Therefore, desorption (elution) experiments were done mainly by changing the pH, i.e., by lowering the pH in this case. In most of the earlier studies, progressive decrease in pH resulted in progressive desorption of the pigments. For testing desorption / recovery of *in situ* adsorbed pigments, the adsorbent bags, from *in situ* experiments, were removed and placed in a Buchner funnel and the un-adsorbed pigments were removed by air-suction. The adsorbed solute was desorbed by inserting the used adsorbent bags in 10ml of 2% aqueous hydrochloric acid kept in a centrifuge tube. The contents were centrifuged at $4^{\circ}C$ for 10min at 10000×g where the desorbed pigments migrated to the supernatant. This desorption step was repeated when found necessary, i.e., if the adsorbent bags appeared still reddish in colour. The pH of the supernatant was adjusted to 5.5 before quantifying the pigments spectrophotometrically. In the same way pigments were also quantified in the extract before and after the addition of adsorbent bags. The quantum adsorbed was calculated and expressed as percent adsorption and percent recovery.

4.2.2.3. *Ex situ* adsorption using column

An initial screening experiment with two different column geometry and different combination of column materials was done. Glass columns of either 1cm diameter or 2cm diameter of various lengths were used. The adsorbent material, before loading into the column, was activated by heating at 110ºC for 1h. To reduce mechanical disturbances at the top of the column material during loading of the pigment extract, the upper surface of the adsorbent in the column was covered with circular polyurethane foam of 3mm thickness as shown in Fig. 4.3. Before loading the pigment extract, the column was washed with 0.1% aqueous HCl and allowed to drain off. The column thus prepared was loaded with 15ml of red beet extract containing an equilibrium concentration of 0.18mgml^{-1} of betalaine (extracted from hairy roots using 0.1% acidified water). The pigment solution was slowly loaded on to the column with the help of a glass rod, and the effluent was collected and estimated for betalaine content. The percent adsorption was calculated based on the difference in the pigment content of the effluent and the extract.

4.2.2.3.1. Desorption

The *ex situ* adsorbed pigment was eluted with different elution solvents and collected as 3ml fractions until the pigment level in the effluent reached a plateau. The ethanol and hydrochloric acid gradient was developed as shown in Table 4.5, where the dilutions to obtain respective levels of each mixture was done using distilled water. Three ml aliquot of each gradient mixture was allowed to flow onto the top of the column material where the gradient mixture and the flow rates were achieved by using HPLC pumps (LC 10ATvp; LC 10AS; Shimadzu). The fractions were individually estimated for betalaine content. Finally all the fractions were pooled and the total volume of the eluent was recorded and the quantity of the pigment eluted was estimated spectrophotometrically and expressed as percent elution.

4.2.2.3.2. Determination of *ex situ* adsorption breakthrough

Adsorption breakthrough can be defined as the situation where the concentration of the compound (pigment in the present context) in the effluent (outlet) is same as that of feed (inlet) sample. Thus adsorption breakthrough is nothing but the consequence of column saturation. Adsorption breakthrough was worked for 2cm × 2cm column of alumina: sand (1:1) pre washed with 0.1% HCl with a void of 7.5ml. Polyurethane foam was placed at the top surface of the adsorbent to avoid mechanical disturbance while loading.

To determine breakthrough at normal flow-rate, the column was loaded with pigment extracted in 0.1% HCl of 0.18mg ml^{-1} betalaine concentration while the level of the pigment extract, 2cm, was maintained constantly above the column material and the effluent was collected in 3ml fractions. The optical density (OD) at 480 and 540ηm was measured for each fraction. Thus, the gravitational flow through the column was considered as "normal flow rate". Pigment loading was continued at the same rate until an OD value equivalent to that of the loading solution was obtained in the effluent fraction. The time point at which OD of the effluent fraction was same as that of the sample was considered as the adsorption breakthrough (s) i.e., all the adsorbent plates in the column were fully saturated with the pigment and will not be able to adsorb any more pigment. Pigment concentration in each eluted fraction, total quantity loaded till the column saturation was achieved and the adsorption breakthrough of the column were calculated. The pigment adsorbed by the column material (mg g^{-1}) at a given flow rate was calculated by

$$Q_S = C_i - C_S / X \quad \text{and} \quad Q_{1/2} = C_{i/2} - C_{1/2} / X \qquad (2),$$

Where, C_i and $C_{i/2}$ are pigment loaded (mg) at `saturation' and `breakthrough' points respectively, Q_S and $Q_{1/2}$ are the total quantity of pigment (mg g^{-1}) adsorbed at saturation and breakthrough point, C_S and $C_{1/2}$ is the quantity of pigment (equilibrium concentration, mg ml^{-1}) present in the effluent (un-adsorbed) at saturation and breakthrough point and X is the quantity of adsorbent (g).

To determine breakthrough at high flow-rates, all the conditions were kept similar to those mentioned for normal flow-rate except that higher constant flow rates were achieved by applying different pressures created at the inlet using a pump with a device as shown in Fig. 4.3. Different effluent flow rates of 0.3, 1, 2, 3 and 5ml per minute were achieved by using 500 KW pump and adjusting the flow rate by adding membrane filters to the silicone rubber tubing fixed to the column inlet.

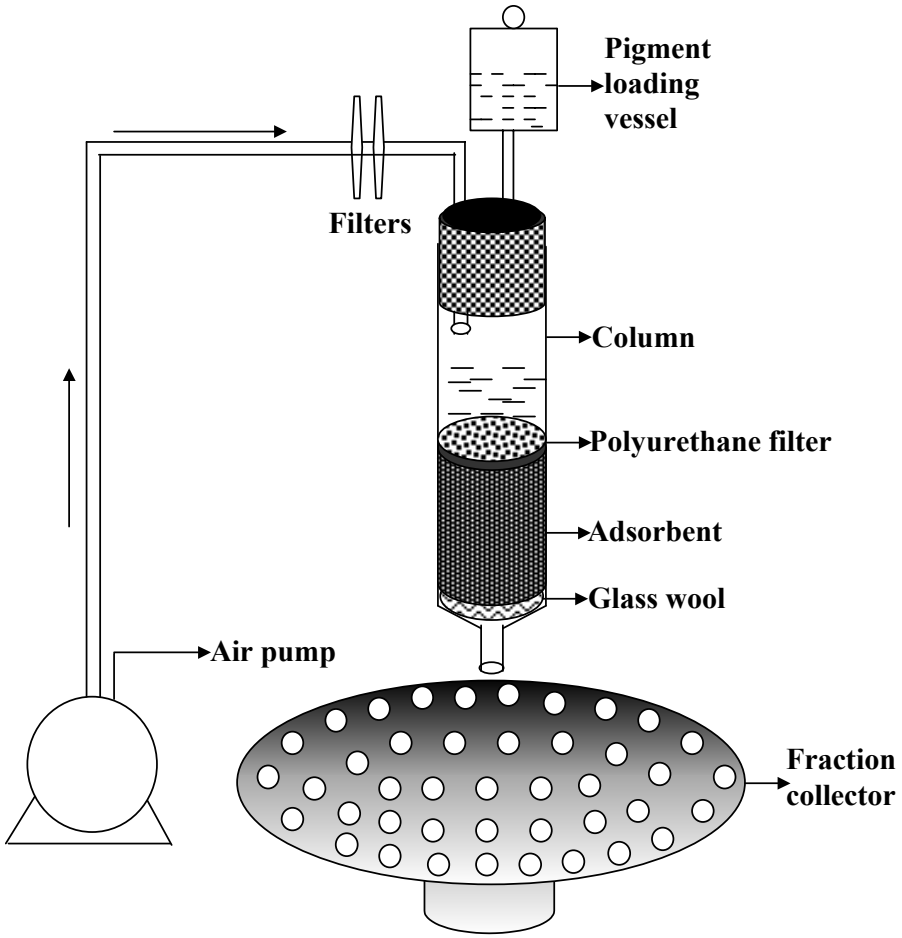


Figure 4.3. Diagrammatic representation of the column set up used for generating different flow rates.

4.2.2.4. Separation of betalaines into betaxanthine and betacyanine

Best *ex situ* adsorption column i.e., Alumina: Processed sand (2:1); 2cm x 2cm was selected and while improving the recovery the separation of betalaines into betaxanthine and betacyanine was also monitored. Various elution solvents used for achieving improved separation were as given in table 4.7.

4.2.2.5. Simultaneous recovery of betalaine and peroxidase by ATPE

Preparation of aqueous two-phase system: ATPS was prepared by mixing the required quantities of polyethylene glycol (of various molecular weights) and ammonium sulphate in the extract and adjusting the final volume to 20ml. After mixing thoroughly, the system was allowed to separate into two phases in a 100ml separating funnel. Volumes of the separated phases were measured. Aliquots of the phases were taken for the enzyme and pigment assays and for determination of protein concentration. The partition coefficient (k) of the enzyme and pigment was determined from the equation $k = (C_t/C_b)$, where C_t and C_b are the equilibrium concentrations in the top phase and bottom phase respectively. Similarly, the recovery of the pigment and the enzyme were calculated using the formula,

$$R = 100\ (Q_p/Q_t)$$

Where, R= recovery in percentage, Q_p = quantity in a particular phase and Q_t = total quantity taken for the experiment.

4.2.2.6. Hairy root culture in bioreactor

A 3L bubble column bioreactor, with a growth chamber containing a basket for supporting the biomass, and a working medium volume of 1.75L was used for the study (Chapter2, Section 2.2.3**).** Hairy root inoculum of 10g grown in shake flask for 4 days using MS liquid medium was placed aseptically in the biomass support basket and oxygen was supplied by bubbling the pre-filtered air at 0.5-2 vvm directly into the bioreactor from the bottom through the air-sparger. Twenty-day-old culture obtained as above was used to study the pattern of pigment release under the influence of CTAB and lipid extract from *L. helveticus*.

4.3. RESULTS AND DISCUSSION

4.3.1. Permeabilization and release of betalaines

4.3.1.1. Physical agents

4.3.1.1.1. Pigment release under the influence of pH

Three different pH used to efflux betalaines from 20-day-old hairy root cultures and the result presented in Figure-4.4, clearly indicates that lower the pH better was the pigment release. Over 70% of the pigment was released within 30min of exposure to the medium of pH 2.0 with no further release later. Whereas, only about 10% of the pigment was released at pH 3.0 and lesser at pH 4.0 in 30min with a very slight improvement up to 240min. However, upon the exposure of hairy roots to different pH of 2.0, 3.0 and 4.0, the released color gradually degraded with the increase in time in pH 2.0 (Fig 4.4). Addition of fresh medium with or without calcium enrichment did not support further growth of hairy roots in case of pH 2.0 treatments for 40min & above. Whereas, those treated with pH 3.0 and 4.0 showed viability and normal growth pattern comparable to non-pH treated controls (data not shown). At pH 2.0 even the viable cultures largely had disrupted cells away from the tips subsequently contributing towards dead suspended particles.

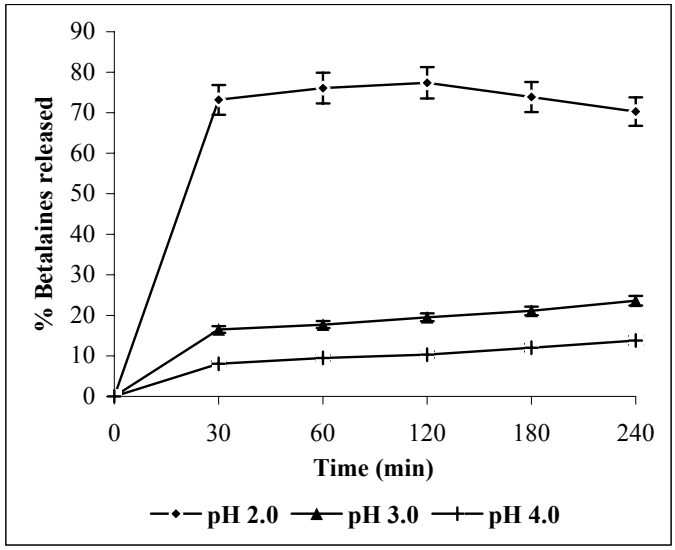


Figure 4.4. Release of betalaines into the medium from hairy root cultures of *Beta vulgaris* under the influence of different pH for different periods.

Thus repeated growth and pigment synthesis may only be expected from the intact tips, while the presence of ruptured cells contribute towards browning resulting in low quality of subsequently extracted pigments. Thus the present study is different from what

have been reported earlier, where repeated synthesis of beet pigments is claimed from the post-permeabilized roots (Mukundan et al. 1998).

Separate observation made for both release and degradation pattern of pigments in the medium and / or water whose pH was adjusted to 2.0, shows a significant difference between the two i.e., the release was faster in water than in to medium (Fig. 4.5A & B). Similarly, there was a faster degradation of the pigment in water of pH 2.0 (Fig. 4.6A & B). This difference may be due to the interaction of ions, which are present in the nutrient medium, acting as osmoticum and membrane protecting agents to certain extent.

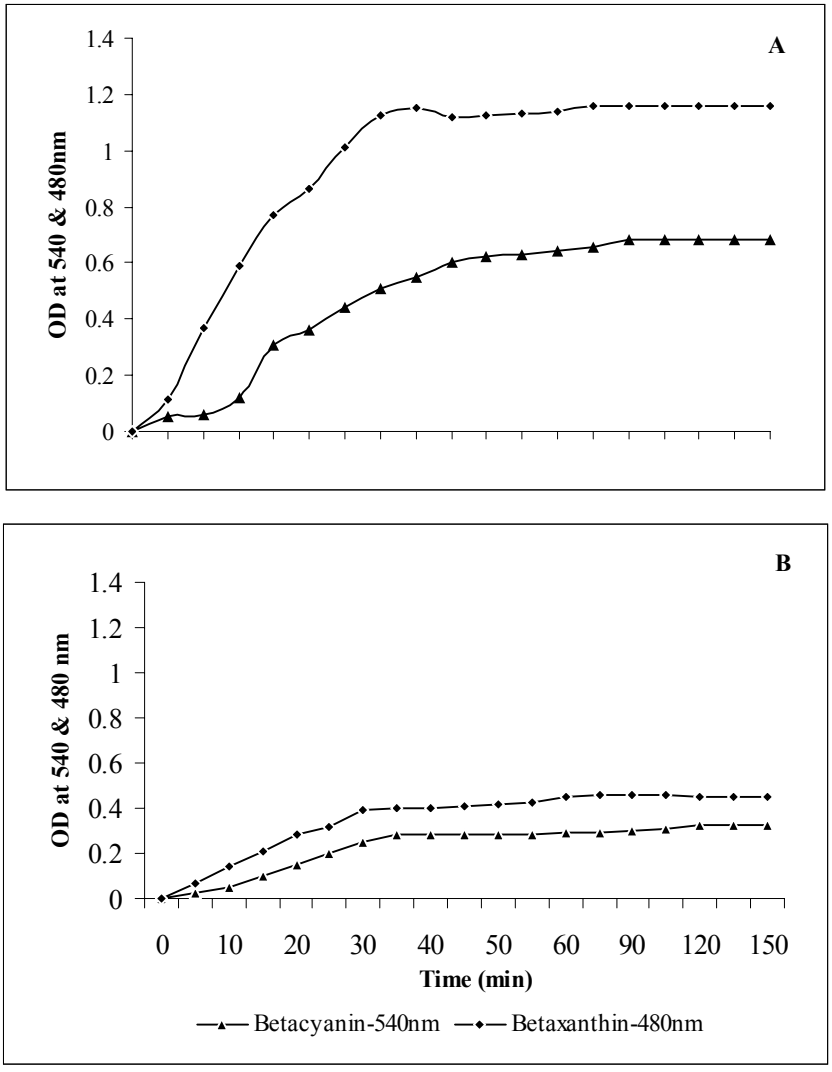


Figure 4.5. Pattern of release of betalaines under the influence of water of pH 2.0 (A) and MS medium of pH 2.0 (B) Data presented as OD.

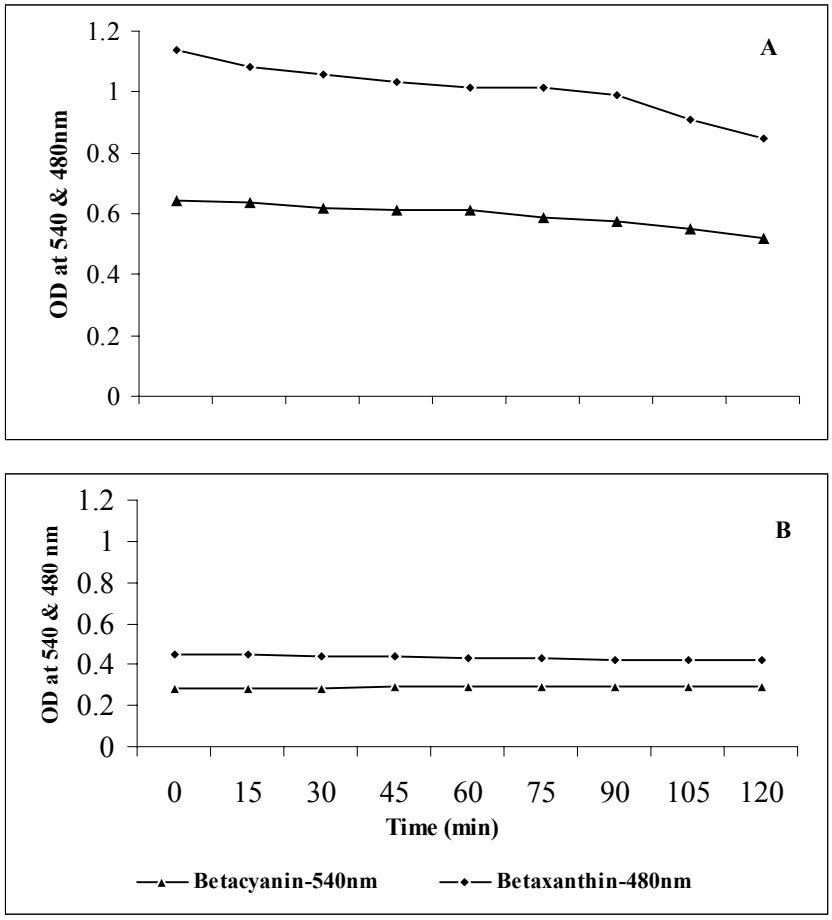


Figure 4.6. Stability of betalaines released at pH 2.0 into water (A) and MS medium (B) using 5g of fresh hairy roots in 25ml of water / medium and incubated on shaker at standard conditions. Data presented as OD.

