# PRODUCTION OF BLUE PIGMENTS FROM THE CALLUS CULTURES OF *LAVANDULA AUGUSTIFOLIA* AND RED PIGMENTS (BETALAIN) FROM THE HAIRY ROOT CULTURE OF *BETA VULGARIS*

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# ABSTRACT

Plants are used to produce many secondary metabolites that are too difficult, expensive or impossible to make by chemical synthesis. Conventional cultivation of plants is of course subject to vagaries of weather, pests and availability of land; hence, the interest in highly controlled culture of plant cells and hairy roots in bioreactors as methods of producing various products. This project focussed on production of blue and red colors of *Lavandula augustifolia* and *Beta vulgaris*, respectively. Callus and suspension cell culture were successfully produced from *L. augustifolia* after extensive trials, but hairy roots could not be generated from this species. In contrast, a successful protocol was developed for consistently producing hairy roots from *B. vulgaris*, but calli could not be generated from this species.

Effects of medium composition on growth of *L. augustifolia* calli and freely suspended cells and production of the blue pigment by the latter, were investigated. Optimal production of callus occurred in full-strength Murashige and Skoog (MS) medium supplemented with 2 mg/l of indole-3-acetic acid (IAA) and 1 mg/l of kinetin. Stable suspension cultures could be produced and maintained in full-strength MS medium supplemented with 1 mg/l each of IAA and kinetin. In suspension culture in full-strength MS medium, the following hormone combinations were tested: (1) 1 mg/l each of indole-3-acetic acid (IAA) and kinetin; (2) 2 mg/l of IAA and 1 mg/l of kinetin; (3) 2 mg/l of IAA and 1 mg/l of benzyl amino purine (BAP); and (4) 2 mg/l each of IAA and BAP. Combination (3) maximized cell growth, but the highest cell-specific production of the blue pigment was seen in combination (2), although pigment production occurred at all hormone combinations. The medium formulation that gave the best production of the pigment in shake flasks was scaled up to a 2 L aerated stirred tank bioreactor, but both the biomass and pigment productivities were reduced in the bioreactor apparently due to the high shear stress generated by the Rushton turbine impeller.

Compared to suspension cultures of *L. augustifolia*, the hairy root cultures of *B. vulgaris* grew extremely rapidly. Hairy roots also produced large amounts of the red pigments. Growth of hairy roots was influenced by the composition of the medium.

Although the full strength MS medium better promoted biomass growth compared to the half-strength MS medium, the final concentration of the biomass and the pigment were nearly the same in both media. Attempts were made to enhance production by using various hormones (i.e. naphthalene acetic acid, BAP, IAA added individually at a concentration of 0.5 mg/l), but none of the hormones proved useful. BAP adversely affected the growth of hairy roots.

In summary, production of pigments by suspension culture of *L. augustifolia* and hairy root culture of *B. vulgaris*, is technically possible, but requires substantial further optimization for enhancing productivity than has been possible in this project.

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# CHAPTER 1

# INTRODUCTION AND OBJECTIVES

# 1.1. Introduction

Plants have been utilised as a source of fats, carbohydrates and protein for centuries. Plants supply almost 80% of natural products (Balandrin and Klocke 1988). Various secondary metabolites including pharmaceuticals, pigments, flavours, fragrances, bio pesticides, food additives and chemicals can be produced from large scale in vitro plant cell cultivation. Metabolites which do not usually occur in nature can also be produced by plant cell cultures. Plants remain the main source for many traditional medicines. In future, more drugs will be produced from plants as a result of the improved understanding of plant metabolism.

## 1.1.1. Secondary products from plant cell cultures

A blue pigment can be obtained from the callus culture of Lavender. These pigments can be used as colouring agent in food instead of artificial dyes. Likewise the red natural beet pigment betalain is produced in *Beta vulgaris* (Beet root). It can be utilised as a natural colorant in drug, food and cosmetic products (Dornenburg and Knorr 1996). Hairy roots have become an alternative source for this pigment because the betalain content is non-uniform in conventionally grown beet root.

## 1.2 Objectives

This project is focussed on the production of blue pigment in suspension culture of lavender and red pigment betalaines from the hairy root culture of *Beta vulgaris* with the following objectives:

1. The generation of lavender callus and transfer to the stable suspension cultures.
2. Optimisation of cell growth and pigment production in lavender suspension cultures using various plant hormones and elicitors.
3. Generation of hairy roots of beet root in different media and the measurement of biomass and pigment production.
4. Identification of the influence of different growth hormones on hairy root growth and production of pigments.

# CHAPTER 2

# LITERATURE REVIEW

# Sub-chapter 1

## 2.1. Tissue culture

The term tissue culture implies the culture of plant cells, tissues and organs under aseptic laboratory conditions in vitro.

### 2.1.1. History of plant tissue culture

Plant tissue culture was started in 1902 by German botanist Gottlieb Haberlandt, who is known to be the father of tissue culture. He could successfully culture palisade cells from leaves of *Lamium purpureum*, pith parenchyma of *Ornithogalum* species and epidermal hair cells of *Pulmonarial* sp. in sucrose supplemented Knop's salt solution (Haberlandt 1902). There was an increase in the size of the cells but they did not divide.

The first commercially produced natural product by plant cell cultivation was a naphthoquinone, shikonin from the cell cultures of *Lithospermum erythrorhizon* demonstrated by Fujita and co-workers (Fujita et al., 1982). Since then many works have been carried out for the industrial preparation of secondary metabolites.

## 2.2. Plant cell cultivation – A principal source of secondary metabolites

Plants provide numerous secondary metabolites including pharmaceuticals, flavours, food additives and agrochemicals (Rao and Ravishankar 2002). Some secondary metabolites obtained from plants are shown in Table 2.1. Nearly 121 drugs used worldwide are extracted from plants (Payne et al., 1991). In the US pharmaceutical industry roughly 25% of the products are derived from plants (Farnsworth 1985). Chemical synthesis of metabolite is generally quite complex and therefore biological sources such as plants will continue to be relied for many secondary metabolites (Pezzutto 1995).