The pH mediated release reported earlier (Mukundan et al. 1998), indicated that only pH 2.0 would assist pigment release, which may be due to the existence of betalaines under zwitterionic state at pH 2.0. In our experiment we have noticed that even pH 3.0 and 4.0 will assist in releasing about 10-20% of the total pigments (Fig. 4.4). Another critical aspect in the present work is consideration of the pH mediated changes of betalaines under low pH conditions and involvement of a correction factor to avoid erroneous results due to change in the absorbance which largely might have been ignored by most of the earlier workers. Though, some of them mentioned about the loss of stability of betalaines at pH 2.0 (Nilson, 1970) this happens mainly in water and not in medium upto 2h. Thus effluxing the pigment at pH 2.0 in to the medium will allow enough time for further processing of released betalaines. In this context, the suggestion of exposing hairy roots to pH 2.0 for 10min followed by transferring to a fresh medium of pH 5.5 for efflux of pigment for next 24h

followed again by one more change of medium (Mukundan et al. 1998) does not appear practical as it needs three medium changes. Also, our experiment clearly indicated that replacing medium of pH 2.0 (30min) with medium of pH 5.8 totally suppressed pigment release in the latter (data not shown).

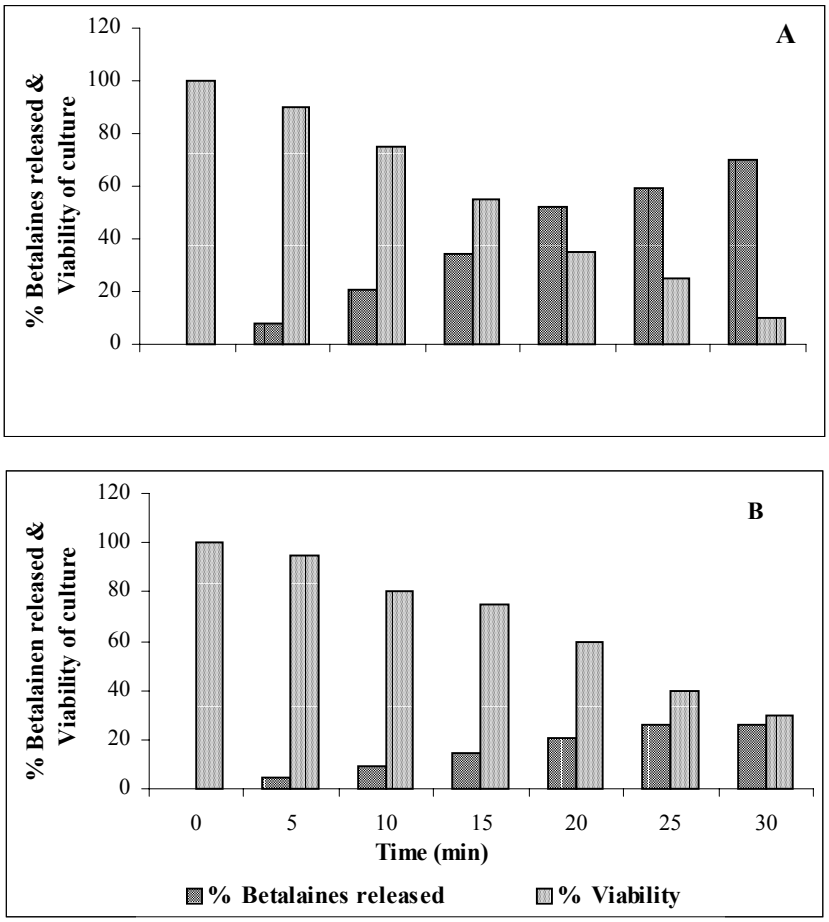


Figure 4.7. Pattern of release of betalaines and viability of cultures under the influence of water (A) and MS medium of pH 2.0 (B) using 5g of fresh hairy roots in 25 ml of water / medium incubated on shaker at standard conditions.

Addition of fresh medium with or without calcium supported the re-growth of 10% of total cultures in the case of pH 2.0 treated hairy roots for more than 30 min unlike the earlier reports where, over 10min exposure to pH 2.0 lead to total loss of viability (Mukundan et al. 1998). It is quite possible that the cells may be severely damaged and the pigment release occurs only from the killed cells, which may also be the reason for the loss of viability of the culture under low pH conditions. A similar observation was made by some of the earlier workers (Mukundan et al. 1998; Kino-Oka et al. 1992). In the present study, the reduction in the treatment period to 5, 10, and 15 and 20min released 4.5, 9.2, 14.9 and 20.7% of pigments with a slight improvement in the viability of plated cultures (Fig. 4.7A & B).

4.3.1.1.2. Sonication

The extent of release of betalaines under the influence of sonication for different periods resulted in cell maceration to different extents with the release of pigment into the medium. Figure-4.8 shows that hairy roots sonicated for 15 seconds released about 8% of the pigment, which remained constant at higher levels of sonication, for 30 and 60 seconds, indicating the resistance of hairy roots to the treatment upto 60 seconds after which the release of pigments increased to 12% in cultures treated for 120 seconds. Post-sonication incubation of cultures in normal MS medium and calcium enriched medium indicated that in all the cases the viability was not lost though the growth rate declined by 50% resulting in the decline of overall betalaine recovery (data not shown). During subsequent sonication, the quantity of betalaines effluxed due to sonication at a given time remained as in first treatment. Thus, by sonicating the hairy roots, a maximum of only 12% of the total betalaines could be recovered without the loss of culture viability during first cycle, whereas after the second harvest viability was totally lost. The frequency of the ultrasonic sound used in the present study had been selected based on the earlier report on effluxing betalaines from suspension cultures of red beet using 1.02 MHz frequency (Kilby and Hunter 1990; 1991). In the present study we found that, 1.02 MHz is too high for permeabilizing the hairy roots since it resulted in complete maceration of hairy root cultures. Therefore, a lower level (0.02 MHz) was tried which was also capable of releasing same amount of pigment reported for red beet suspension cultures (Kilby and Hunter 1990; 1991). Bubble formation during sonication mediated pigment release, as reported earlier, also confirms involvement of cavitational events. Since the cavitational events are coupled with

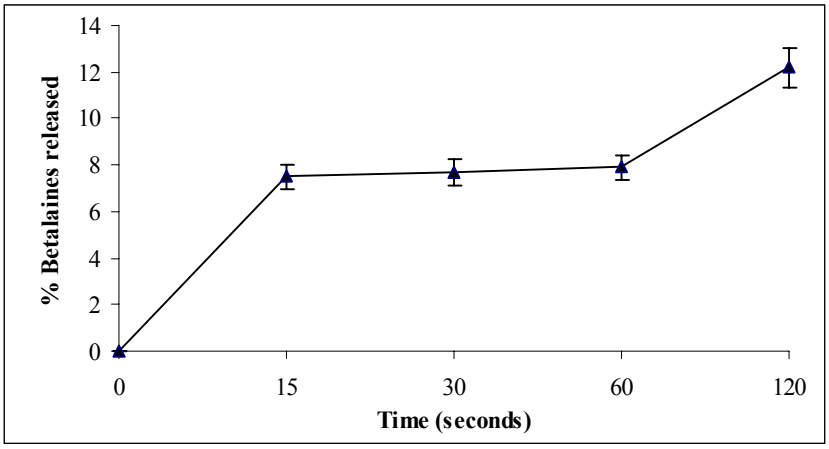


Figure 4.8. Release of betalaines from hairy root cultures of *Beta vulgaris* sonicated at 0.02 MHz for different time periods.

the release of the free radicals (Kilby and Hunter 1991), sonic effluxing of the pigment may not be good for food applications. The sonicated cultures contained a lot of suspended ruptured cells and could be reused only once as the hairy roots lost the viability after the second round of sonication. This is in contrast to the suspension cultures (Kilby and Hunter 1990; 1991), which were amenable for repeated sonication. Calcium treatment did not have any beneficial effect on viability.

4.3.1.1.3. Temperature

Figure-4.9 shows the pattern of release of beet pigments under different temperatures during the time course of 30min and 60min. The release of betalaine was directly proportional to the temperature increase at a given treatment period. As high as 45% of the betalaine was released in 30min in 50ºC followed by about 35% and about 5% at 45 and 40ºC respectively. The pigment release reached a plateau after 30min and was about 50% at 50ºC in 60 minutes. Though there was an increasing trend of pigment release at 40 and 45ºC the quality of released color was poor with a brown tinge, which was also confirmed in HPLC analysis. It should be noted that a high temperature of 50ºC shows a declining trend and all the treated cultures lost their viability and the calcium treatment did not result in any improvement in the viability in contrast to the earlier report (Dilorio et al. 1993). Though, temperature treatment resulted in the efflux of over 50% of pigments, it may not be a suitable effluxing method considering its lethal effect on hairy root cultures and adverse effect on the leached color. Our culture did not respond positively to the calcium treatment, unlike existing reports on its role in improving the viability of the culture (Dilorio et al. 1993).

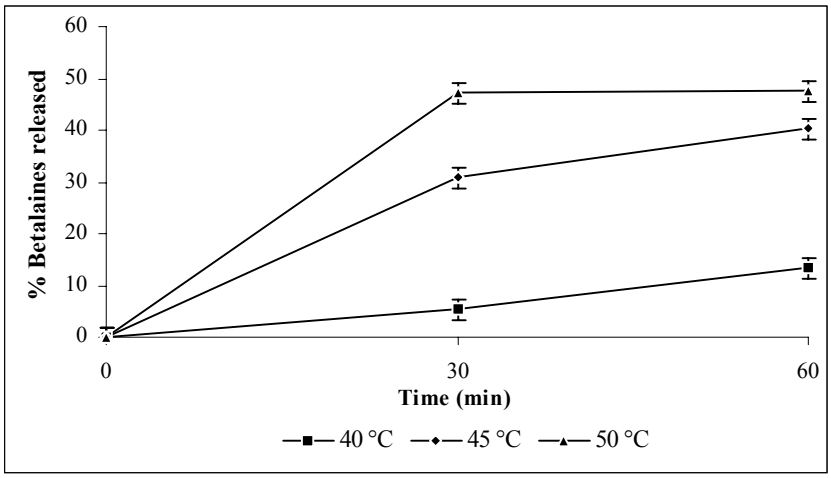


Figure 4.9. Release of betalaines from hairy root cultures of *Beta vulgaris* under the influence of different temperature for different periods.

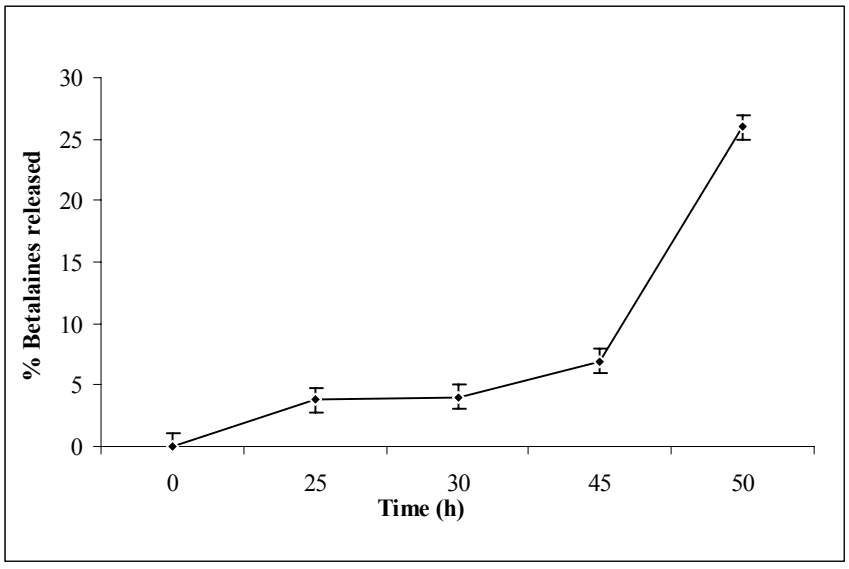


Figure 4.10. Release of betalaines from hairy root cultures of *Beta vulgaris* under the influence of oxygen stress induced by cessation of gyration for different periods.

4.3.1.1.4. Oxygen stress and osmotic stress

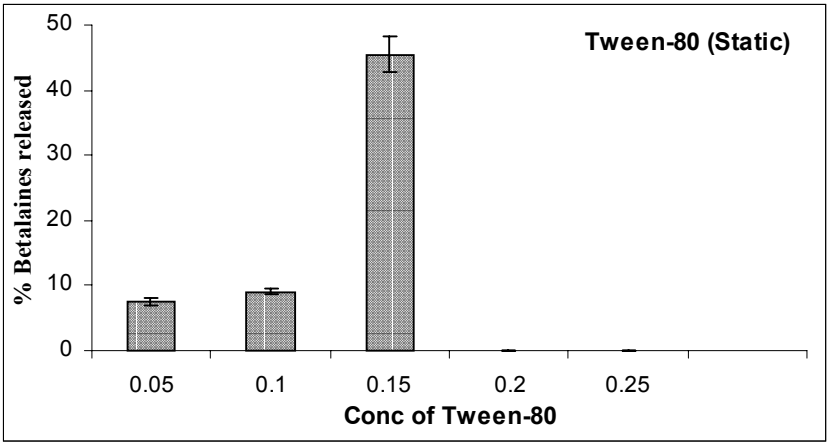
As depicted in the Figure-4.10 the release of betalaines under oxygen stress imposed by cessation of gyration was gradual up to 45h leading to a mere 5% efflux of total

betalaines. However, there was a steep increase after 45h resulting in the release of about 25% of the betalaines in 50h of period under 2000Lux of light condition, with an associated loss of culture viability. There was no release of pigments under dark condition even after 50h. Though oxygen starved hairy root cultures released pigments, in contrast, to other reports (Taya et al. 1992), it resulted in the complete death of hairy root cultures and there was an associated browning of the released pigments almost instantaneously indicating poor stability of the released pigments. The osmotic stress on the other hand did not result in the release of pigment even at as high as 5M-sorbitol level in the medium for 24h.

4.3.1.2. Influence of chemical agents

The release of betalaines under the influence of Tween-80 was observed only after 48h, where 0.05% and 0.1% imparted almost similar effect with a marginal improvement in the latter with a drastic increase of betalaine release at 0.15% treatment in static cultures, and the further higher levels totally suppressed the release, where the roots turned dark (Fig.4.11). At 0.15% nearly 46% of betalaines was released into the medium in 48h. Whereas, in agitated medium, though the pattern of release remained almost similar to the static cultures, the release was retarded by ten times.

Fig. 4.12 Indicates the pattern of release of betalaines under the influence of Triton X-100, wherein both static and agitated conditions effectively induced pigment release. Highest level of Triton X-100 used (0.2%) released over 70% of the pigment into the medium within 2h, which remained constant upto 4h and declined very slowly in case of static treatment and more slowly in agitated cultures. The decline was significant after 4h and by 8th hour there was nearly 12% loss (of the total released) in agitated medium indicating the degradation of the leached pigment.



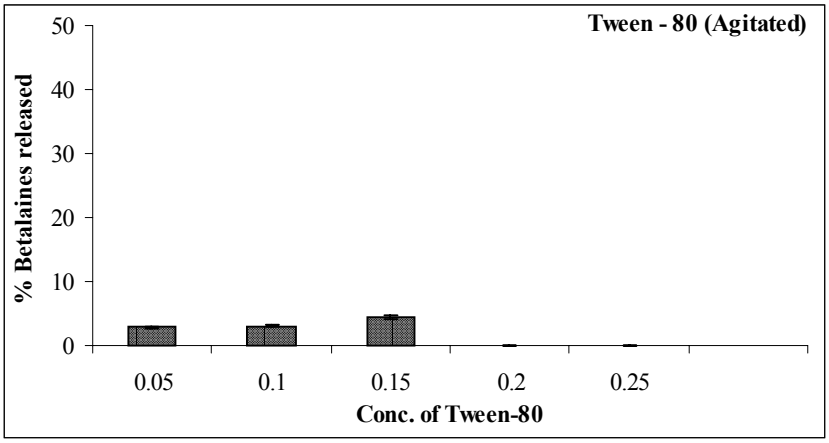
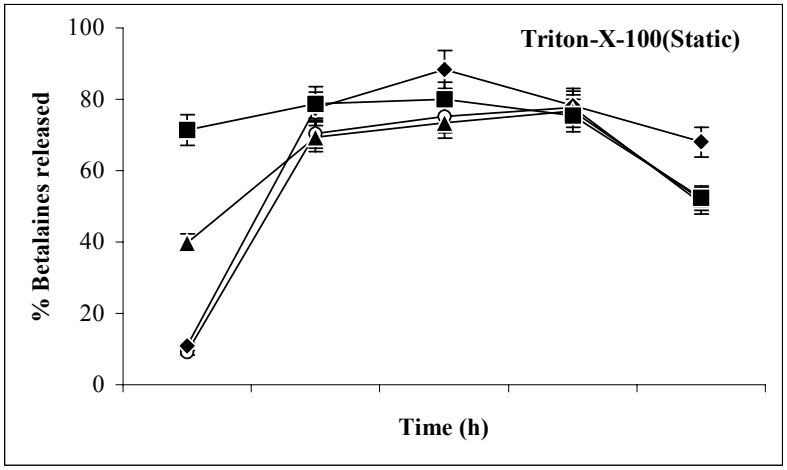


Figure 4.11. Pattern of pigment released into the medium after 48h under the influence of different levels of Tween-80 from red beet hairy roots in static and agitated culture conditions. The results are the means ± S.E of two independent experiments, each with six replicates.

In static medium the degradation was rapid after 12h of release. A lower concentration of 0.15% released about 40% of the pigment in 2h, which increased further leading to nearly 60% of the pigment released into the medium, and the pigment degradation occurred after 12h, almost similarly in both static and agitated medium. Further lower levels of Triton X-100, 0.05% and 0.1%, caused good pigment release of upto 65%, only after 4h treatment. In these low concentrations loss of pigment due to degradation was negligible under both static and agitated conditions upto 12h of treatment and the degradation occurring subsequently accounted for nearly 15% loss of the released pigment (Fig. 4.12).



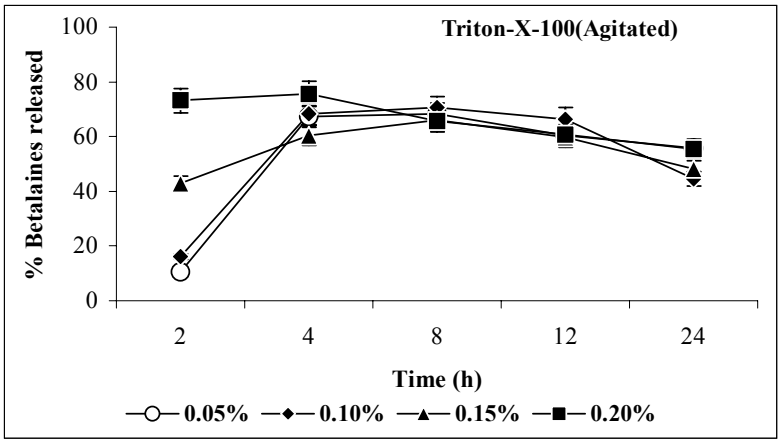


Figure 4.12. Pattern of pigment released into the medium under the influence of different levels of Triton X – 100 from red beet hairy roots in static and agitated culture conditions. The results are the means ± S.E of two independent experiments, each with six replicates.

CTAB, a cationic detergent and an efficient food-grade cell wall permeabilizing agent used extensively for permeabilizing yeast cells (Joshi et al. 1987), was initially tried at levels similar to that of Triton X-100, which resulted in immediate release of pigment into the medium, probably due to dissolution of phospholipid bilayer, as observed by earlier workers (Chao et al. 2000). Therefore, lower concentrations such as 0.0005% to 0.05% were tried which resulted in the pattern of release as compiled in Fig. 4.13. The highest concentration, i.e., 0.05% resulted in over 90% of pigment release within the first hour which remained constant till the 4^{th} hour in static medium and later (6^{th} to 8^{th} hour) declined, probably due to degradation (data not presented for these periods). However, even after 24h, over 60% of the total pigment (i.e., nearly 80% of the released pigment) was still remaining in the spent medium. Similar was the pattern of pigment release, in agitated medium with a lower level of release at respective time intervals and a similar pattern of degradation as well. Whereas in the case of 0.01% CTAB treatment, in static cultures, nearly 70% of the betalaines leached in 1h which increased upto 78% (of the total BN content) by the end of 4^{th} hour, declining at later periods as observed earlier. Similar treatment (0.01% CTAB) in agitated cultures, by the end of first hour, caused only about 50% of the pigment release into the medium and nearly 80% of the total pigment release by the end of 4^{th} hour. As in the earlier treatments, here too there was a loss of pigment in the medium with prolonged incubation periods observed upto 24 hours (Fig. 4.13).

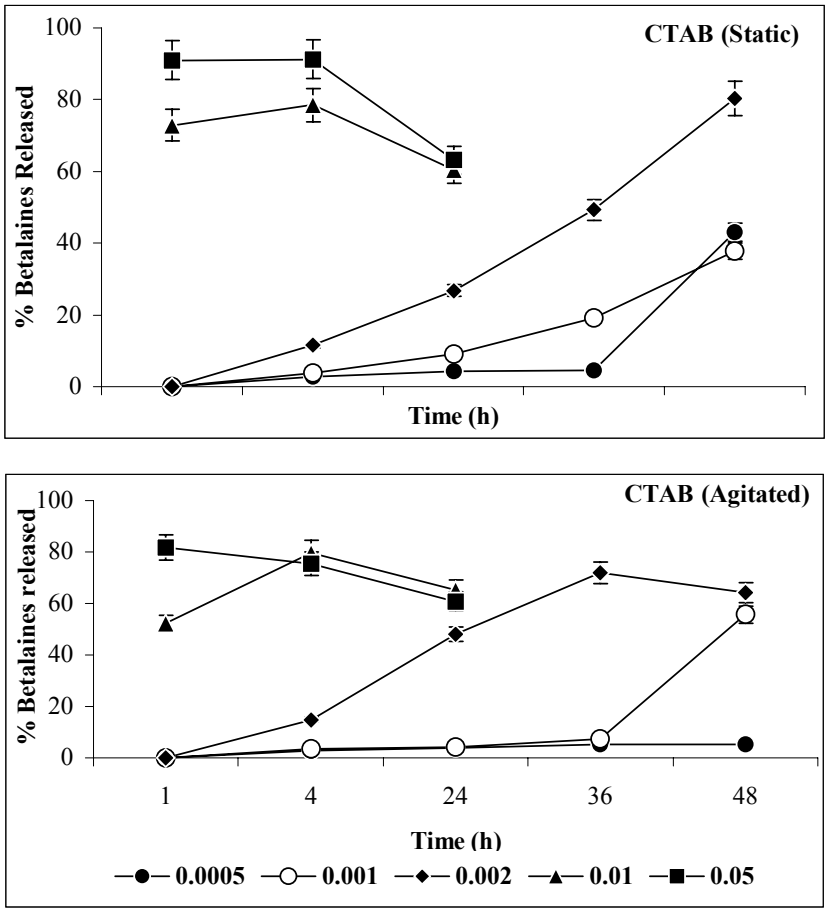


Figure 4.13 Pigment release from hairy roots of *Beta vulgaris* under the influence of different levels (% w/v) of cetyl trimethylammonium bromide (CTAB) in static and agitated culture conditions. The results are the means ± S.E of two independent experiments, each with six replicates.

Among the lower concentrations, 0.002% supported a steady and continuous time-bound release upto 48 hours reaching a maximum release of over 80% in case of static cultures whereas in agitated cultures, a slightly higher level of pigment was recorded by the end of 36th hour with a narrow decline at a later stage. At further lower concentrations (Fig. 4.13), a very low release of less than 20% pigment was observed at the end of 36th hour only in static conditions but not in agitated cultures. However, at a later stage of 48h, a level of over 50% release was observed in case of agitated cultures whereas at similar period much lesser level of pigment release was observed in static condition, with an exception at a very low level of 0.0005% treatment in agitated condition, where the pigment release was negligible throughout the treatment period.

4.3.1.2.1. CTAB-mediated pigment release in the bioreactor

Addition of the best level of CTAB i.e., 0.002% to check the pattern of pigment release in a bubble column reactor resulted in time bound pigment release. A highest amount of pigment release being 80% was observed at the end of 48th h, preceded by about 30 and 44% pigment release after 24 and 36h after treatment respectively. A trend of decline in pigment content was observed after 48 hours (Fig. 4.14).

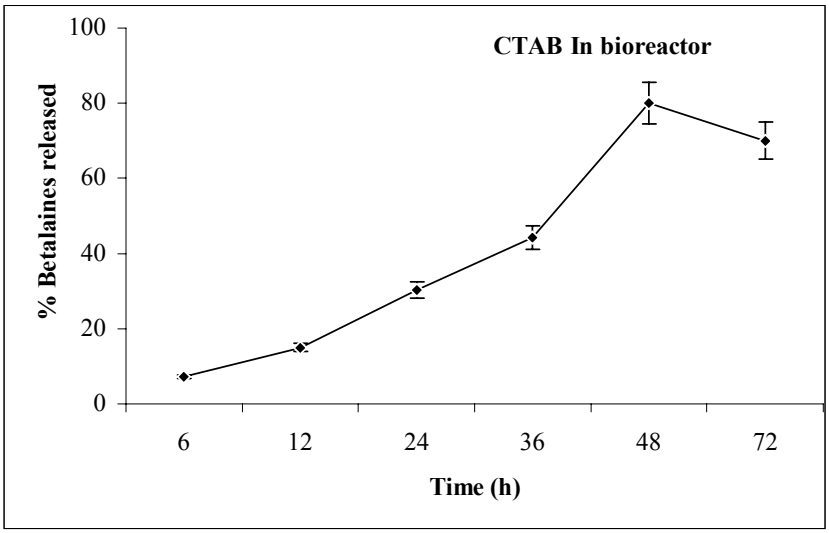


Figure 4.14. Pattern of pigment release from red beet hairy roots, grown in bubble column bioreactor, under the influence of 0.002% cetyl trimethylammonium bromide (CTAB). The result is the mean of three separate trials and the vertical bars represent SE of mean of six replicates.

4.3.1.3. Influence of biological agents

4.3.1.3.1. *Candida versatalis*

Addition of different levels of culture broth of *C. versatalis* (0.5 OD at 660ηm) though resulted in a dose-dependent release of pigment initially upto 24h, by the end of 48h all the treatments under static condition resulted in 90% pigment release (Fig. 4.15).

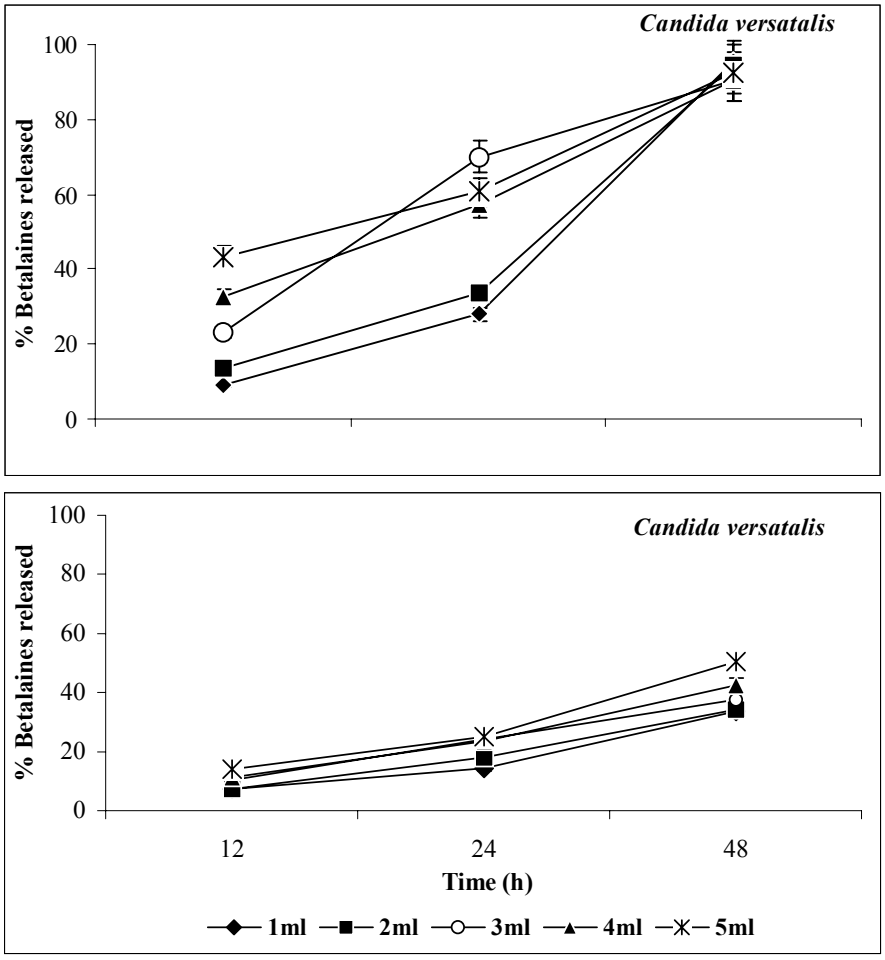


Figure 4.15. Pattern of pigment release from red beet hairy roots treated with different volumes of culture broth of *Candida versatalis* in both static and agitated culture conditions. The results are the mean values ± S.E of two independent experiments with six replicates in each.

However, in agitated cultures, a much lower level of about 50% release occurred in the highest dose, with other doses mainly causing 30 – 40% release by the end of 48th h (Fig. 4.15). Important observation here was the uniformity in the effect at 48th h static cultures where lower levels also resulted in 90% pigment release (Fig. 4.15), but not so in the case of agitated cultures. An exceptional value of about 70% release was observed (even upon repetition of the experiment) on 24th hour in treatment with 3ml of *C. versatalis* broth in static culture.

4.3.1.3.2. *Saccharomyces cereviseae*

Treatment with different levels of culture broth of *S. cereviseae* (0.5 OD), similar to that of *C. versatalis,* resulted in varied release of pigment with the highest of about 55% in case of highest dose (5ml) followed by about 40 and 30 in case of 4ml and 1ml treatments respectively with an apparently low effect at 3ml causing only about 25% of the total pigment release at the end of 24th h in static cultures only (Fig. 4.16). Subsequently a steady time dependent increase of pigment release in case of 5ml and 1ml only was evident where over 80% and 60% pigment release occurred in respective cases. Whereas, the treatments with 4ml and 2ml broth resulted in the release of about 50% of the total pigment by the end of 48th h with a further decline later. The cultures treated with 3ml broth released about 25% of the total pigment by the end of 24th h which remained constant throughout under static condition (Fig 4.16). In agitated cultures, the pattern of release of pigment with the above treatments was altogether different with no release by the end of 48th h. Further, about 35% of the pigment release was observed in case of the cultures treated with 5ml and 3ml broth and rest of the treatments resulted in the release of less than 30% of the total pigment by the end of 72nd h in case of cultures maintained under agitated condition.

4.3.1.3.3. *Lactobacillus helveticus*

Addition of different levels of culture broth of *L. helveticus*, as in other microbial treatments, resulted in a dose-dependent release of pigment in a manner almost similar to that with *C. versatalis* only in static cultures, with no any pigment release in agitated cultures even after 48h. However, the significant difference in the pattern of release in static cultures, when compared to that of *C. versatalis*, was the leaching of 90% of the pigment by the end of 24th h in 5ml with a narrow increase at a later stage (Fig. 4.17). The effects of lower concentrations of *L. helveticus* resulted in the same pattern of pigment release as observed in *C. versatalis*.

The biological agents have been tried for the first time for pigment recovery, with a good level of success. The food-grade microorganisms used in the present study were efficient in pigment release probably because they are known to synthesize bio-surfactants (Singh, 1994), in which case the latter would act as permeabilizing agents. Even in the case of biological agents the pigment release was higher in static cultures, indicating that under oxygen stress the hairy roots are highly vulnerable to agents of physical, chemical or biological origin and give away the membrane integrity. Both C. versatalis (Fig. 4.15) and

L. helveticus (Fig. 4.17) required only about 48h treatments to permeabilize beet cells releasing over 90% of the pigment into the medium. Whereas a long period was required for S. cereviseae to cause nearly the same amount of pigment release (Fig. 4.16) observed in other two treatments. In this case, in the agitated medium, there was absolutely no release of pigment until 48h indicating that beet hairy root cells are capable of evading certain microorganisms under well-aerated (congenial) conditions.

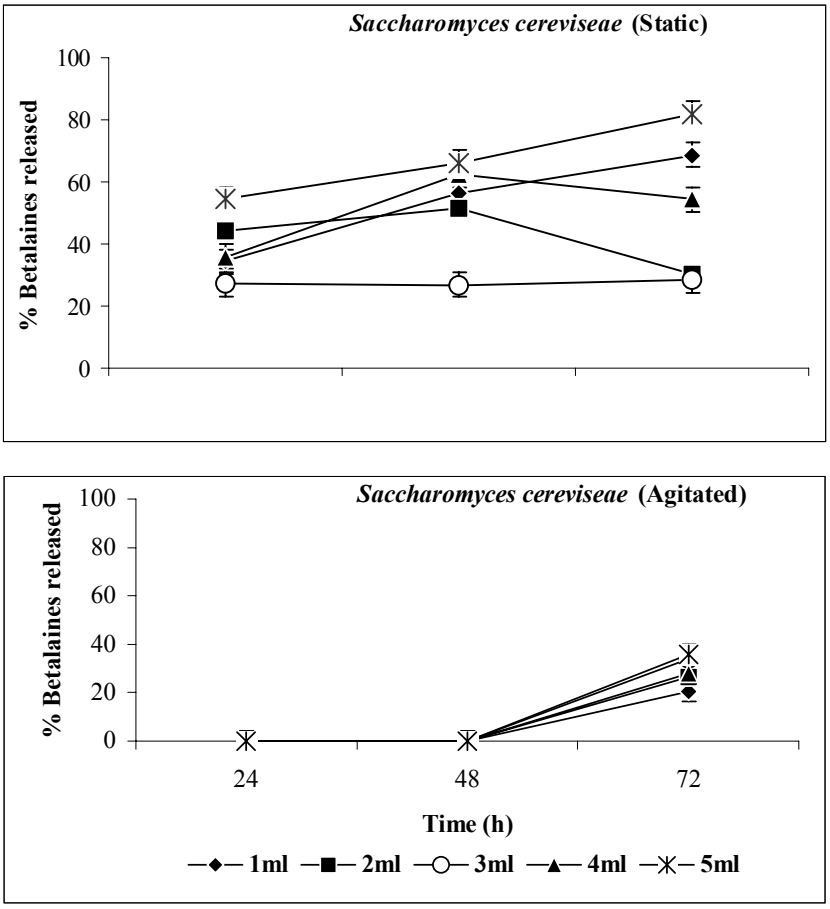


Figure 4.16. Pattern of pigment release from red beet hairy roots treated with different volumes of culture broth of *Saccharomyces cereviseae* in both static and agitated culture conditions. The results are the mean values ± S. E of two independent experiments with six replicates in each.

It should be noted that, the microbial cells also could multiply in the medium their application is restricted to batch cultivation. As these food grade microorganisms are well known nutraceuticals known to impart additional sensory properties to the food products, a new line of study for microbial products with natural pigment might emerge based on the present observations. For example, minimally processed fresh vegetable products such as

pickled vegetables where lactic acid is a component, one can impart natural red colour effluxed using L helveticus.

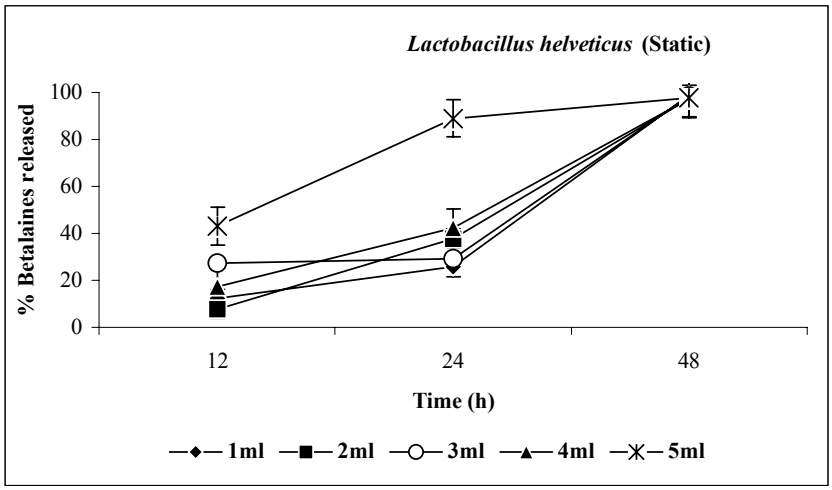


Figure 4.17. Pattern of pigment release from red beet hairy roots treated with different volumes of culture broth of *Lactobacillus helveticus* in static culture condition. The results are the mean values ± S. E of two independent experiments with six replicates in each.