**Table 2.1. Major secondary metabolites produced from plant cell culture**

| Product | Plant | Uses |
|---|---|---|
| Ajmalicine | *Rauwolfia serpentina* | circulatory stimulant |
| Azadiractin | *Azadiracta indica* | Insecticide |
| Betalaines | *Beta vulgaris* | Food colorant |
| Colchicine | *Colchicum autumnale* | Antitumour |
| Diosgenin | *Dioscorea deltoidea* | Raw material for active steroids |
| Reserpine | *Rauwolfia serpentina* | Tranquilizer |
| Shikonin | *Lithospermum erythrorhizon* | Antibacterial |
| Taxol | *Taxus* species | anticancer agent |

Compared to production in whole plant the use of callus culture and hairy roots has the following advantages.

1. It is independent of climate and other environmental factors.
2. The cultures are free from pests and microbial attack as a sterile environment is used.
3. Many useful products which are absent in parent plants can be produced.
4. Some economically important plants are difficult to grow naturally because the life cycle may be too long. This difficulty may be overcome by cell cultures.
5. Cell cultures minimise the reliance on land.
6. Crop production is generally related to the growing season but cell culture is not.
7. Reduced labour cost (Vanisree et al., 2004).

The secondary metabolite production from plant cell culture has certain drawbacks. Low growth and genetic instability of the cells are the major biological problems which result in low metabolite production. Cell aggregation, poor mixing and oxygen transfer are some technological problems. The plant cells are sensitive to shear stress and it creates many problems in the large scale cultivation of plant cells.

## 2.3. The steps involved in secondary metabolite production from plants

1. Selection of a high producer plant.
2. Callogenesis (in vitro callus culture) or generation of hairy roots.
3. Suspension culture development of callus cells or hairy roots.
4. Application of strategies to enhance yield.
5. Scaling up.
6. Mass cultivation in bioreactors.
7. Downstream processing (extraction and purification of compounds).

### 2.3.1. Callus culture

Callus is an unorganised mass of cells. It is often produced on the wounds of organised tissues. So it consists of undifferentiated cells which are parenchymatous. Callus growth can be induced from plant explants like stem, roots, leaves, etc. In the process of dedifferentiation parenchymatous cells of explants play the major role especially when the isolated explants contain only differentiated cells. So in callus formation the mature cells revert to the juvenile state. In the initial stages, the wounded parts only divide but later on the complete explants start dividing and produce the mass of cells.

The supply of growth hormones is essential for callus induction; auxin, cytokinin or both. Some plants require light and some produce callus in dark. Usually 25-28$^0$C is the required temperature. After getting a suitable size of callus, it can be transferred to a fresh medium. This process is called sub culturing. So, in the process of production of secondary metabolites, the callus culture remains as the primary step for subsequent plant cell culture.

### 2.3.2. Cell suspension cultures

A cell suspension culture is usually produced by transferring a suitable amount of friable calli into a liquid medium. This will be stirred continuously usually by a shaker. The suspension culture has proved to be the most effective alternative for the production of secondary metabolites because of the ease in the observation and control of growth parameters. The initial callus is the critical factor for the establishment of a successful culture. A friable callus is transferred to liquid culture in shake flasks. Shakers should be having a speed control. Continuous shaking of the culture medium first exerts a small pressure which helps to break the callus into single cells and subsequently distributes the cells uniformly and also serves for the proper exchange of gases (Dixon 1985).

The suspension cultures should be sub cultured every 15 days. This is done by removing the cells by sieving and transferring them to a fresh medium. The plant cell culture passes through different phases like lag, exponential and stationary phase exactly as the microbial culture.

It is easy to control environmental and cultural parameters in cell suspension cultures. So it has become the most acceptable way for secondary metabolite production. But still there are some factors like aggregation of cells, growth of cell on walls and rheological properties of broth which make the mass cultivation problematic.

## 2.4. Various steps to enhance secondary metabolite production

### 2.4.1. Screening and selection of cells with high productivity

The most important step in secondary metabolite production from in vitro plant cell cultures is the selection of cells that can produce the maximum amount in both callus culture and hairy root culture. Because of the heterogeneity, repeated screening is found to produce more effective cell lines with high production (Ogino et al., 1978). For example, in *Beta vulgaris* yellow callus culture, repeated screening increased the betaxanthin production upto 1.8 fold on 48$^{th}$ subculture to yield 45 mg/g DW whereas 25 mg/g dry weight was the production in the first subculture (Trejo-Tapia et al., 2008).

### 2.4.2. Optimisation of nutrients in the medium

The secondary metabolite production is greatly influenced by the composition of the cell culture media (Stafford et al., 1986). The constituents that are usually optimised are carbon source, nitrate and phosphate.

**2.4.2.1. Sugar (carbon source) levels**

The most commonly used sugar source in plant cell culture media is sucrose. But for the production of ajmalicine from *Catharanthus roseus* cells, the better source is glucose (Schlatmann et al., 1985).The secondary metabolite production is found to be affected by the changes in carbon source concentration. For instance rosmarinic acid production showed an improvement in *Zataria multiflora* Boiss callus cultures by the increase in carbohydrate concentration. The maximum amount of rosmarinic acid (158.26± 1.086mg/g DW) was produced when 90 g/l of glucose was supplied when compared to 60 g/l which gave a yield of 131.350±0.568 mg/g DW (Francoise et al., 2007). Similarly in *Coleus blumeii* cultures, the rosmarinic acid yield was about 0.8g/l with a sucrose concentration of 2.5 %( w/v) but a sucrose concentration of 7.5 %( w/v) gave 3.3g/l (Misawa 1985).

**2.4.2.2. Nitrate concentration**

Nitrate is an important nutrient in plant cell culture media. The cell growth and the secondary metabolite production are immensely influenced by the ratio of ammonium and nitrate. Shikonin and betacyanin production are enhanced by high nitrate and low ammonium concentration and high levels of both ions had a positive effect on berberine and ubiquinone production (Ikeda et al., 1977; Fugita et al., 1981; Bohm and Rink 1988).