4.3.1.3.4. Treatment with killed dry cells and cellular fractions of *L. helveticus*

Among the different fractions of *L. helveticus*, it was the free lipid fraction, obtained from medium supernatant, that exhibited rapid and efficient pigment release where 64% and 82% pigment was recorded in the medium by the end of 8th and 12th h respectively in static culture condition (Fig. 4.18). Subsequently, up to 24h, there was a narrow improvement in the pigment content of the medium without any indication of pigment degradation.

Other cell fractions were less efficient in inducing pigment release, where total insoluble carbohydrate fraction showed no cell permeabilizing property, the wall-bound lipid showed meager pigment release by 12th h onwards and the whole dry cell powder caused over 10% pigment release by the end 24h. The trend was almost similar for all the other fractions in both static and agitated conditions (Fig. 4.18). The free lipid fraction under agitated condition released about 47% and 60% of the pigment by the end of 8th and 12th h respectively (Fig. 4.18). Here the pigment release continued steadily accounting for 81% by the end of 24th h, which is nearly the same as recorded for 12h under static condition.

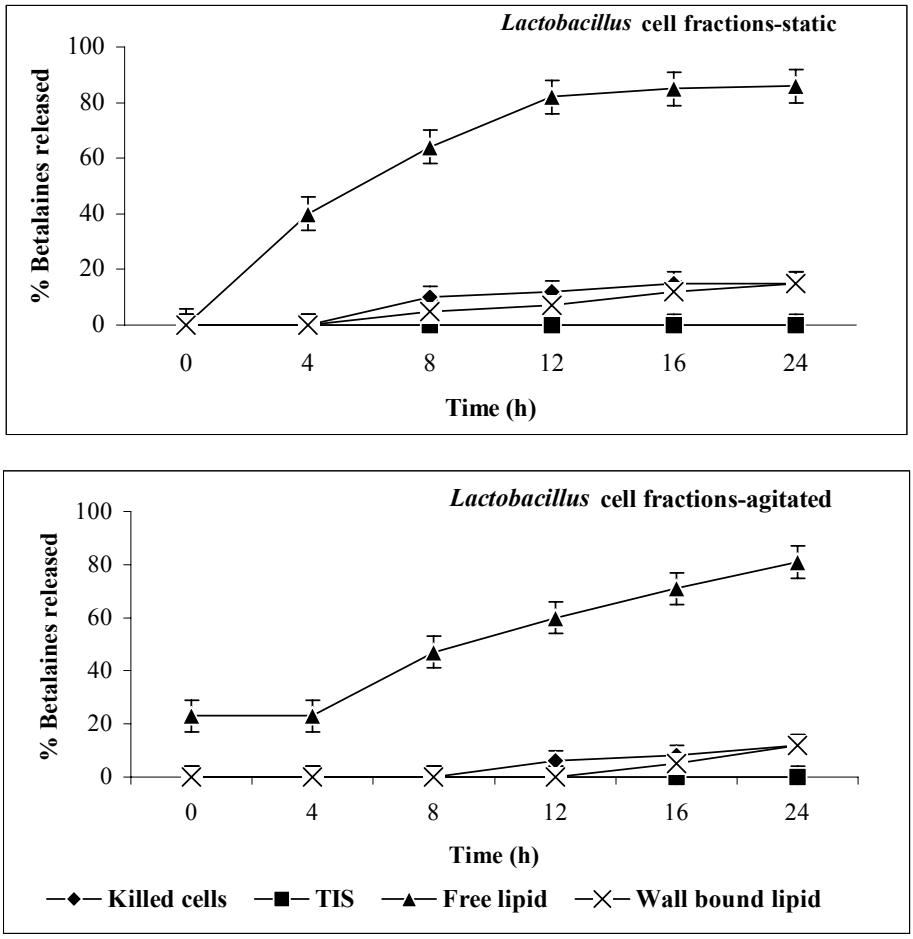


Figure 4.18. Pattern of pigment release from red beet hairy roots treated with *L. helveticus* cell fractions such as killed cells, total insoluble sugars (TIS), free lipid and cell wall bound lipid under static and agitated culture conditions. The results are the mean values ± S. E of two independent experiments with six replicates in each.

4.3.1.3.5. Lipid-mediated pigment release in the bioreactor

Addition of free lipid fraction of *L. helveticus* into the bubble column reactor resulted in time bound pigment release. A highest amount of pigment release being 84%, observed by the 12th h of treatment, preceded by about 50 and 19% pigment release after 8 and 4 h of treatment respectively. No loss of pigment was observed upto 24h after the highest release on the 12th h (Fig. 4.19).

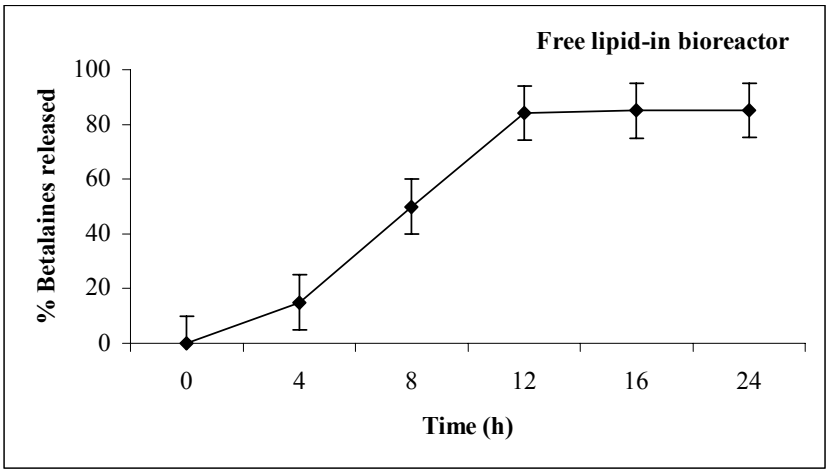


Figure 4.19. Pattern of pigment release under the influence of free lipid fraction of *L. helveticus* from red beet hairy roots grown in the bubble column bioreactor. The result is the mean of two separate trials.

4.3.1.4. Viability of the treated cultures

Viability of permeabilized hairy roots was checked both by plating the permeabilized cultures on basal MS solid medium or by transferring to the liquid medium. While higher levels of Tween – 80 and Triton X-100 affected the viability; the roots were viable at all levels of CTAB both in plating test as well as in the bioreactor. The hairy roots permeabilized using cell components of *L helveticus* showed good viability, both in plating test as well as those in bubble column bioreactor, the latter treated for 6h using lipid fraction of *L. helveticus* bioreactor, replacing the permeabilized cultures with fresh medium was also tried. Table 4.1 summarizes the observation on the viability recorded as 'yes' for growth of fresh root tips from permeabilized cultures. The root tips of the effluxed cultures treated with lowest concentrations of triton-x-100 (0.05%), CTAB (0.0005%) and free lipid surfactants had almost similar texture as that of the control when subjected for scanning electron microscopy (Fig 4.20) which further justifies the viability of the cultures.

Table 4.1. Viability of beet hairy roots treated with different effluxing agents

Treatment	Concentration (% w/v)	Duration (h)	Viability
Oxygen stress	*	48	Yes
Tween-80	0.05	48	Yes
	0.10		Yes
	0.15		Yes
	0.20		No
Triton X-100	0.05	36	Yes
	0.10		Yes
	1.15		Yes
	0.20		No
	0.30		No
CTAB	0.0005	50	Yes
	0.001		Yes
	0.01		Yes
	0.05		Yes
	0.10		Yes
Fractions of *L. helveticus*			
Dry cell powder	0.1	12	Yes
Free lipid	variable	-	Yes
Bound lipid	variable	-	Yes
Total carbohydrate	0.1	-	Yes
Free lipid in bio-reactor	variable	6	Yes

* Oxygen stress was imposed by cessation of gyration

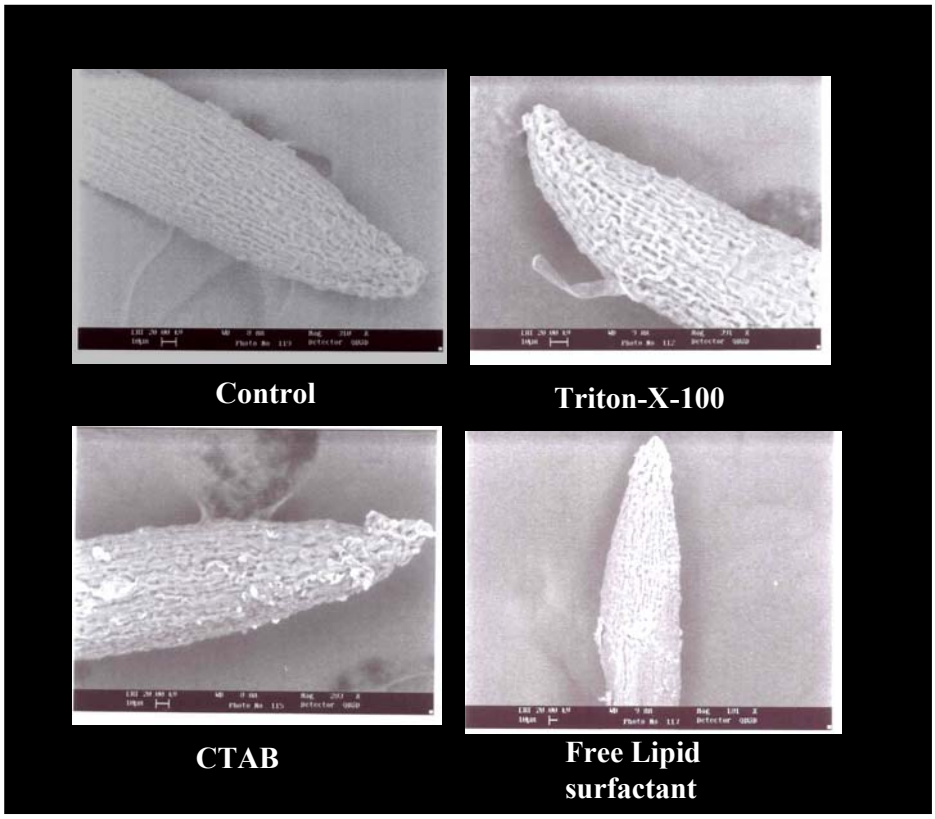


Figure 4.20. Scanning electron photomicrographs of the hairy root cultures treated with lowest concentrations of Triton-X-100 (0.05%), CTAB (0.0005%) and freelipid fraction of *L. helveticus*.

4.3.1.5. Repeated recovery of pigment under the influence of CTAB

Repeated effluxing using CTAB for increasing the productivity was assessed. It was found that beet hairy roots effluxed repeatedly thrice, twice and once resulted in the yield of 4, 3.6, and 1.26 fold higher betalaines respectively, when compared to control. Treatment with additional calcium resulted in a narrow improvement in viability, leading to 4.3, 5.32, and 2.55 fold higher pigment yield in thrice twice and once effluxed cultures respectively (Fig 4.21). The results presented in the figure 4.21 clearly indicate that the cultures effluxed for twice when treated with additional calcium resulted in the total yield 5-fold higher pigment. This increase in pigment yield may be because of the role of calcium in cell wall repair and thus re-growth of hairy roots and further synthesis of pigments. The role of calcium in repair of membrane well-known, the sonicated cell suspension cultures of carrot

were subjected for repeated effluxing after treatment with calcium (Kilby and Hunter, 1990; 1991; Dilorio et al, 1993).

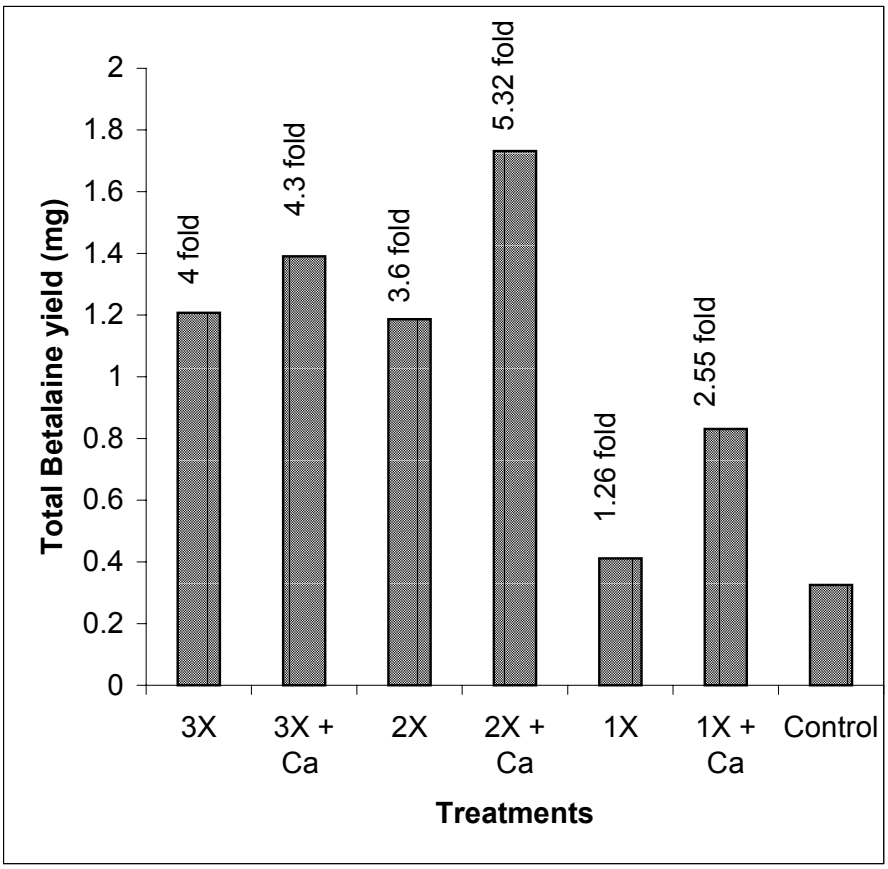


Figure 4.21. Total yield of betalaines from the hairy root cultures subjected for repeated effluxing using CTAB (0.005% w/v). The cultures grown for 20 days were subjected for repeated effluxing up to three times (1X, 2X, 3X) at an interval of one-week period. A set of effluxed cultures was also treated with additional calcium (3-folds that present in MS medium), to study the improvement in re-growth and pigment synthesis. The values are average of six replicates.

4.3.1.6. Influence of calcium channel modulators on the release of pigments

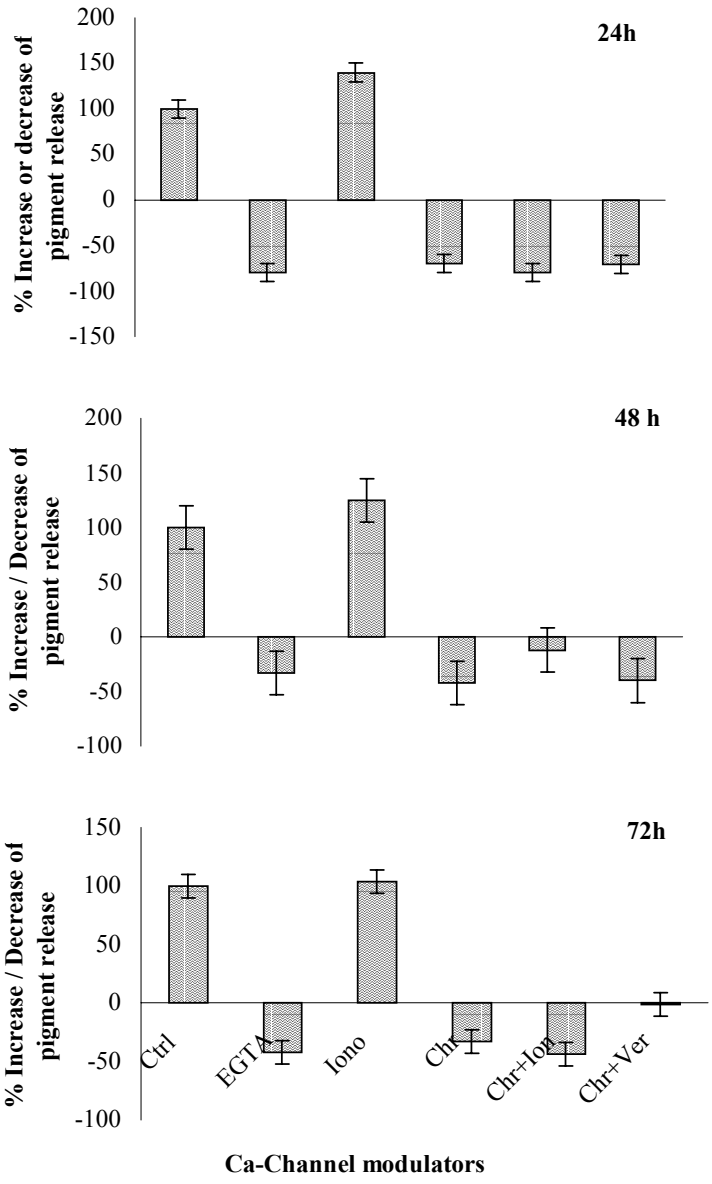


Figure 4.22. Influence of calcium channel modulators on Triton-X-100 mediated pigment release from hairy root cultures. 20 day old hairy root cultures were treated with various calcium channel modulators such as EGTA (Ethylene glycol tetraacetic acid), Ionophore A23187 (Ion), Chlorpromazine (Chr) and verapamil (Ver) were subjected for effluxing using Triton-X-100 after 24, 48 and 72 h. The pigment released was monitored, quantified and expressed as 5 increase or decrease of pigment released when compared to control cultures not treated with Ca^{2+}- channel modulators but subjected for effluxing. The values are the average of six replicates and the vertical bars indicate (± SE) standard error of mean.