**2.4.2.3. Level of Phosphate**

The level of phosphate ions has different effects on various secondary metabolite productions. The betacyanin production was increased by low level of phosphate concentration from *Beta vulgaris* callus cultures (Bohm and Rink 1988). But when the phosphate concentration was doubled in *Lavandula spica* callus culture, it produced more pigment and cell growth (Trejo-Tapia et al., 2003).

#### 2.4.2.4. Concentration of growth hormones

In plant cell cultures, both the secondary metabolite production and callus growth are significantly affected by the auxins and cytokinins concentration and type (Mantell and Smith 1984). For example, Indole 3 acetic acid and kinetin increased the biomass production and anthocyanin content in *Daucus carota* cell suspension cultures (Narayanan et al., 2005). 2,4-Dichlorophenoxy acetic acid enhanced carotenoid production from *Daucus carota* suspension cultures (Mok et al., 1976) but the reverse effect was found in nicotine production from *Nicotiana tabacum* (Sahai and Schuler 1984).

#### 2.4.2.5. Addition of precursors

The addition of precursors or compounds that are structurally related to the secondary metabolite is found to increase the production of metabolites. The precursors sodium cinnamate and cinnamic acid could enhance the flavonoid accumulation in *Scutellaria baicalensis Georgii* suspension cultures (Martin and Dusek 2007).

### 2.4.3. Optimisation of cell culture environment

By manipulating culture conditions such as illumination, temperature, pH of the medium, variations have been found in secondary metabolite production.

#### 2.4.3.1. Light

The effect of illumination on product formation varies in different cell cultures. In *Daucus carota* cell cultures, more anthocyanin was produced when cultured in light (Seitz and Hinderer 1988). Dark environment was suitable for cell cultures of *Citrus lemon* to produce more monoterpenes (Kreiger et al., 1988).

**2.4.3.2. Temperature**

For plant cell cultures, usually a temperature of 17-25$^0$C is used. But it varies for each species. In the cell culture of *Lavandula vera*, maximum cell growth was found at 30$^0$C and rosmarinic acid production was maximum at 28$^0$C (Ikeda et al., 1977; Pavlov et al., 2004). 27.5$^0$C was found to be the optimal temperature for the production of ajmalicine from callus cultures of *Catharanthus roseus* (ten Hoopen et al., 2002).

**2.4.3.3. pH of the medium**

pH also plays an important role in cell cultures. Usually before autoclaving the medium pH is adjusted to between 5 and 6. High pH leads to the browning of callus.

**2.4.3.4. Size of the inoculum**

One of the most important factors which affect the growth of the cell suspension cultures is the inoculum size. The division of the cells in the culture stops when the cell concentration reaches a critical value. In most of the cell cultures an inoculum size of about 10-15% (v/v) has proved to be optimal.

**2.4.3.5. Aeration and agitation**

For large scale cell cultures aeration and agitation are vital factors. For instance a supply of dissolved oxygen $dO_2$ up to 40% helped to increase the saponin accumulation in the bioreactor suspension cultures of *Panax ginseng* (Thanh et al., 2006*)*. In Muscat grape suspensions when carbon dioxide was added it enhanced the

monoterpene production and also promoted the linalool synthesis (Ambid and Fallot 1981). But in many cases carbon dioxide inhibited the secondary metabolite production.

For example carbon dioxide decreased the ginsenoside yield from *Panax ginseng* root cultures in bioreactors (Ali et al., 2008).

## 2.4.4. Permeabilization

The plant cell is protected by plasma membrane and cell wall. Usually plant cells store most of the secondary metabolites in vacuoles. Cell permeabilization helps to create pores on the cell membrane so that the products can easily be released. Examples of two permeablizing agents are dimethyl sulfoxide and chitosan (Beumont and Knorr 1987). Ultrasonication and electroporation are two methods to permeabilize cells (Brodelius et al., 1988).

## 2.4.5. Elicitation

Elicitors are chemicals which can trigger biochemical or physiological responses in plants and then lead to the production of secondary metabolites (Zhao et al., 2005).

There are biotic and abiotic elicitors. Glycoprotein and cell wall materials of fungi are some biotic elicitors. UV radiation and some chemicals can be considered as abiotic (Dicosmo et al., 1987; Funk et al., 1987; Furze et al., 1991; Johnson et al., 1991; Robbins et al., 1991; Rajendran et al., 1994; Rao et al., 1996; Guo et al., 1998). UV light enhanced the flavonoid production in *Passiflora quadrangularis* callus cultures (Antognoni et al., 2007). The production of artemisinin, an antimalarial endoperoxide, from the hairy root cultures of *Artemisia annua* was increased 6 times (1.8 µg $mg^{-1}$ dry weight) by the addition of 150 mg/l chitosan. Similarly 0.2mM methyl jasmonate and yeast extract (2 mg/ml) also increased the artemisinin yield to 1.5 and 0.9 µg/mg dry weight respectively (Putalun et al., 2007).

## 2.4.6. Cell immobilization

The secondary metabolite production can be improved by the organization of plant cells. For this organization the process of immobilization is applied. Immobilization is the process of confinement of cells or enzymes on a support by adsorption, entrapment or covalent linkage. The plant cell immobilization technique that was first described by Brodelius et al in 1979 was the calcium alginate gel entrapment of viable cells of *Catharanthus roseus, Morinda citrifolia* and *Digitalis lanata*. There are many techniques for immobilization. The most popular method is the one in which cells are entrapped in a gel. These gels usually polymerise around the cells (Novais 1988). The most commonly used gel is Calcium alginate because they are very simple and non toxic. The immobilisation by calcium alginate gel stimulated the podophyllotoxin production in cell cultures of *Juniperus chinensis* (Premjet and Tachibana 2004). Gelatin, carageeenan, agar, agarose and polyacrylamide are the other gels which have been used (Nilsson et al., 1983).