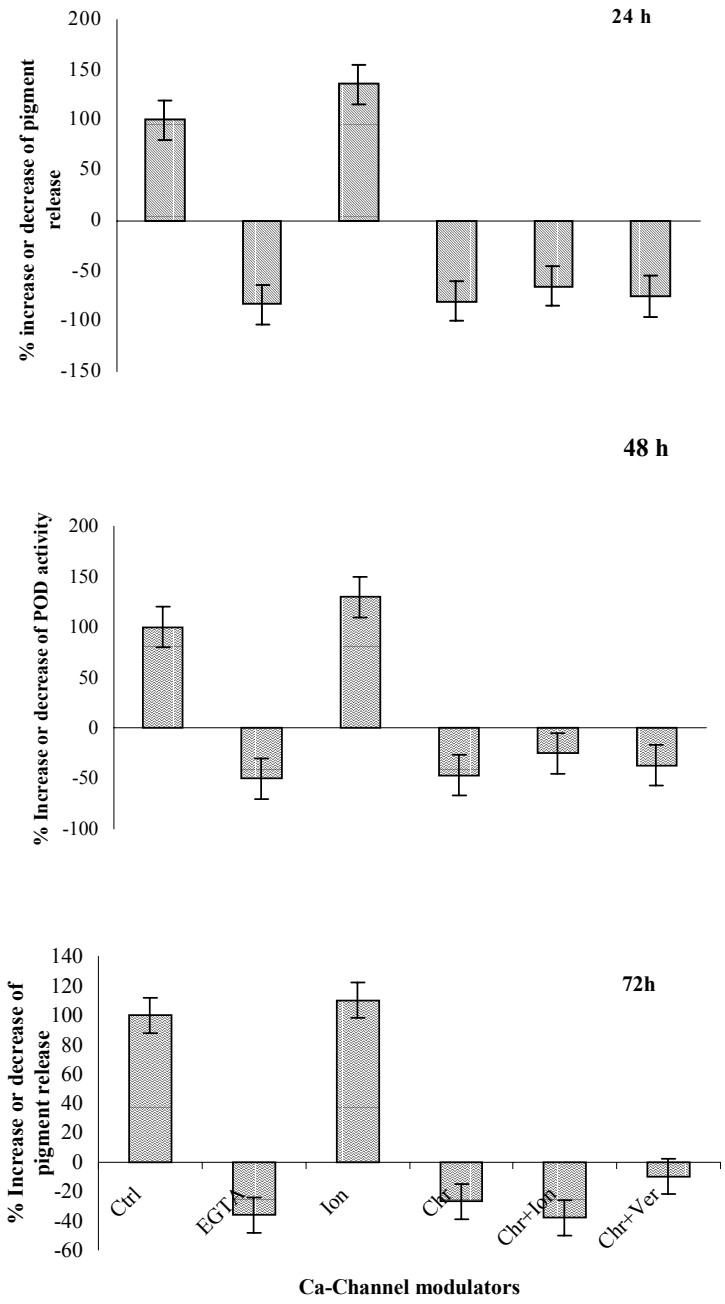


Figure 4.23. Influence of calcium channel modulator on CTAB mediated pigment release from hairy root cultures. 20 day old hairy root cultures were treated with various calcium channel modulators such as EGTA (Ethylene glycol tetraacetic acid), Ionophore A23187 (Ion), Chlorpromazine (Chr) and verapamil (Ver) were subjected for effluxing using CTAB after 24, 48 and 72 h. The pigment released was monitored, quantified and expressed as 5 increase or decrease of pigment released when compared to control cultures not treated with Ca^{2+}- channel modulators but subjected for effluxing. The values are the average of six replicates and the vertical bars indicate (± SE) standard error of mean.

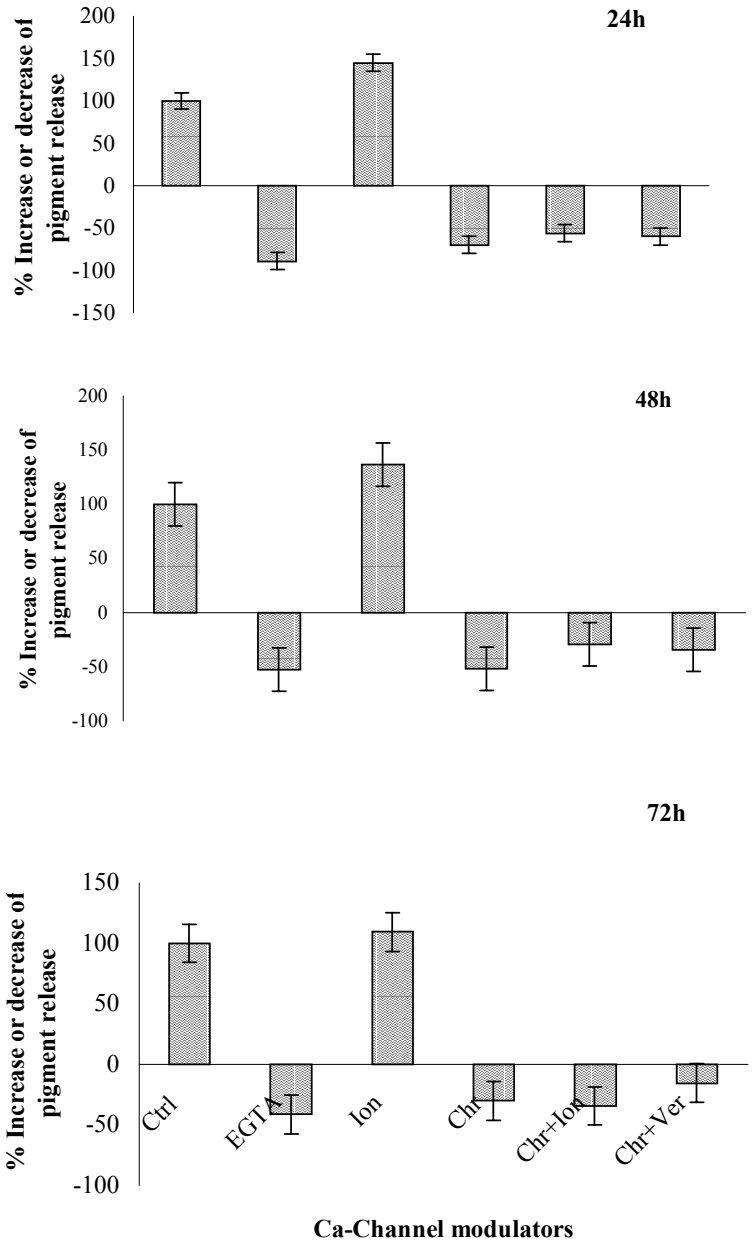


Figure 4.24. Influence of Calcium channel modulators on free lipid surfactant mediated pigment release from hairy root cultures. 20 day old hairy root cultures were treated with various calcium channel modulators such as EGTA (Ethylene glycol tetraacetic acid), Ionophore A23187 (Ion), Chlorpromazine (Chr) and verapamil (Ver) were subjected for effluxing using freelipid fraction of *L. helveticus* after 24, 48 and 72 h. The pigment released was monitored, quantified and expressed as 5 increase or decrease of pigment released when compared to control cultures not treated with Ca^{2+}-channel modulators but subjected for effluxing. The values are the average of six replicates and the vertical bars indicate (± SE) standard error of mean.

The results presented in the figures 4.22-4.24 show that the most of the Ca^{2+}-channel modulators are effective when subjected for effluxing after 24h. When effluxed using

Triton-X-100 (0,005% W/V) the EGTA (calcium chelator) appeared to suppress about 80%, 33%, 42% of the total pigment released after 24, 48 and 72h respectively after treatment. The calcium channel enhancer Ionophore A23187 caused an increase in pigment release accounting to about 40%, 25% and 4% as compared to control after 24, 48 and 72h of treatment. The treatment with calmodulin inhibitor the chlorpromazine resulted in the suppression of pigment release by about 70%, 42% and 35% after 24, 48 and 72h of treatment respectively. The effect of chlorpromazine was more pronounced when the same was used along with the ionophore especially at 24h. **A similar trend was observed with the cultures treated with chlorpromazine along with verapamil a calcium channel blocker (Fig 4.22).**

The CTAB (0.005%) mediated pattern of pigment release studied in presence of calcium channel also showed treatment dependent response i.e., suppression of pigment release in the presence of calcium channel and calmodulin antagonists while enhancing the pigment release in the presence of calcium channel enhancer. Similar to Triton-X-100 mediated pigment release most of the calcium channel modulators appeared to be most effective at 24h of treatment (Fig 4.23).

Compared to Triton-X-100 and CTAB, the calcium channel modulators had more influence on the free lipid surfactant mediated pigment release. EGTA caused more suppression of pigment release causing about 90%, 52% and 41% decrease in pigment release compared to control cultures (not treated with EGTA). The ionophore A23187, similar to EGTA caused highest enhancement of pigment release compared to its effect on Triton-X-100 and CTAB causing 45%, 36.4% and 9.4% increased pigment release (Fig 4.24). Whereas the effect of other calcium and calmodulin antogonists had similar effect as that of the Triton-X-100 and CTAB mediated pigment release. All in all, the results showed various levels of involvement of calcium-calmodulin mediated signaling processes in chemical and free lipid surfactant mediated pigment release from the hairy root cultures of red beet. Involvement of Ca^{2+} channel in chemical and biosurfactant mediated effluxing of betalaines has been demonstrated for the first time in the present study. However in the plant cell cultures such studies have been very well documented in different processes especially in defense signaling during host pathogen interactions. Strong evidence for the role of Ca^{2+} has been demonstrated in cowpea epidermal cell death after challenging with the fungus *Uromyces*, which was further hypothesized because of the diffusion of peptide elicitors from the invading fungi (Gelli et al, 1997). The Ca^{2+} involvement was observed in triggering the defense responses when plant cell suspensions were treated with microbes or

elicitors (Levine et al. 1996; Atkinson et al. 1996; Jabs et al. 1997; Zimmerman et al. 1997; Somssich and Hahlbrock 1998; Piedras et al. 1998). The similar mechanisms may be operating when the beet hairy root cultures were treated with the permeabilizing agents at very low concentrations. This was more evident when the cultures were treated with free lipid surfactants as the effect of Ca^{2+} channel modulators was more pronounced.

4.3.2. Adsorption and recovery of betalaines

4.3.2.1. *In situ* adsorption.

Both the pigments (betaxanthine and betacyanine) being the derivatives of betalamic acid, exhibit similar properties except for their adsorption maxima and absence of glycosidic group in betaxanthine (Von Elbe, 1979). Among the different individual adsorbents tried without methanol pre-treatment (Table 4.2), alumina showed maximum adsorption of 68.2%, of which only about 22% could be desorbed from the adsorbent ultimately resulting in the recovery of about 15% of the total pigment that is loaded / effluxed. Similarly, silica gel alone showed 22.6% adsorption of which though 77% desorbed into acidified water, the overall pigment recovery of the unit operation of *in situ* adsorption was only 17.45%. When alumina and silica were mixed in equal ratio, adsorption percentage of betalaines increased significantly showing 97% adsorption resulting in a recovery of 71.4% although the desorption was only 73.6% in this case. XAD-2 and XAD-4 were poor adsorbents though vastly used by other workers for plant secondary metabolites (Payne and Schuler 1988). Both cornstarch and dextrin white showed almost similar adsorption capacities (about 36%) with dextrin white being more amenable for desorption leading to a recovery of 43.5%. In general, the materials which had poor adsorption capacity invariably showed high desorption property indicative of low surface energy / adsorption isotherms for betalaines. Maltodextrin, which is extensively used for encapsulation of natural pigments (Choi et al. 2001), was less efficient than cornstarch and dextrin white. Cyclodextrin, with its cyclic arrangement of dextrin molecules is generally capable of holding a vast number of natural flavour and pigment molecules (Furuya and Yoshi 2000), was a poor adsorbent of betalaines, capable of holding only 17.1% of the pigment from the medium. These observations clearly indicate the involvement of specific interactions between adsorbent and adsorbate where the net energy plays an important role rather than contour of adsorbent particles as has also been recorded by other such studies (Payne and Schuler 1988; Komaraiah et al. 2002).

Table 4.2. Efficacy of different adsorbents for *in situ* adsorption and recovery of betalaines*.

Adsorbent**	% Adsorption	% Desorption	% Recovery of total betalain
Silica gel	22.60	77.20	17.45
Silica: Alumina (1:1)	97.00	73.60	71.39
Alumina	68.20	21.80	15.00
Wheat starch	20.50	75.01	15.38
Corn starch	36.40	35.20	12.80
Maltodextrin	30.30	6.80	2.06
Cyclodextrin	17.90	28.60	5.12
XAD-2	0.00	0.00	0.00
XAD-4	6.70	100.00	6.70
Dextrin white	35.40	43.50	15.40

*** In 25ml medium containing equilibrium concentration of 0.13mg ml^{-1} betalain**

**** Total weight of adsorbent in each case = 3g.**

The adsorbents are sometimes pre-treated with methanol before use (Sim and Chang 1993; 1997; Payne and Schuler 1988), as it is well known that such a treatment cause changes in adsorption energy (Snyder, 1968). When the above adsorbents were pre-treated with methanol, there was a significant improvement in adsorption in case of alumina and silica (Table 4.3) where alumina showed a very high adsorption of about 97%. Similarly, the nonionic resins XAD-2 and XAD-4 that adsorbed negligible quantities of pigment before pretreatment, adsorbed substantial levels of about 46% and 42% respectively, upon methanol pre-treatment. However, with the increase in adsorption capacities of these materials, there was concomitant reductions in pigment recovery during desorption process leading to a poor yield of betalaines after desorption. All in all, silica gel, wheat starch and corn starch that were capable of moderate adsorption and desorption, did not change much even after

methanol pre-treatment. In general, this primary screening experiment clearly indicated that alumina, silica and the mixture of both without any pretreatment were useful for *in situ* recovery of betalaines effluxed into the medium from hairy root cultures of red beet.

Table 4.3. Adsorption of betalaines* *in situ* using different adsorbents pretreated with methanol.

Adsorbent**	% Adsorption	% Desorption	% Recovery of total betalain
Silica gel	26.36	47.10	12.42
Silica:Alumina (1:1)	79.00	27.50	21.73
Alumina	97.20	0.60	0.58
Wheat starch	32.72	44.40	14.53
Corn starch	21.80	58.30	12.71
Maltodextrin	31.80	4.14	1.32
Cyclodextrin	33.60	14.90	5.01
XAD-2	45.90	0.77	0.35
XAD-4	42.20	0.73	0.31
Dextrin white	26.80	47.40	12.70

*** In 25ml medium containing equilibrium concentration of 0.13mg ml^{-1} betalaine**

**** Total weight of adsorbent in each case = 3g.**

Based on these observations, the three adsorbents were selected and further work on the effect of the time `*t*' was established, as the `*t*' value is needed to accomplish complete adsorption of a particular concentration of solute from the medium (Doran, 1995). Two grams each of the three adsorbents were kept in bags and introduced into a solution of betalaines 2.02mg 5ml^{-1} and incubated on a gyratory shaker (100rpm). The pigment remaining in the medium was recorded after every 10 minutes for a total period of 70 minutes. The observations presented in Fig. 4.25 shows that, irrespective of the adsorbent, maximum adsorption occurred within the first 10 minutes and was completed within 20 minutes with no net adsorption later, as was evident by the constant level of pigment in the solution after 10min. This being similar for silica and alumina used in other studies (Snyder,

1968), only activated carbon was observed to exhibit higher '*t*' values of 75min (Shekina et al. 2002) to 800 h (Nilsson, 1970).

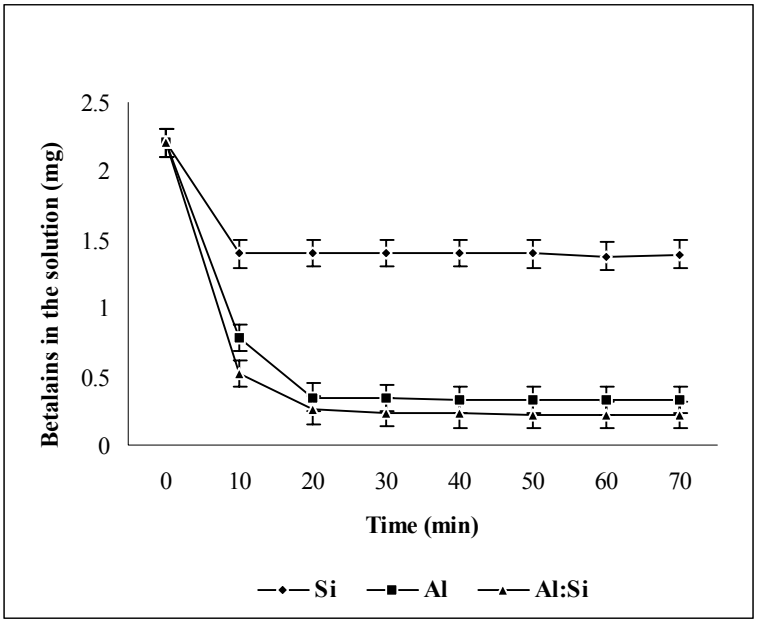


Figure 4.25. Time course of betalaine adsorption by different adsorbents where the data represents the amount of betalain remaining in the medium, which is an average of six replicates.

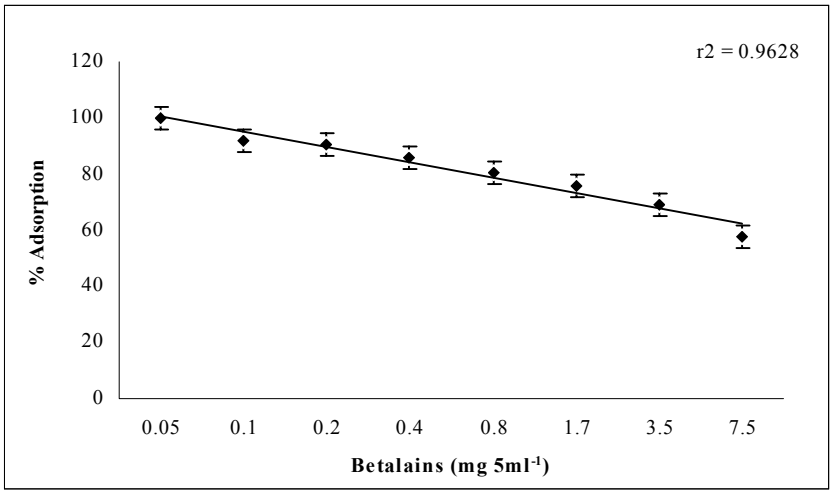


Figure 4.26. Effect of different equilibrium concentration of betalaine as a function of adsorption process by Al:Si (1:1). Data is the mean of seven replicates.

Once the adsorption time was known, the effect of solute concentration on the adsorption process was found out using the mixture of silica and alumina in a similar experimental model. Two grams of adsorbent was added to 5ml medium containing different concentrations of pigment and incubated on a gyratory shaker for 30min. Here, as expected,

maximum adsorption occurred at the least solute concentration with about 60% getting adsorbed at a very high solute level (Fig. 4.26).