## 2.4.7. Product removal

In many cases the amount of secondary metabolites produced are very low. This is because when the products get discharged into the medium, enzymatic or non enzymatic degradation may take place or the substance may be volatile or sometimes its production may be inhibited by feed back inhibition. If an artificial site for the collection of the products could be added, that increases the product formation. For this the medium can be integrated with a liquid or solid phase that removes the metabolite. Such culture systems are called three phase systems. Beiderbeck and Knoop (1987) reported that overall production could be enhanced by taking away and sequestering the product in a non biological screened off area (Beiderbeck and Knoop 1987). For this, either adsorbents like activated charcoal, XAD-2, XAD-4, XAD-7 and polyethylene-glycol could be added or dissolved. These adsorbents create a special phase for the accumulation of the products. Adsorption with XAD-7 could successfully reduce the feed back inhibition of plumbagin synthesis in *Plumbago rosea* immobilized cells (Komaraiah et al., 2003).

## 2.5. *Lavandula augustifolia* (Hidcote)

## 2.5.1. Habitat

This is an aromatic shrub that belongs to the family *Lamiacea*e which usually grows in the Mediterranean region. The common name is English lavender. It has green leaves and dark purple flowers. Figure 2.1 shows the flowers of *L.augustifolia*.

Figure 2.1. Flowers of *Lavandula augustifolia*

## 2.5.2. Uses

1. A blue pigment is produced from the callus cultures which finds application as colouring agent for food, pharmaceuticals and cosmetics (Nakajima et al., 1990).
2. Its aromatic oil is extensively used in toiletries and perfumes (Boelens 1986).
3. It is an ornamental plant because of its bright, beautiful flowers.
4. Its medicinal properties include as a curative for indigestion, headache and muscular pains.

## 2.5.3. Production of blue pigments from the callus culture of Lavandula

Some experiments have been carried out for the extraction of blue pigment from this plant callus. *Lavandula augustifolia* callus cultures produce yellow pigments which combine with $Fe^{2+}$ in the liquid medium to give dark blue pigments. These pigments are very useful in food, cosmetics and pharmaceutical industry as colouring agents (Nakajima et al., 1990). In recent years, these colours find more use because of the limitation in using artificial dyes. *L.augustifolia* plants do not produce these pigments naturally. So tissue culture has a great significance. But in vitro production of the pigments was very low. By altering the medium components and conditions, improvements in the productivity was found (Dornenburg and Knorr 1997; Trejo-Tapia et al., 1999).

In 2003, Trejo-Tapia et al carried out experiments on *Lavandula spica* callus cultures. They tried different media compositions by eliminating ammonium and adding L-cysteine. They found that a medium composition with an inoculum size of 10 g/l fresh weight, 2.5mM phosphate, 14.1mM nitrate, and an iron concentration of 1mM was the best for cell growth and blue pigment production. But when the sucrose concentration varied, the maximum pigment production was achieved at a sucrose level of 90g/l but a level of 30g/l gave the best cell growth.

Blue pigments were produced from the *Lavandula vera* cells immobilized with calcium alginate in the presence of L-cysteine under illumination. But when the cells were cultivated in the dark, brown pigments were accumulated. The precursor components of blue pigments 1-methylethyl 3,4-dihydro-7,8-dihydroxy-5-methoxy-3,10-dimethyl-4-oxo-4H-naphtho[2,3-b]pyran-3-butanoate were found to be present in these brown pigments which combined with $Fe^{3+}$ ions in the medium to produce blue colour (Nakajima et al., 1990).

## 2.6. Bioreactors for large scale secondary metabolite production

In the production of secondary metabolites, one of the major steps is the scaling up of the cultures into bigger volumes (Godoy-Hernandez et al., 2000). The plant cells cannot be grown in the bioreactors used for microbial culture because the cells are different in the pattern of growth and nature. Slow growth rate, low content of metabolites, low product secretion and genetic instability are the major problems found when plant cells were transferred from shake flasks to bioreactor (Zhong 2001).
There are different types of bioreactors used for different plant cell culture. Some of the most commonly used bioreactors are mentioned below.

### 2.6.1. Stirred tank bioreactor (STR)

This is the most commonly used bioreactors. In this type, there is an impeller which allows homogenous transfer of oxygen and nutrients. The first commercially produced secondary metabolite Shikonin from the cell cultures of *L.erythrorhizon* was cultured in stirred tank reactor of capacities 200L and 750L (Tabata and Fujita 1985).

### 2.6.2. Air-Lift bioreactor

This is quite similar to STR but it does not have an impeller. Thereby it exerts less stress on the cells. This bioreactor is more suitable for plant cell culture because it supplies less oxygen to the cells which are slow growing. The Air-Lift bioreactor is composed of a draught tube. The density of the medium is reduced by the continuous air sparging from the bottom of the reactor.

### 2.6.3. Bubble Column Bioreactor

They are vertical in shape usually composed of a cylindrical vessel with large diameter. They are devoid of any moving parts. So it is easy to retain a sterile environment. Bubble lift bioreactor was successfully used for the production of cell

clusters from the callus of *Lavandula augustifolia* 'Munstead' in MS medium supplemented with 0.23 µM 2,4-dichlorophenoxy acetic acid) and 2.22 µM benzyl adenine and 1.89 µM abscisic acid and subsequent regeneration of plants in basal MS medium containing 1.14 µM indole 3 acetic acid (Wang et al., 2007).

### 2.6.4. Rotating Drum Bioreactor

This bioreactor is composed of a drum on rollers which rotates horizontally. This is connected to a motor. Proper mixing and aeration are facilitated by the rotation of the drum.

### 2.6.5. Spin Filter Bioreactor

This reactor is fitted with a spin filter on the agitator. The major advantage of this bioreactor is that it can retain cells even at a dilution rate higher than the maximum specific growth rate.

## 2.7. Techniques of cultivation

### 2.7.1. Batch cultivation

This is the traditional way of plant cell culture. Here plant cells are cultivated in a 'closed system' supplied with some amount of medium. The major draw back of batch culture is there will be the depletion of nutrients after some time. This seems to affect the production of the metabolite and growth of cells (Sajc 2000).

### 2.7.2. Fed-Batch cultivation

In fed-batch cultivation, fresh nutrients are supplied periodically. This helps to avoid the inadequacy of nutrients. For producing ginseng from *Panax notoginseng* cell suspension cultures, fed-batch cultivation has been used (Jhang et al., 1996).

## 2.7.3. Continuous cultivation

In this type of cultivation, medium is continuously fed into the culture so that a constant growth environment is maintained (Gaden 1959). For the cultivation of *Podophyllum hexandrum* (Farkya et al., 2004) and *Catharanthus roseus* (Park et al., 1990) this technique has been used. The maintenance of long term sterility is the major problem associated with continuous cultivation.

# Sub-chapter 2

## 2.8. Hairy root culture

*Agrobacterium rhizogenes* is a gram negative soil bacterium that belongs to the family Rhizobiaceae (Chilton et al., 1977). It induces a disease called hairy roots in plants. When this bacterium infects a wounded plant part, it transfers the T DNA of Ri (root inducing) plasmid which is induced by acetosyringone produced from the wounded plant part. This causes the production of hairy roots (Nilsson and Olsson 1997). Agrobacterium uses the unusual amino acids called opines secreted by hairy roots as food (Weising and Kahl 1996).

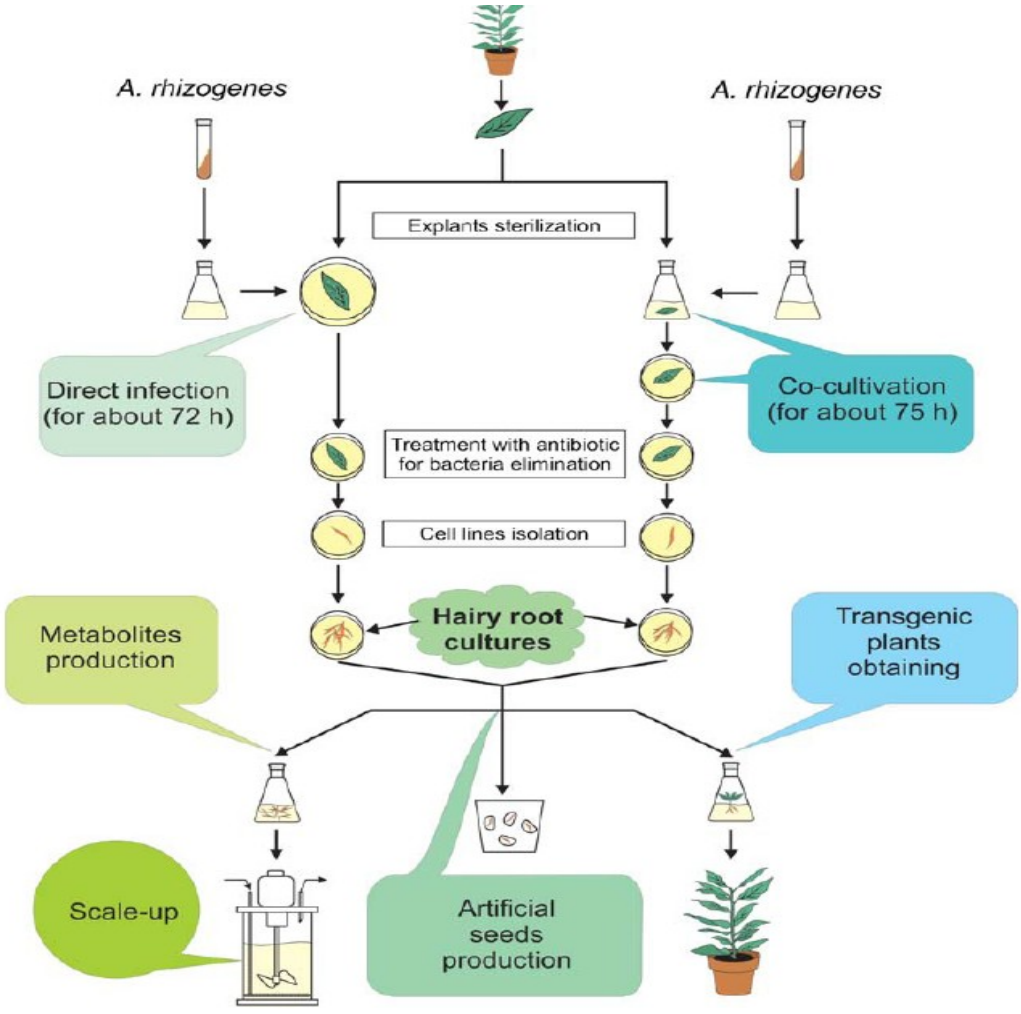


Figure 2.2. Flow chart for the production of hairy roots (Pavlov et al., 2007).

Figure 2.2 shows the main steps involved in hairy root induction and their application.

## 2.8.1. Advantages of hairy root culture

1. Fast growth in a hormone free media.
2. Profusely branched.
3. Genetic stability.
4. Abundant and stable production of secondary metabolites (Rhodes et al., 1990; Shanks and Morgan 1999).
5. Hairy roots produce many metabolites which are produced in very less quantity in normal roots.

Because of these properties, hairy roots have become an alternative source for the production of many valuable secondary metabolites including medicinally important enzymes.

Some metabolites produced by hairy root cultures are shown in Table 2.2.