Assuming that complete *in situ* adsorption occurs at the least pigment concentration, the adsorbent required to remove different equilibrium concentrations of pigment was calculated and presented as the `*q*' value using equation (1). As shown in Fig. 4.27, the predicted values (curve) matched well with the experimental values (dots) with a constant `*q*' value of 0.17mg g^{-1} solute. By applying the same formula (1), one can also predict the effect of volume where the adsorbent required linearly increases with the increase in dilution of pigment in the medium. However, to check whether the adsorption process recorded here follows the standard adsorption phenomenon such as *Langmuir isotherm* (Doran, 1995), the following equation was applied and the adsorption equilibrium was calculated.

$$\frac{C_e}{q_e} = \frac{1}{Q_o b} + \frac{C_e}{Q_o} \qquad (3),$$

where C_e is the equilibrium concentration (mg ml^{-1}) and q_e is the amount adsorbed (mg g^{-1}) at equilibrium. Q_o and b are *Langmuir's* constants related to adsorption capacity and energy of adsorption respectively (obtained by plotting x/m and log x/m against different equilibrium concentrations). The plots of C_e/q_e Vs C_e, though initially linear for the selected adsorbent, the overall adsorption follows *Langmuir isotherm* model for betalain adsorption (Fig. 4.28). The values of Q_o and b were found to be 0.174mg g^{-1} and 0.9, respectively which were calculated from the slope and intercept of *Langmuir* plot.

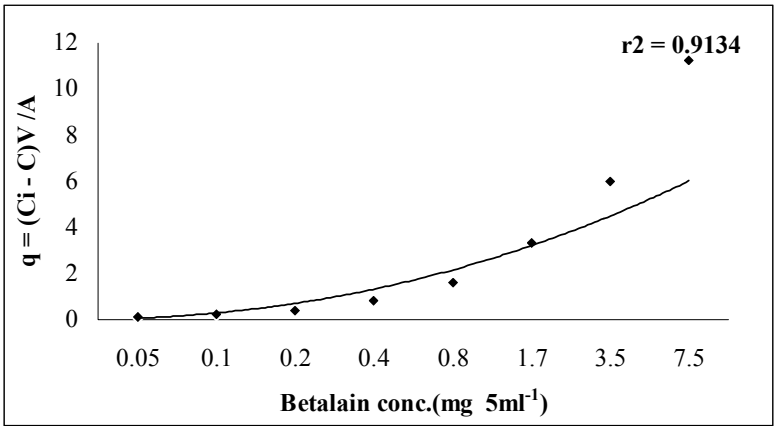


Figure 4.27. Quantity of adsorbent (q) required for complete removal of different equilibrium pigment concentrations. Curve shows the predicted value and the dots represent experimental value of at least six replicates.

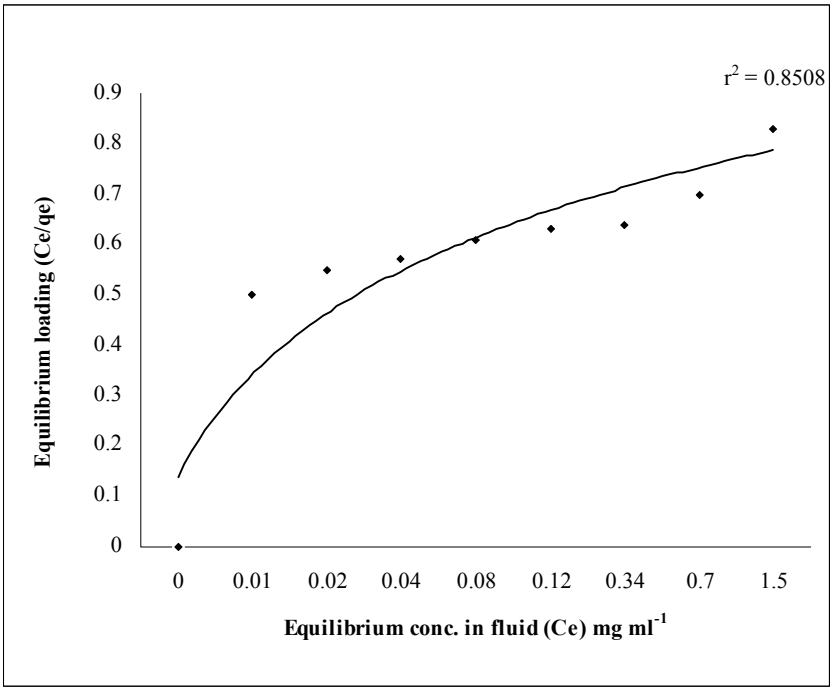


Figure 4.28. Langmuir plot for betalaine adsorption. The curve represents predicted value and the dots represent experimental data of six replicates.

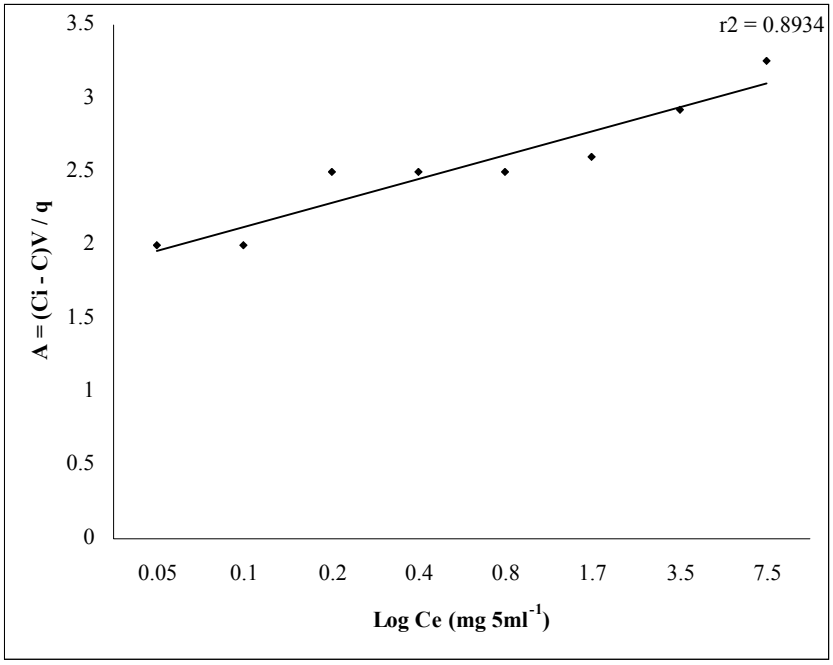


Figure 4.29. Plot showing experimental and calculated values of the amount of adsorbent required to remove different equilibrium concentrations of betalaine. The line shows predicted value and the dots represent experimental data of the mean of ten replicates.

Based on the above properties, the quantity of adsorbent required to recover different levels of betalaines using silica: alumna mixture was found by plotting $(C_i - C)$ V/q versus equilibrium concentration of betalaines. Fig. 4.29 shows the linearity of this relationship with the calculated values (line) correlating with the experimental values (dots). All in all, the observations made in the present study for *in situ* recovery of betalaines are new and very useful for rapid recovery of pigments that are leached into the medium.

Table 4.4. Adsorption and recovery of betalaines* in a column (*ex situ*) containing different adsorbents having same geometry.**

Column material	% Adsorption	% Recovery***
Amberlite	0	0
Alumina	96	0
Alumina: Silica (2:1)	94	47
Alumina: Silica (1:1)	100	62
Alumina: Silica (3:2)	100	55
Alumina: Silica (7:3)	100	54
Silica gel	100	70
Alumina: Sand (2:1)	97	84
XAD-2	0	0
XAD-4	0	0

*** 24.4 mg of total pigment in 15 ml was loaded.**
**** Column containing adsorbent occupying 3.5 cm height and 2.0 cm internal diameter.**
***** Recovered by eluting the adsorbed pigment using 2% (v/v) aqueous HCl and the pH after elution was immediately adjusted with 1% NaOH to 5.0 before quantifying the pigment concentration by spectrophotometry.**

4.3.2.2. *Ex situ* adsorption and recovery

Extensive studies have been conducted on the recovery of pigments by passing through adsorption columns where, pigment passes through a series of adsorption plates leading to an array of interactions taking place between the adsorption material, pigment and

the solvent (Snyder, 1968). In such cases column geometry plays a significant role. In the present study, keeping the column geometry constant having 3.5cm long and 2cm inner diameter (=11ml), an initial screening was done for different adsorbents as listed in Table 4.4. The resins Amberlite XAD-2 and XAD-4 did not show any adsorption, as the optical density of the effluent was same as that of the loaded sample. All other adsorbents showed various levels of adsorption where alumina alone showed 96% adsorption with no desorption occurring when eluted with 2% HCl water. However, when used in combination with other vastly used adsorbents such as silica and activated sand, the adsorption property of alumina changed. The low level of 94% adsorption was of that of alumina: silica at a ratio of 2: 1 respectively. A similar ratio of alumina with sand showed 97% adsorption and a highest level of 84% desorption. Other alumina: silica combinations, though showed complete adsorption (100%), desorption was low (Table 4.4).

Table 4.5. Solvent gradient developed using ethanol and HCl for elution of betalaines from the column.

Ethanol (%)	Acid (HCl) (%)
10	0.5
20	0.5
30	0.5
40	0.5
50	1.5
60	2.0
70	3.0
80	3.5
90	4.0
100	5.0

For many practical and industrial applications, it becomes necessary to select the best adsorbent as well as the column geometry for a given set of conditions. Column geometry is known to influence adsorption / desorption properties of the solutes. The observations listed in table 4.6 indicate that neither adsorption nor desorption/elution were dependent on volume of adsorbent, instead, the column geometry played a crucial role. While the percent adsorption was not significantly affected, the elution time and elution volume varied. Small columns of 4.7cm^3 and 6.28cm^3 of adsorbent volume were found ideal having best adsorption coupled with early product recovery in low volumes of eluent (Table 4.6). Since 2cm x 2cm column caused 99% adsorption with 70% elution in less eluent volume in 60min, this column was selected for further experiments aimed at improving the desorption of the solute. Of the different types of elution solvents used, as shown in Table 4.7, the solvent number 5, which is a gradient mixture of ethanol and HCl resulted in 100% recovery of the solute in less elution volume and elution time (1h).

Table 4.6. Effect of column geometry on *ex situ* adsorption of betalaines* and their recovery using alumina: sand (2:1) as adsorbent and 2% HCl as eluent.

Column size			**% Adsorption**	**% Recovery**	**Eluent vol (ml)**	Elution time **(min)**
Length (cm)	**Dia (cm)**	**Vol. of adsorbent (cm^3)**				
2.0	2	6.28	99.0	70.00	70	60
3.5	2	11.03	97.0	84.00	100	60
6.0	1	4.71	99.3	27.15	80	60
6.0	2	18.84	98.7	31.30	110	45
12.0	1	9.42	97.0	85.70	100	180

*

24.4mg of total pigment was loaded. Elution and pigment quantification were as mentioned for Table 4.4.

Table 4.7 Effect of different elution solvents on recovery of betalaines* from column**

% Adsorption	% Recovery	Elution solvent***	Eluent vol (ml)	Elution time
99.00	70.0	1	70	1.0h
99.86	69.0	2	65	1.5h
99.30	81.0	3	60	1.5h
99.20	44.8	4	65	1h 40 min
98.50	100.0	5	50	1.0h

*** 24.4mg of total pigment was loaded**
**** Column had adsorbent Alumina: Sand (2:1) occupying 2 cm length and 2 cm diameter of internal space.**

*****1. 2% HCl**
2. Ethanol gradient with 2% HCl
3. Fractions 1-10 Ethanol gradient with 2% HCl, Fractions 11-14 Ethanol gradient with 4% HCl, Fractions 15-20 Ethanol gradient with 5% HCl
4. Ethanol gradient with 0.5-5% HCl
5. Fractions 1-9 Ethanol gradient with 0.5% HCl; Fractions 10-20 Ethanol gradient with 1.5-5% HCl
For ethanol gradient details, see Table 4.5.

In addition to fixed parameters, it also becomes necessary to predict the column performance under changed process conditions. A comparison of theoretical values with the available experimental values, followed by simulations makes such predictions possible (Doran, 1995). Therefore, in the present study the column capacity for highest pigment adsorption was found out. Generally, the column is said to have reached a state of adsorption equilibrium with no any adsorption energy remaining at the surface of the adsorbent. This starts happening sequentially from top to bottom of the column ultimately resulting in "adsorption breakthrough" where the concentration of pigment at inlet as well as at outlet remains constant. The time point when the effluent optical density starts steeply raising, the column is said to have reached a saturation point or the break point. The

experimental model used in the present study has been explained for calculating the breakthrough points. An equilibrium concentration of pigment ($C_e = C_i = 0.18$mg ml^{-1}) was loaded onto the fixed bed column where several variables such as liquid velocity, pigment concentration at the mobile phase (C_m) and bed length, i.e., the distance traveled by the pigment extract were considered at different feed flow rates and the solute concentration at the outlet was recorded at fixed time intervals. Results compiled in Figs 4.30– 4.38 indicate that the flow patterns followed sigmoid curves and reached plateau immediately after the breakthrough point. At any constant equilibrium concentration the very slow flow rate (0.02ml sec^{-1}) revealed breakthrough at 100min whereas a high flow rate resulted in early breakthrough. To properly design and operate fix-bed adsorption processes, the adsorption capacity, i. e., the breakthrough curves must be known so that about 50% of the breakthrough value can be considered as the column adsorption capacity. An understanding of the equilibrium relationship that determine the extent to which material can be adsorbed onto a particular surface will help quantify the adsorbent required for a specific unit operation of separation. When an adsorbent and adsorbate (betalaines) are at equilibrium, there occurs a defined distribution of betalain-pigments between solid and fluid phases and no further net adsorption occurs and hence, the system can be considered to have reached an equilibrium status. Several adsorption equilibrium data for various inorganic / organic compounds are available as adsorption isotherms (Choi et al. 2001). However, no such data is available for natural pigments such as betalaines. In the present study where alumina-sand column was used as the stationary phase, the isotherm of only alumina was considered as sand having zero adsorption was used only for improving the flow rate. The constant relationship 'k' between the quantum of pigment adsorbed onto alumina that had been calculated earlier for in situ ($r^2 = 0.9$) was almost similar for *ex situ* column adsorption of different flow rates (Fig. 4.30-4.32). Here, increase in flow rate resulted in reduction of x/m value. This behaviour of liquid-solid adsorption is different from gas-solid adsorption where, in the latter, increase in pressure (flow rate) generally causes increase of x/m value. For normal flow rate (0.02ml sec^{-1}), using equation (2), Q_S and $Q_{1/2}$ were calculated, for which the flow details and elution pattern are plotted in Fig. 4.30. $Q_S = C_i - C_s/X$ and $Q_{1/2} = C_{i/2} – C_{1/2} / X$ which are 16.9mg g^{-1} and 8.86mg g^{-1} respectively.

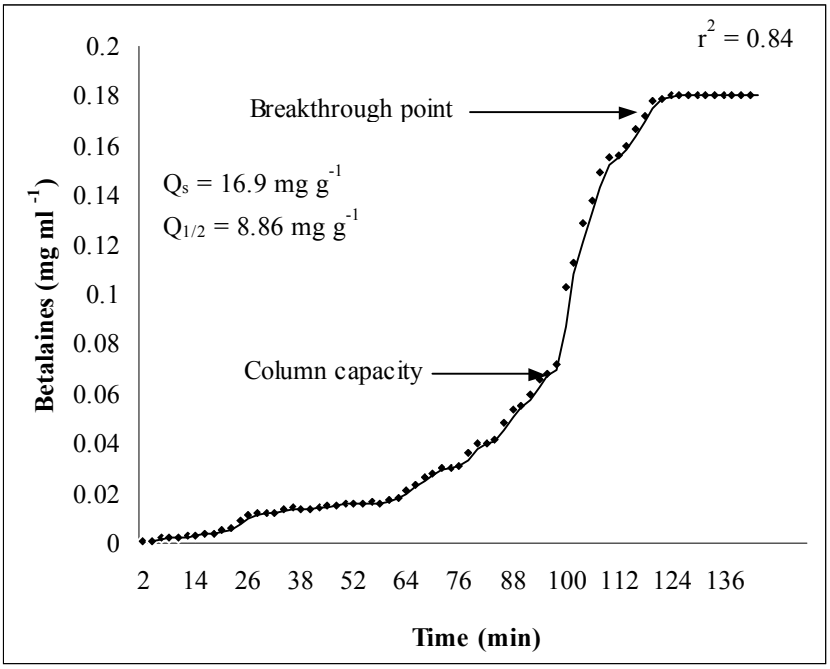


Figure 4.30. Breakthrough curve for betalaine adsorption at normal flow rate (0.02ml sec^{-1}), dots represent experimental values and the line shows the calculated values. The data is the average of three experiments.

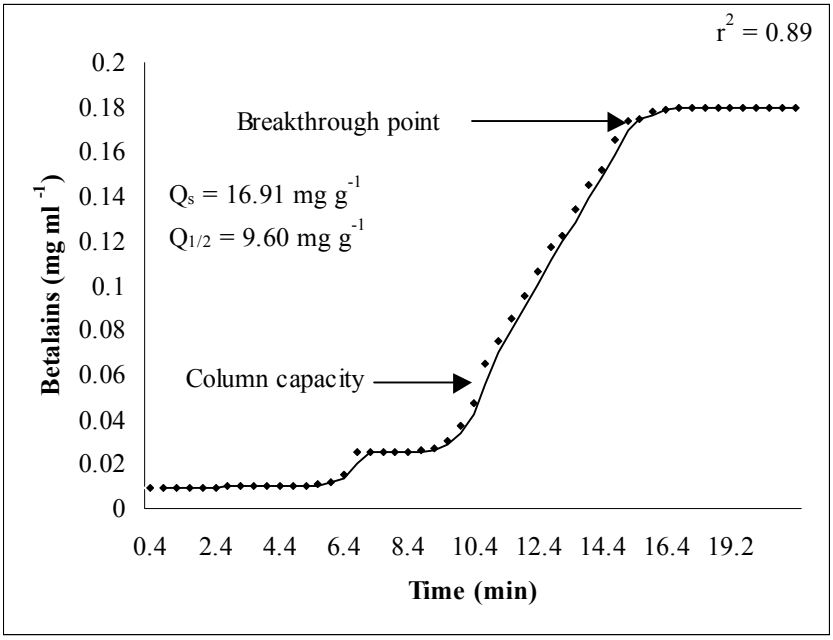


Figure 4.31. Breakthrough curve for betalaine adsorption at moderate flow rate (0.3ml sec^{-1}) where dots represent experimental values and the line shows the calculated values. The data is the average of three experiments.