## Table 2.2. Metabolites produced by hairy root cultures

| **Secondary metabolite** | **Hairy root culture** | **Biological activity** | **Reference** |
|---|---|---|---|
| Ajmalicine | *Rauwolfia micrantha* | Antihypertensive | (Sudha et al., 2003) |
| Betalains | *Beta vulgaris* | Colorant | (Weathers et al., 2005) |
| Artemisinin | *Artemisia annua* | Antimalarial | (Pavlov and Bley 2006) |
| Syringin | *Saussurea involucrata* | Antifungal | (Fu et al., 2006) |
| Taxol | *Taxus brevifolia* | Anticancer | (Huang et al., 1997) |

## 2.8.2. Beet root

Beet root or *Beta vulgaris* is a common vegetable that belongs to the family Chenopodiacea. It is considered as a major source for the pigment betalaines. This pigment consists of red-violet betacyanins and yellow betaxanthins which are soluble in water. Betanin, which is the major component of betacyanin is approved as a food colorant by the European Union (Moreno et al., 2008). They are also used in cosmetics and drugs (Dornenburg and Knorr 1996).

### 2.8.3. Hairy roots from beet root

Some experiments have been carried out on beet root hairy root cultures. Atanas Pavlov et al (2005) cultivated beet root hairy root in 50ml MS medium with 30g/l sucrose in the dark and they got maximum cell growth on $12^{th}$ day of cultivation which was 1.4g/flask accumulated dry biomass. The maximum betalain amount achieved was 42.2mg/flask which was on day 15 of cultivation (Pavlov et al., 2005). Moreno et al(2008) reported that continued pigment accumulation from *Beta vulgaris* hairy root cultures can be obtained by brief exposure to acidic medium (pH 2) and then return to standard medium (Moreno et al., 2008).

## 2.9. Consumption of Sugar

Sucrose has proved to be the most suitable carbon source for the production of secondary metabolites. Sucrose was hydrolysed completely into glucose and fructose on $6^{th}$ day of hairy root cultivation (Pavlov et al., 2005). This rapid hydrolysis was found to be proportional to the growth of hairy roots and betalain production (Pavlov et al., 2005).

## 2.10. Changes in major nutrients

MS medium contains many nutrients which are essential for the growth of hairy roots.

### 2.10.1. Nitrogen source

A nitrogen source is very essential for the pigment production and growth of hairy roots. Ammonium and nitrate compounds are the major nitrogen sources. These ions are used up swiftly in the beginning of the cultivation.

## 2.10.2. Phosphate ions

Experiments proved that phosphate ions also play an important role in the hairy root growth as well as secondary metabolite production. The growth and pigment production were found to be related with the consumption of phosphate ions (Pavlov et al., 2005). Figure 2.3 shows the phosphate and nitrate consumption profile of *Beta vulgaris* hairy roots as per literature.

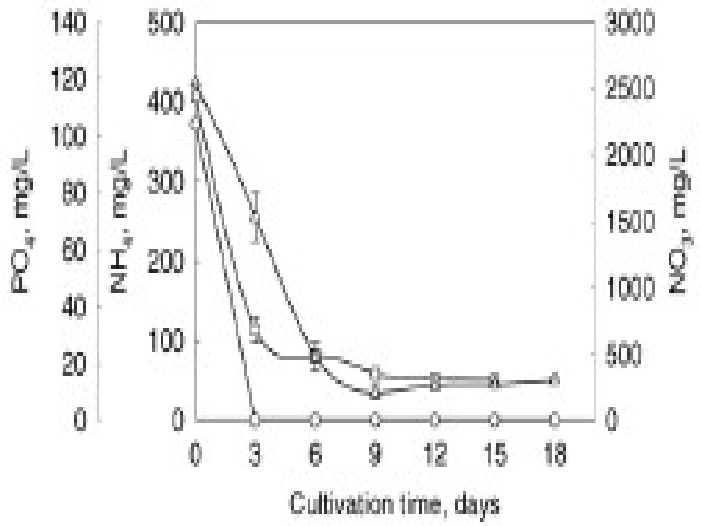


**Figure 2.3.** Phosphate (Δ), ammonium (○) and nitrate (□) consumption profiles of *Beta vulgaris* hairy roots (Pavlov et al., 2005)

## 2.11. Role of plant hormones on hairy root culture

Different plant growth regulators seem to have different effects on biomass production and secondary metabolite formation from hairy roots. Much research has been carried out to investigate the effect of endogenous and exogenous plant growth hormones on the hairy root growth. According to Crozier et al (2000), these externally applied hormones influence the secondary metabolite formation and cause changes in the morphology (Crozier et al., 2000).When auxin was added to *Lithospermum flavum* root cultures the coniferin production was increased (Lin et al., 2003). On the contrary, in the hairy roots of *Artemisia annua,* the secondary product (artemisin) accumulation was repressed by the addition of indole 3 acetic acid (IAA) while naphthalene acetic acid (NAA) increased the production (Weathers et al., 2005).

Likewise cytokinin also has different effects on different hairy root cultures. For example, addition of cytokinin could not produce any effect on the growth and secondary metabolite production in *Hyoscyamus muticus* hairy root cultures (Vanhala et al., 1998), but Saurwein et al. (1992 ) reported that an improvement in the production of alkaloid and growth was seen in *Hyoscyamus albus* hairy root cultures by the addition of cytokinin and auxin in the ratio 1:1 (Saurwein et al., 1992). Phenolic biosynthesis was increased in the hairy root culture of *Panax ginseng* by the addition of benzyl amino purine and kinetin (Jeong et al., 2007).

# CHAPTER 3

# MATERIALS AND METHODS

# Sub-chapter 1

# Callus culture of *Lavandula augustifolia* 'Hidcote'

## 3.1. Plant material of Lavandula

The lavandula seeds were obtained from Garden Stuff Ltd, Christchurch, New Zealand.

## 3.2. Sterilization and planting of seeds

The seeds were washed under running tap water. Then were cleaned with the detergent Savlon and washed again with sterile water. Then the seeds were surface sterilised with 70% v/v ethanol for 1 minute followed by soaking the seeds in 3% (v/v) sodium hypochlorite for 10 minutes. Then the sterilant was washed off using sterile distilled water (3´ 50ml) inside the laminar air flow hood. Then the seeds were inoculated in sterile bottles containing Muraschige and Skoog (MS) medium with sucrose 30g/l and 1.8g/l gelrite and kept in an incubator at $25^0$C under continuous illumination of 70 μmol $m^{-2}$ $s^{-2}$. The media had been sterilised at $121^0$C for 15 minutes. The seeds started germinating after one week. The composition of Muraschige and Skoog (1962) medium is shown in Table 3.1.