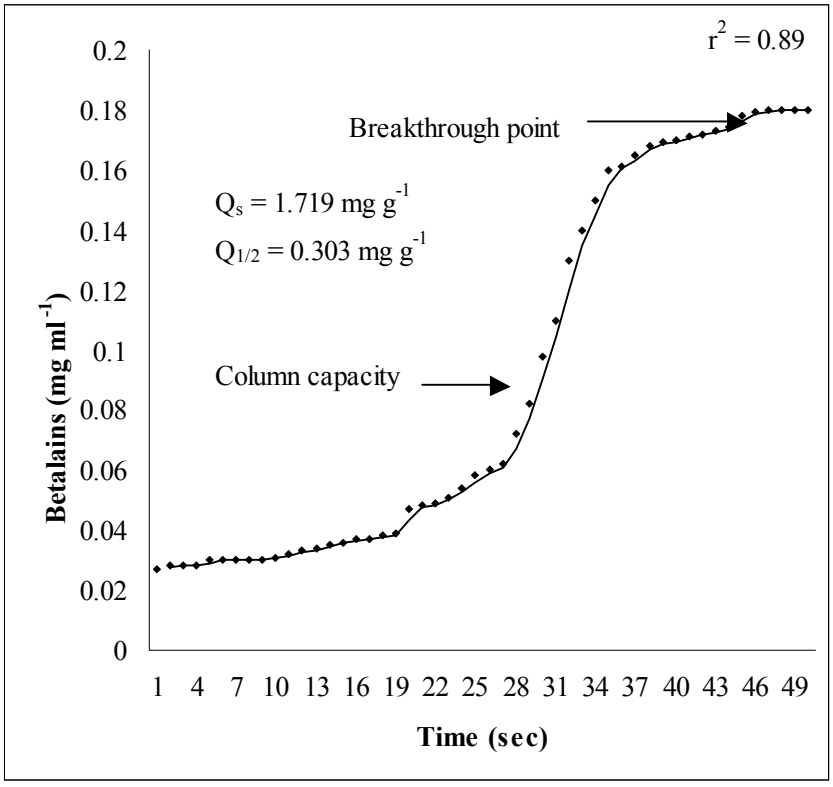


Figure 4.32. Breakthrough curve for betalaine adsorption at a flow rate of 3.1ml sec^{-1} where dots represent experimental values and the line shows the calculated values. The data is the average of three experiments.

The higher flow rates of 0.3mlsec^{-1} and 3.1ml sec^{-1}, though resulted in early breakthrough, the net adsorption at saturation ($Q_S = C_i - C_S / X$) were 16.91mg g^{-1} and 9.60mgg^{-1} respectively (Fig. 4.31), indicating that this is the ideal flow rate. Further higher flow-rate of 3.1ml sec^{-1} (Fig. 4.32) resulted in Q_S and $Q_{1/2}$ values of 1.72mg g^{-1} and 0.30mgg^{-1} respectively. The low net adsorption values in higher flow rates are due to lesser contact period where the `t' value is lower than the ideal `t' value of 20min (see Fig. 4.25). A similar observation has been made for benzoic acid adsorption using activated charcoal (Chern and Chein 2001).

4.3.3. Separation of betalaines into betaxanthine and betacyanine

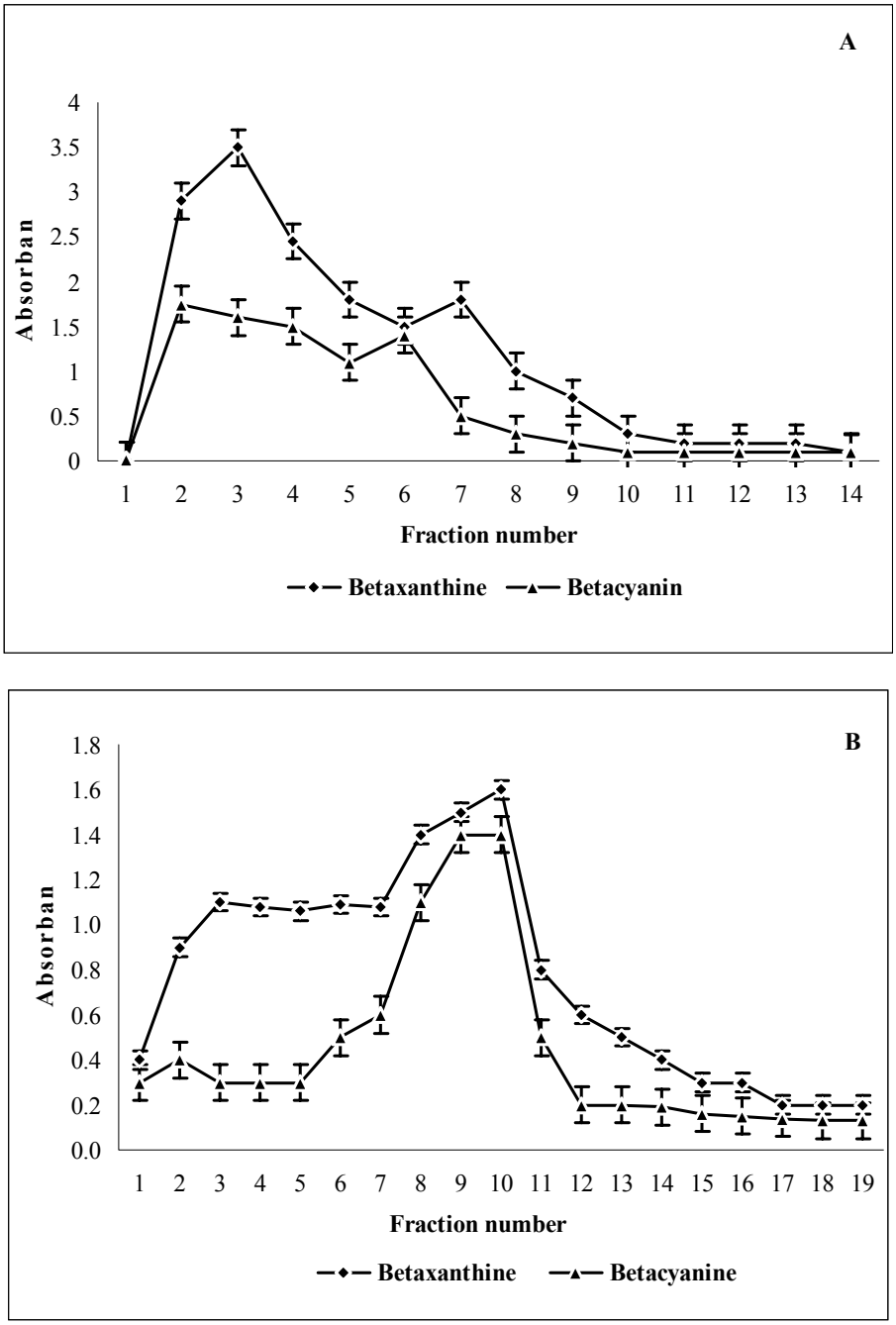


Figure 4.33. Elution profile of the betalaines adsorbed onto alumina: processed sand (2:1) column when eluted with EtOH gradient having 2% HCl (A) and an acid gradient of 0.5-5% (B). The values are the mean ± SE (standard error) of at least six replicates.

Development of an *ex situ* column for the separation of the two components of betalaines i.e., betaxanthine and betacyanine is an important step in the production of an economically viable online production system for continuous production. Various adsorbent

column materials and the elution solvents were employed to separate betalaines into betaxanthine and betacyanine. After the initial screening of various column materials, the combination of alumina: processed sand (2:1) (2cm × 2cm column) that resulted in the maximum pigment adsorption and de-sorption was chosen for further studies on separation of betalaines by using various elution solvents. A little separation initially was observed when the pigment was subjected for elution with EtOH gradient in water with 2% HCl (Table 4.7). However, except one or two initial fractions most of the fractions appeared to have the mixture of both betaxanthine and betacyanine visibly which was further confirmed by spectrophotometrically (Fig. 4.33A). Therefore the acidity was changed from 2% to a gradient of 0.5-5% which also the resulted in almost similar separation (Fig. 4.33B). However a contrasting result was obtained where little separation was obtained in last fractions (Fig. 4.34A) when the acid gradient was split into three ranges i.e., with EtOH gradient, the fractions 1-10 were eluted with 2% HCl, the fractions 11-14 were eluted with 4% HCl and fraction 15 onwards the pigment was eluted with 5% HCl. In this case there was absolutely no separation initially with a slight separation at the last fractions. However, the best separation was obtained when the adsorbed pigment was eluted with the solvent No. 5 (Table 4.7), the fractions were eluted into three separate groups (Fig. 4.34B) i.e., initial fractions from 1-8 had only betaxanthine, followed by betacyanine (fractions 9-17) and a mixture of both (18-20). The initial fractions appeared bright yellow coloured followed by purplish red and a yellowish red (Fig. 4.35). The separated fractions were further pooled into three groups such as pool-1 comprising of all the yellow fractions (betaxanthines), followed by pool-2 comprising all the purplish red fractions and pool-3 comprising of all the mixed fractions (Fig 4.35). HPLC analysis of the pooled fractions showed single peak each i.e., pool-1 and pool-2 indicating the presence of pure betaxanthine and betacyanine. However, the mixture showed three peaks of which two had different retention times than that of either of the purified fractions indicating the presence of additional xanthine and

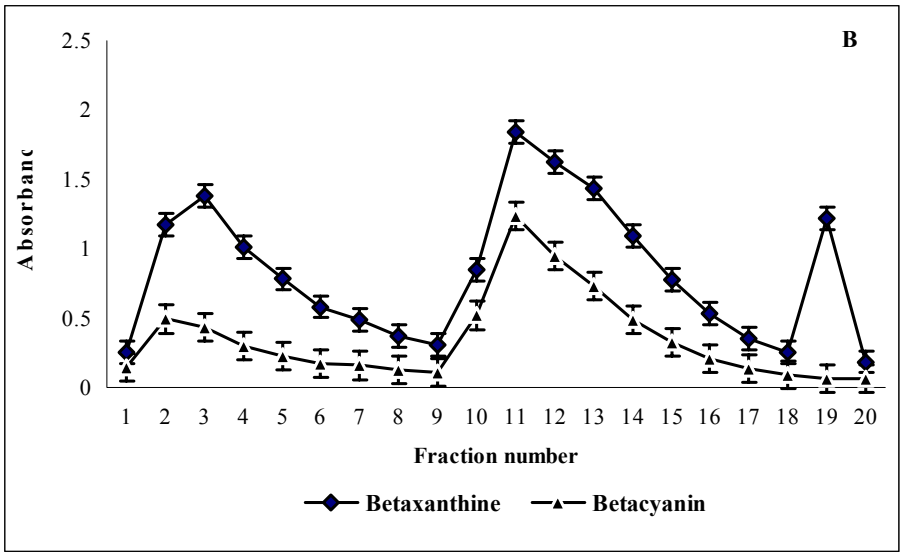


Figure 4.34. Elution profile of the betalaines adsorbed onto alumina: processed sand (2:1) column when eluted with EtOH gradient and ranges of acidity, i.e., the fractions 1-10 were eluted with 2% HCl, the fractions 11-14 were eluted with 4% HCl and fraction 15 onwards the pigment was eluted with 5% HCl. The values are the mean ± SE (standard error) of at least six replicates.

cyanine pigments which may be structurally slightly different. Aronoff and Aronoff (1948) separated the beet pigments in an extract on a talc-siliceous earth column into at least 11 coloured bands. However, several investigators have reported the separation of beet pigments to several fractions by electrophoresis (Lindstedt, 1956; Wyler and Dreiding, 1957; Peterson and Joyshyn, 1960). But since long time there are no attempts for the separation of betalaines from hairy root cultures at commercial scale. Therefore, the present study reports for the first time an *ex situ* column for the separation of betalaines in to two pure fractions i. e., betaxanthine and betacyanine with a possibility of integration of the same into an online production and recovery system (Fig 4.37).

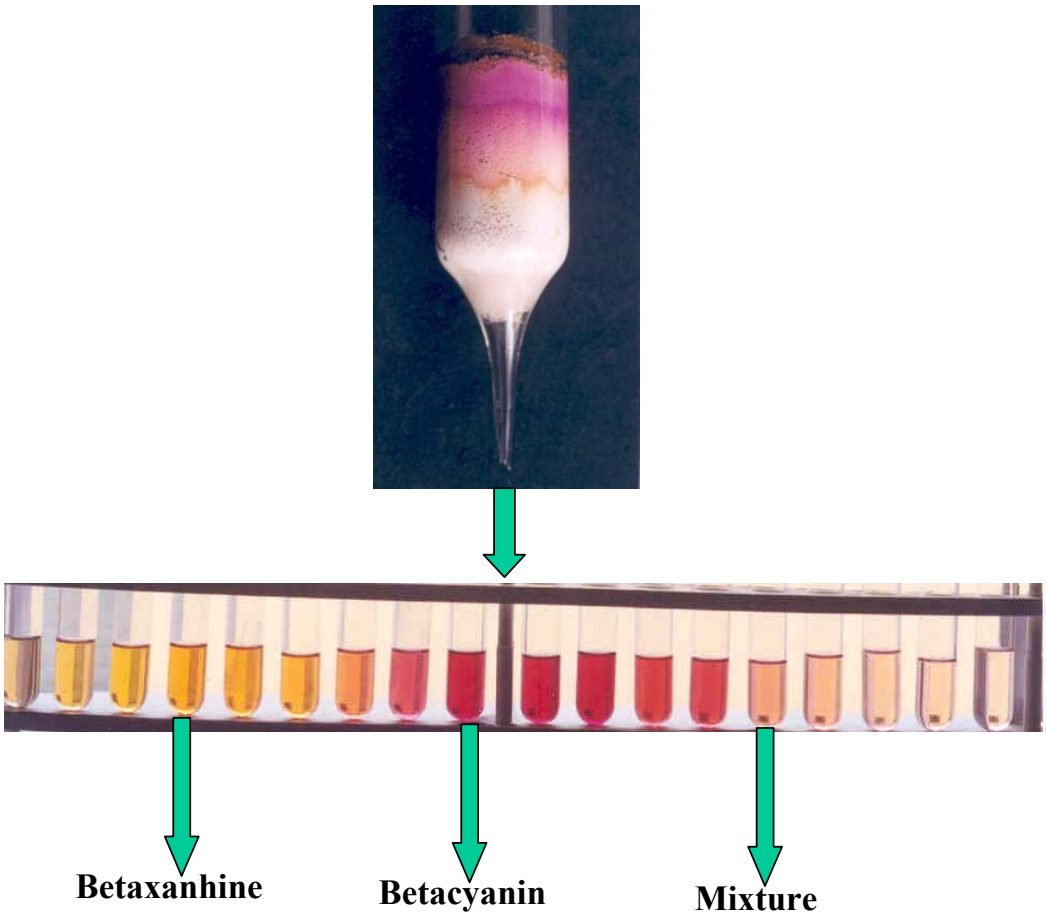
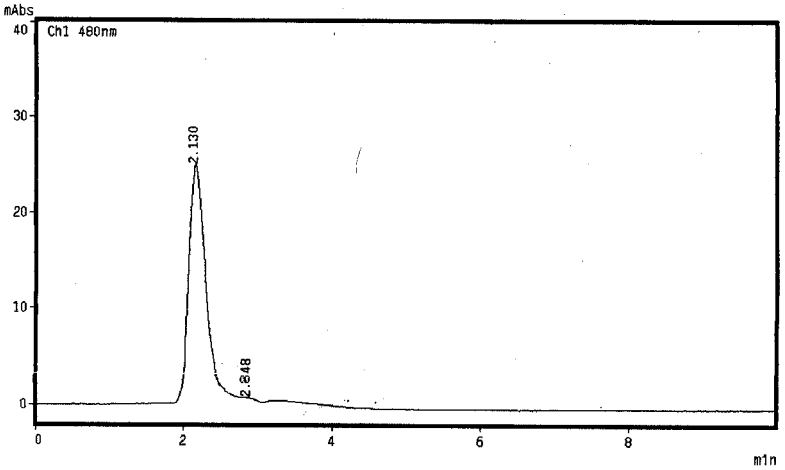
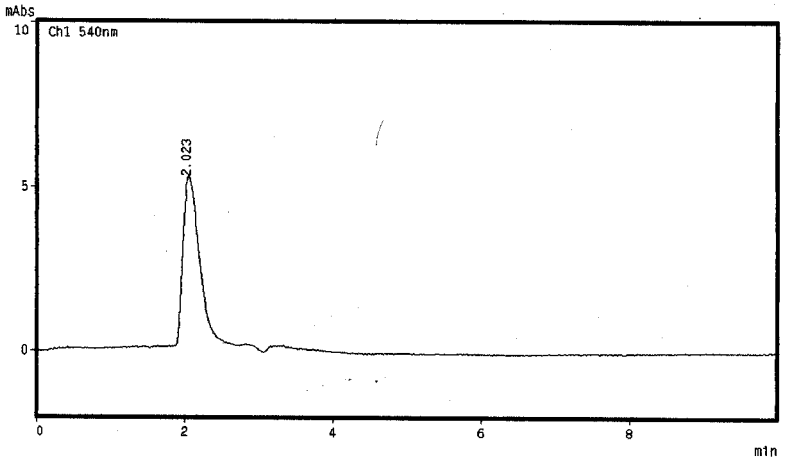


Figure 4.35. Alumina: Processed sand column with the fractions separated in to betaxanthine, betacyanine and Mixture

(A)



(B)



(C)

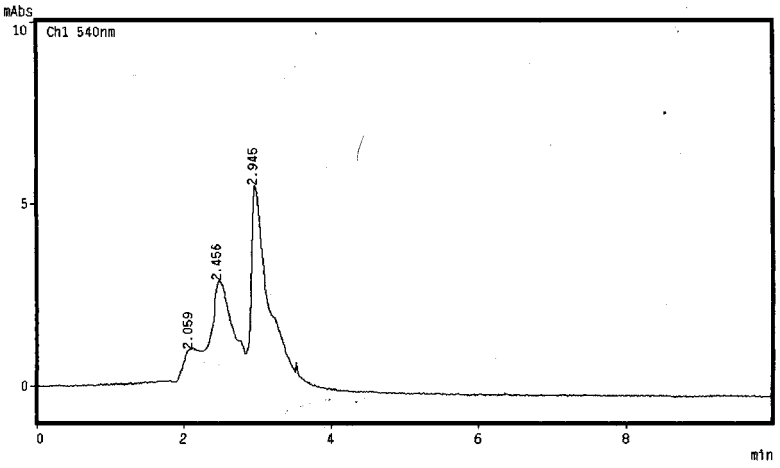


Figure 4.36 HPLC profiles of the pooled column fractions indicating the purity of separated pigment such as betaxanthine (A); betacyanine (B) and the mixture (betalaine).