## 3.3. Development of callus cultures

The callus cultures were produced from the hypocotyl fragments of plantlets. Hypocotyl portions were harvested, cut in pieces with a sterile scalpel and placed in sterile petri plates containing Murashige and Skoog medium (1962) with 3% sucrose, 1.8g/l gel rite and two different hormone combinations: indole-3 acetic acid (IAA) 2ppm + 1ppm kinetin or 1ppm IAA +2ppm kinetin. The plates were incubated at $25^0$C under an illumination of 11μmol$m^{-2}s^{-2}$ measured using a photometer (LI CDR, model LI- 189, USA).

## 3.4. Subculturing of callus cultures

The callus cultures were subcultured on fresh plates every two weeks into MS medium containing 2mg/l IAA and 1mg/l kinetin with 30g/l sucrose and 1.8g/l gelrite.

**Table 3.1. Composition of Murashige and Skoog medium** (Muraschige and Skoog 1962)

| **Component** | **Concentration in the medium mg/l** |
|---|---|
| **Macronutrients** | |
| $NH_4NO_3$ | 1650 |
| $KNO_3$ | 1900 |
| $MgSO_4.7H_20$ | 370 |
| $CaCl_2.2H_2O$ | 440 |
| $KH_2PO_4$ | 170 |
| **Micronutrients** | |
| $H_3BO_3$ | 6.2 |
| $MnSO_4.4H_2O$ | 22.3 |
| $ZnSO_4.7H_2O$ | 8.6 |
| KI | 0.83 |
| $Na_2MoO_4.2H_2O$ | 0.25 |
| $CuSO_4.5H_2O$ | 0.25 |
| $CoCl_2.6H_2O$ | 0.025 |
| Fe-EDTA-Na salt | 40 |
| Nicotinic acid | 0.5 |
| Thiamine HCl | 0.1 |
| Pyridoxine HCl | 0.5 |
| Glycine | 2 |
| Myoinositol | 100 |
| Sucrose | 30000 |

## 3.5. Suspension cultures

Friable white calli were used to produce suspension cultures. About 2 grams of freshly harvested calli were put in 50 ml of sterile (121º C, 15 min) full strength MS medium containing 30g/l sucrose, 1mg/l IAA and 1mg/l kinetin and MS medium with 2 mg/l IAA and 1 mg/l kinetin without agar in 250ml flasks. The cultures were incubated

at 25ºC on a shaker agitated at 100 rpm and and kept illuminated at 11µmolm$^{-2}$s$^{-2}$. The cultures were maintained by subculturing into fresh MS medium containing 1 mg/l IAA and 1 mg/l kinetin every two weeks.

## 3.6. Callus culture in MS media with different hormone concentrations

The cells (about 0.3g fresh weight) were cultured in 250ml shake flasks containing 30ml of sterile MS medium in the following strengths and hormone combinations:

1. Full MS with the hormone combination 1 mg/l IAA and 1 mg/l kinetin.
2. Full MS with the hormone combination 2 mg/l IAA and 1 mg/l kinetin.
3. Full MS with 2 mg/l IAA and 1mg/l BAP (benzyl amino purine).
4. Full MS media with 2 mg/l IAA and 2 mg/l BAP.

The flasks were shaken at 100rpm at an illumination of 11 µmolm $^{-2}$s$^{-2}$ at 25$^{0}$C. The experiments were conducted in triplicate.

## 3.7. Batch cultivation of Lavandula cells in 2 L stirred tank bioreactor

Batch cultivation was carried out in a 2L stirred tank bioreactor (New Brunswick Scientific Co, Edison, New Jersey model, Q 544000, ) with a working volume of 1.5 L. Basic MS medium with a hormone concentration of 2ppm IAA and 1ppm kinetin (that is, the medium that had the maximum production in shake flask culture) was used for the cultivation. The impeller agitation speed was 100rpm and a light intensity of 11µmolm$^{-2}$s$^{-2}$ was provided. The pH of the medium was adjusted to 5.8 before autoclaving at 120ºC for 15 minutes. pH was recorded throughout the experiment. Temperature was maintained at 25 $^{0}$C by circulating chilled water from chiller unit into

the cooling finger of the bioreactor. Water was passed at 5$^{0}$C through the exit gas condenser to reduce the broth evaporation. Temperature was controlled automatically. The samples (30ml) (as two separate 15 ml samples for duplicate values) were collected every three days to analyze for dry weight, sugar content, phosphate and nitrate concentration.

## 3.8. Analysis

The pigment and cell growth were determined every three days.

### 3.8.1. The blue pigment

This was measured by the following method. A 3ml portion of the culture broth was taken. It was centrifuged at 10000g for 5 minutes (HITACHI, high speed refrigerated centrifuge, himac CR 22 G II). The supernatant was discarded. To the pellet 3ml acid methanol (1% HCl) was added. The mixture was centrifuged again at 10000g for 5 minutes. Then 200 micro litres of 3M NaOH was added. Then the absorbance was measured spectophotometrically (HITACHI, U-2000 spectrophotometer) at a wavelength of 590 nm against a blank of 3 ml acid methanol (1% HCl) and 200 microlitres of 3 M NaOH. 590nm is considered as the maximum absorbance of blue pigment at alkaline pH (Nakajima et al., 1990).