4.3.4. Aqueous two phase extraction for simultaneous recovery of pigment and enzyme

As discussed in the previous chapters, the red beet hairy roots can be used for the production of two important products i.e., the betalaines and Peroxidase. Therefore, developing a process for simultaneous recovery of the pigment betalain and the enzyme Peroxidase is an attractive strategy. Aqueous two-phase systems are often used for large-scale continuous separation of proteins, natural pigments and removal of contaminants from fermentation broths, as they can rapidly produce an initial purification (Hart and Bailey, 1991). ATPE can be used as a preliminary step in the purification of proteins and has been applied for the commercial purification of several products, including recombinant products. Hence the main aim of the present study is to attempt for simultaneous recovery of pigment and the enzyme by using aqueous two phase (ATPE) system and check whether it is possible to partition the two products into different phases such as bottom and top phase. The preliminary set of experiments conducted by using different phase compositions such as various molecular weight polyethylene glycol (PEG) and a constant ammonium sulphate concentration. The results presented in table 4.8 clearly indicate that there was better phase formation with increase in molecular weight of the PEG and the lowest molecular weight PEG used did not form any phases. Overall from the results it is clear that the Peroxidase tends to move towards the aqueous and salt rich bottom phase. However, the distribution of the pigment was more or less even in both phases with a degradation of loss of pigment upto 30-60% (table 4.8). Keeping in view the separation behaviour the idea is to partition the maximum quantity of the enzyme to bottom phase and the pigment to the top phase. From the table it is clear that of the various mixtures, the ATPE system J appeared best (with a better trade-off) for the portioning and recovery of betalaines and Peroxidase. The ATPE system 'J' resulted in the recovery of only about 25% betalaines in the top phase and about 137% POD. The increase in recovery of the POD above 100% may be because of the enhancement of the activity of the enzyme because of amplification of the activity due to its purification in the salt phase (Miranda et al, 1995; Srinivas et al, 1999). When the partitioning behaviour is considered, the partitioning of pigment to the top phase appeared to increase with increase in the molecular weight of PEG though there was a considerable amount of degradation of the pigment (table 4.8). On the other hand for POD no definite trend was observed with respect to molecular weight of the PEG. However, the enzyme appeared to concentrate in the salt-rich bottom phase with enhancement in the total activity. The retention of considerable amount of pigment in the bottom phase and the enzyme in the top phase may however be improved further by studying various parameters such as various

concentration of NaCl, different pH and temperature as reported elsewhere (Srinivas et al, 1999; Miranda et al, 1995). It can be noted from the results that though there was a high partition coefficient for the pigment (0.9), in certain phase systems, there was a drastic reduction in the recovery of the pigment possibly because of the degradation of the same.

Table 4.8. Effect of phase composition on the partitioning and recovery of betalaines and peroxidase.

System			Recovery of the pigment (%)		Recovery of peroxidase (%)		Partition co-efficient	
PEG (%W/V)		Amm. Sulphate (%W/V)	Top phase	Bottom phase	Top phase	Bottom phase	Top phase	Bottom phase
PEG 1500								
A	4	13	**Did not form phasees**					
B	6	13						
C	8	13						
D	10	13						
PEG 4000								
E	4	13	12.7	26.9	30.1	187.4	0.5	0.2
F	6	13	12.1	26.4	33.0	226.5	0.5	0.1
G	8	13	19.7	22.7	49.7	206.9	0.9	0.2
H	10	13	18.8	18.8	57.2	179.5	0.9	0.3
PEG 6000								
I	4	13	25.7	41.1	21.0	124.9	0.6	0.2
J	**6**	**13**	**25.5**	**38.1**	**42.4**	**137.7**	**0.7**	**0.3**
K	8	13	10.8	30.0	27.7	124.4	0.4	0.2
L	10	13	10.3	68.0	33.1	120.6	0.2	0.3

Most of the earlier studies on ATPE have concentrated only on one product i.e., either enzyme or the pigment (Srinivas et al. 1999). The present study is the first report where ATPE is attempted for the recovery of two products simultaneously from a single system. As observed in the present study concentration of the peroxidase in the bottom salt rich phase was also reported for horse radish peroxidase and peroxidase from the leaves of *Ipomea palmetto* (Srinivas et al. 1999) form this behavior it may be concluded that the enzyme may be hydrophilic. However no reports are available for the application of ATPE for the portioning and recovery of betalaines. It is well known that the betalain pigments are also hydrophilic and hence there needs to be extensive study taking various kinetic

parameters in to consideration for arriving at a congenial strategy for their simultaneous separation.

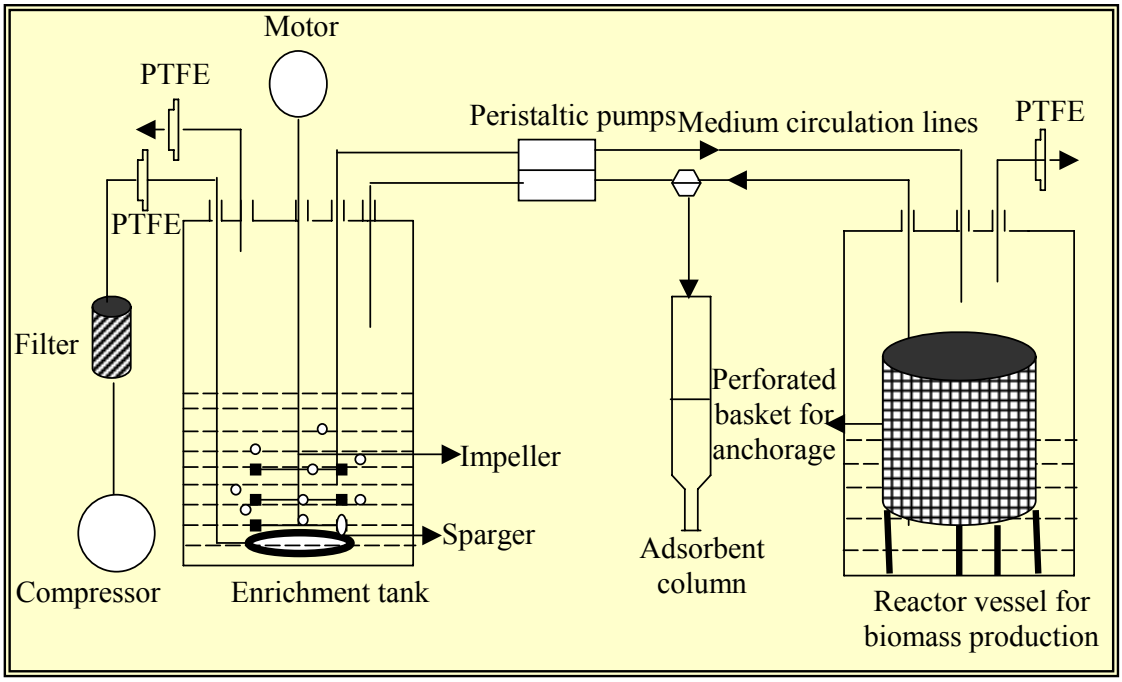


Figure 4.37. Online bioreactor model with an attached air enrichment tank and an *ex situ* column where, the biomass grown can be directly subjected for effluxing the betalaines. The effluxed can be passed through the adsorbent column leading to the recovery of pure betaxanthine and betacyanine.

All in all a large body of information has been generated on various down stream processing aspects such as in situ release of pigments by non-destructive methods, followed by adsorption and recovery by desorption. An attempt has also been made for the application of aqueous two- phase extraction (ATPE) technology for the simultaneous recovery of the betalaine and POD. A prototype reactor model for continuous production and online recovery of betalain and POD has been suggested (Fig. 4.37).

Chapter highlights

- **Various cell-permeabilizing agents were screened and a best strategy was developed**
- **A process for *in situ* and *ex situ* adsorption and recovery of betalaines has been developed**
- **An *ex situ* adsorption column was developed for separation of betalaines into pure betaxanthine and betacyanine**
- **An attempt was made for developing a method for simultaneous recovery of betalaines and peroxidase**
- **Basic information such as involvement Ca^{2+} - channel in chemical mediated effluxing of betalaines was also generated**

Plant kingdom is an indispensable source for a number of chemical substances, which may be primary or secondary metabolites. While the primary metabolites are directly synthesized and encoded by their respective genes or by basic photosynthetic process the secondary metabolites in plants are derived from basic photosynthates with modifications to produce simple to complex molecules. The plant secondary metabolites have been grouped in to a number of classes such as phenolics, terpenes, steroids and alkaloids according to their biosynthetic pathways. 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 still of natural plant origin.

Conventionally, plant secondary metabolites are obtained from field grown plants. However, recently plant cell cultures have been exploited for the production of various secondary metabolites of food and pharmaceutical importance. Of the various plant parts 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. 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).

Among the *in vitro* culture systems, the cultured cell suspensions are not gained momentum because of their instability and non-uniformity of product formation. Hence the differentiated organ cultures such as hairy root cultures' are widely studied. Hairy root cultures are used as alternative production systems for secondary metabolites due to their tremendous potential of higher growth rate and uniform product formation. Being organized, they are amenable for scaling-up in bioreactors is an added advantage. The metabolite pattern found in hairy roots is similar, if not always identical to that of plant roots. 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.

In addition to secondary metabolites, the hairy roots are the source of number of primary metabolites such as enzymes. The hairy root cultures of many plant specie have

been reported to produce appreciable levels of enzymes such as peroxidase. Secondary metabolites are highly sequestered in to the cell vacuole. Therefore, the approaches for reverse sequestering the vacuole stored secondary products in to the cell exterior are an important step towards the development of an online production system. Various methods have been proposed for this purpose. Since more than 50% of the production cost in any production system goes for down stream processing it is important to optimize the conditions for product recovery. With the available literature and background information one would very well appreciate the commercial potential of hairy root cultures for the production of various secondary and primary metabolites.

Objectives

In view of this background a series of experiments were designed and executed to achieve the following objectives. The data generated from these studies form the core matter of this thesis. The specific objectives set for the present study are given below

- To establish different clones of hairy roots using different strains of *Agrobacterium rhizogenes* and double transform the superior clone with additional *rol* genes and characterize the hairy root clones so obtained for growth, morphology and pigment synthesis.
- To study the effect of different culture volumes on the performance of hairy root cultures.
- To study the growth and pigment formation in a bioreactor for improved mass-transfer.
- To study off-line and on-line recovery of pigments.
- To screen hairy root clones for the production of peroxidase enzyme and select the best clone for further elicitation of production and partial purification and characterization of the enzyme.
- To integrate biomass production and product recovery.

Highlights of the findings

Entire work carried out and the results obtained are summarized under the following headings.

Establishment and characterization of clones

Ten hairy root clones of red beet (*Beta vulgaris*) were induced using different strains of *Agrobacterium rhizogenes* viz., A4, A 2/83, A 20/83 and LMG-150, accordingly the clones were named as A4(1), A4(2), A4(3), A 2/83(1), A 2/83(2), A 2/83(3), A 20/83(1), A

20/83(2), A 20/83(3) and LMG-150. Further, the clones were characterized based on their growth performance, capacity for pigment synthesis, endogenous auxin content, T-DNA copy number and ISSR genetic marker. The clone LMG-150 that produced highest biomass and betalain pigment was selected as the better performing or best clone and was used in all further studies. However, significant differences in morphological and physiological characteristics were observed among the ten clones that depended mainly on the *A. rhizogenes* strain. The growth and pigment synthesis in all the clones were directly proportional to the endogenous auxin level but not to T-DNA copy number despite the known fact that T-DNA harbors the *rol* genes, which influence endogenous phytohormone levels. In the clone obtained from LMG-150 that produced highest biomass and pigments levels, it was found that the enzyme peroxidase was involved in regulating the endogenous auxin pool. In addition an attempt was also made to double transformation of the best clone for putting additional *rol* genes. The double transformed clone with additional *rolABC* produced highest biomass and pigment synthesis. Since the double transformant showed unstable productivities, the best clone LMG-150 was considered for all further studies.

Optimization of growth conditions and scale up

The superior clone was studied for further optimizing the two important growth conditions such as medium volume, carbon source and scale up. Among the different culture volumes studied, the hairy root grown well in 250 ml flask containing medium with decrease in biomass production in higher volumes. Further, to find out the reason for the poor performance in higher volumes the hydrodynamic pressure exerted on hairy roots at different position in the flask was calculated and found that there was increase in hydrodynamic stress with increase in culture volume the results are same with increase in shaker speed. Upon subjecting to treatments containing different sugars (3% w/v) it was found that sucrose was rapidly utilized, followed by maltose, and a very limited use of glucose, but the other hexoses – fructose, lactose, xylose and galactose or glycerol totally suppressed both growth and betalaine synthesis. No habituation or adaptability to maltose or glucose occurred, evidenced by the lack of growth upon re-culture in respective medium. Glycerol, was not taken up alone, but was utilized to a considerable extent in the presence of low levels of sucrose for growth only but not betalaine synthesis. Red beet hairy root culture did not exogenously hydrolyze sucrose to hexoses, as there were only traces of reducing sugar present in the medium soon after inoculation, without an increase later, confirmed by HPLC. There was an increase in medium osmolarity in the presence of fructose indicating the exudation of certain compounds from the roots. Red beet hairy roots appear useful as a

model system to study sugar metabolism / signaling due to their sensitivity to different sugars that may directly link to morphological changes and betalaine synthesis. Scale up studies using different bioreactors had shown that the bubble column reactor with an attached medium-containing vessel for air enrichment gave better growth and biomass production when compared to bubble column reactor alone.

Studies on peroxidase enzyme

The genetically transformed roots of red beet have been shown, for the first time, to produce very high levels of peroxidase (POD; EC 1.11.1.7) accounting for $1.21x10^6$ Units L^{-1}. Each clone differed significantly from the others in growth, hormone dependency and POD production where LMG-150 produced highest biomass (140g FW L^{-1}) as well as POD (ranging from 8000-9000 U g^{-1} FW and $1.18x10^6$ U L^{-1} with a specific activity of 600 U mg^{-1} protein) on hormone-free medium, both in shake-flask as well as in bioreactor with a further enhancement to $1.21x10^6$ U L^{-1} upon the addition of extra calcium chloride (5 mM). PAGE with active staining showed 5 distinct bands of R_m 0.06, 0.16, 0.25, 0.38 and 0.46 in the biomass and bands at R_m 0.06, 0.16, 0.25 and one extra band of R_m 0.575 in the spent medium where isozymes of R_m 0.38 and 0.46 were totally absent. The pH optima and other properties were grossly comparable with the standard horse-radish POD (HRP) with better thermal stability than HRP. In an effort to elicit further the POD activity, the cultures were contacted with biotic elicitors such as dry cell powders of microbial cultures (0.1– 0.5% w/v) and the respective culture filtrates (1-5% v/v). Similarly, abiotic elicitors, particularly metal ions (2-8 folds of that present in the nutrient medium), plant hormone Thidiazuron (at 0.25-1 ppm) and other bio-molecules such as Glutathione (at 0.5 mM-10 mM) and Methyl jasmonate (at 20-100 μM) were used. It was observed that dry cell powder of *Candida versatalis* significantly elicited the enzyme activity (3.52-fold higher than the control) followed by glutathione (3.44-fold) and *Rhizophus oligosporus* (3.09-fold). Among abiotic ones, thidiazuron, Mg and Ca salts elicited 2.49, 3.03 and 2.8 fold activities respectively. While most of the biotic elicitors were effective when added on 15^{th} day of culture, the abiotic elicitors were effective when added on 20^{th} day. Combination of highly effective elicitors indicated that glutathione (1mM) and dry cell powder of *R. oligosporus* caused 4-fold enhancement in enzyme activity, accounting for $10.9x10^6$ U L^{-1}. Therefore, the present source appears to offer a cheaper and additional alternative for the commercial production of POD. Apart from this, one of the isozymes of peroxidase was purified by anion exchange chromatography (AEC). The isozyme had the molecular weight of approximately 45kD, and showed optimum activity at pH 5 and stability over a range of pH 3-9. The enzyme showed

highest affinity to H_2O_2 like other plant peroxidases. Among the hydrogen donors the enzyme had highest affinity to orthodianisidine hydrochloride.

Downstream processing

Various physical chemical and biological agents were studied in detail for the permeabilization and the release of pigment where, the chemical agent CTAB has been selected as best to recover the max quantity of the pigment. Some studies on the involvement of calcium channel in the pigment release through chemical agents were also made. Among the biological agents Lactobacillus *helveticus*, S*accharomyces cereviseae* and *Candida utilis,* as well as cell fractions of *L. helveticus*. The free and wall bound lipid fractions of biotic agents were also tried for the recovery of betalains with or without oxygen stress. It was found out that the free lipid surfactants of the biological agents are most ideal for the permeabilization of hairy root cultures, as they are known to add some functional property for the released pigment, which is used in food and pharmaceutical agents. Once the pigment released it is very important that the pigment is recovered completely. The mechanism involved in pigment release was partially elucidated where calcium calmodulin signaling pathways were found to mediate the release of betalaines from vacuoles.

Among the different adsorbents studied for *in situ* recovery Alumina: silica (1:1) appeared ideal showing *in situ* adsorption of 97% in a unit time of 30min. accounting for *in situ* recovery of 71.39% of the total betalaine effluxed. *Ex situ* recovery of betalain was done using various combinations of alumina-silica and processed sand and different column geometries indicated that alumina with processed sand at 2:1 ratio (weight / weight) and a minimum column material of 2cm height and 2cm diameter was good enough to cause 97% pigment adsorption from a solution containing 1.6mg ml^{-1}. Desorption and recovery of pigments *ex situ* from columns were affected by various elution mixtures, where, a gradient elution with ascending levels of HCl-ethanol in water resulted in 100% recovery of adsorbed pigments in a significantly lesser volume of eluent in a short period of 1h. Since the hairy roots produce both pigment and the peroxidase aqueous two-phase extraction (ATPE) strategy was studied for the simultaneous recovery of both pigment and the enzyme.

Conclusions

All in all, the present investigation has resulted in finding out the feasibility of transforming red beet to obtain singly and doubly transformed cultures resulting in hairy roots capable of simultaneous production of both pigment (betalaines) and an enzyme (peroxidase) where extensive basic information developed on *in situ* product release, recovery and kinetic parameters for scale-up of the process would be of immense use for

further commercial applications of such systems. The high sensitivity of red beet hairy roots for different sugars and the presence of several isoforms of peroxidase make them very attractive for more basic work elucidating various mechanisms involved in root biology.

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