### 3.8.2. Cell growth

The fresh weight and dry weight of the cells were measured. The cell suspension (30 ml for shake flask culture and 15 ml for bioreactor culture) was filtered through a pre weighed filter paper by vacuum filtration. Then the cells were washed with 15 ml (bioreactor) or 30 ml (shake flask) distilled water two times. Then the cells with the filter paper (GFC, Whatman filter paper, 90 mm, Catalogue no: 1822080) were weighed in an aluminium boat of known weight and the fresh weight was calculated. For the dry weight measurement, the aluminium foil was dried overnight and weighed. Then the

filter paper with the callus was dried on the foil overnight at 70ºC in an oven (WATVIC, Catalogue number: IM24, Clayson laboratories, New Zealand). This was then cooled to
room temperature in a desicator for 30 minutes and weighed again. Then the dry weight of the callus was calculated.

### 3.8.3. Specific absorbance

The specific absorbance of the blue pigment was calculated as the absorbance per gram dry weight of the callus ($A_{600}/g^{-1}$).

### 3.8.4. Sucrose analysis

During the bioreactor experiment, after the cells were removed, the media were filtered through sterile 0.22µm syringe driven filter unit (Miller- GS, catalogue no. SLGS033SS) and stored in -70° C freezer (Forma Scientific, model 8338, S/N 81899-538). After the final day of the experiment, these samples were thawed and analysed for sucrose, phosphate and nitrate. High Performance Liquid Chromatography (HPLC) (Dionex Corporation) fitted with P680 HPLC pump, ASI-100 automated sample injector and thermostatted column compartment was used to measure the sucrose content. About 10µl of samples were injected into the HPLC machine. The system was equipped with Sugar Pack 1 column (Waters, Milford, Mass).The temperature of the column was set at 80ºC. 0.47µm sterile membrane filtered (White, Gridded, Catalogue number: HAWG047S1, Millipore Corporation, Bedford) calcium EDTA (ethylene diamine tetraacetic acid) with a concentration 500milligram per litre of deionised water was used as the mobile phase. The flow rate was 600µl/minute

### 3.8.5. Measurement of phosphate and nitrate

Phosphate and nitrate ion concentrations in the media were measured by Ion exchange chromatography. The machine was fitted with a conductivity detector; anions (nitrate and phosphate) were separated on ICS 2000 Anion column, Ionpa CAS11HC

Dionex) (4×250mm). 32 millimolar Potassium hydroxide was used as the eluent. The pump pressure was set at 2300 psi. The temperature of the column was 30° C and the sample flow rate was 1ml/ minute.

## Sub-chapter 2

### Hairy root culture of *Beta vulgaris*

## 3.9. Plant material of Beet root

Seeds were purchased from Garden Stuff Ltd, Christchurch, New Zealand. The seeds were washed with tap water for 5 minutes. Then were cleaned with the detergent Savlon and washed again with sterile water until the soap particles were washed off. They were then immersed in 70% ethanol v/v for 30 seconds. Surface sterilization was done after this using the sterilant, 5% (v/v) sodium hypochlorite for 10 minutes. Then the sterilant was removed by washing in the sterile distilled (3× 50ml) inside a laminar air flow hood. Then the seeds were inoculated in sterile bottles containing sterile full strength MS medium with sucrose 30g/l and 1.8g/l gel rite using sterile scalpel and kept in an incubator at $25^0$C under continuous illumination of 70 µmol $m^{-2}$ $s^{-2}$. The germination started after one week.

## 3.10. Steps involved in hairy root induction

1. Three week old sterile leaves were harvested from plantlets. Then incisions were made on these leaves.
2. They were then infected using a 48 hour old culture of *Agrobacterium rhizogenes* strain Imp 5794 purchased from Landcare Research, Auckland, and co-cultivated in sterile MS solid medium for two days. The bacteria were cultured in yeast mannitol broth (YMB) agar medium (sterile, 121ºC, 15 minutes) at 30ºC and maintained by sub culturing into fresh plates every one month and preserved at 4ºC. The composition of the YMB medium is given in Table 3.2.
3. After two days the explants were transferred into sterile MS agar bottles containing 500mg/l cefotaxime (antibiotic) to kill the bacteria and incubated at $25^0$C in dark.
4. They were sub cultured after one week into the same medium. Antibiotic was eliminated from the medium after two sub cultures.

5. Hairy roots started being produced from the infection site after two weeks.
6. After three passages in agar medium the roots were excised and cultured into100 ml sterile (121ºC, 15 minutes), half strength MS liquid media in 500ml flasks agitated at 100rpm in the dark.

## Table 3.2. Composition of YMB medium

| Component | Concentration g/l |
|---|---|
| Magnesium sulphate | 0.2 |
| Potassium phosphate dibasic | 0.5 |
| Agar | 15 |
| Mannitol | 10 |
| Sodium chloride | 0.1 |
| Yeast extract | 0.4 |

## 3.11. Hairy root experiment in half and full strength MS media

In the experiment, about 0.25 gram fresh weight (for each flask) of hairy root was inoculated separately in 100ml half strength and full strength MS liquid medium in 500 ml conical flasks. Then they were shaken at 100 rpm at $25^0$C in the dark and the experiment was carried out for18 days.

## 3.12. Hairy root culture with the addition of plant growth regulators

The experiments were conducted using 0.25 gram fresh weight of hairy root in 100ml of MS media with the following concentrations of auxins and cytokinins:

### 3.12.1. Auxins

a. 0.5 mg per litre of naphthalene acetic acid (NAA)
b. 0.5ppm indole, 3 acetic acid (IAA)

### 3.12.2. cytokinin

c. 0.5 ppm BAP (benzyl amino purine)

## 3.13. Betalain extraction

The hairy roots were filtered from the broth using a 90mm GFC, Whatman filter paper (Catalogue no: 1822090). The roots were then washed using sterile distilled water (2× 100 ml). The excess water was wiped off with a sterile tissue paper. The pigments were extracted from the fresh hairy roots using 50% v/v ethanol (in sterile water). For this about 10 ml of ethanol per 1 gram fresh weight of hairy roots was added and the roots were ground using a mortar and pestle. The homogenate was centrifuged at 10000g for 10 minutes (HITACHI, high speed refrigerated centrifuge, himac CR 22GII). Then the supernatant was collected. This procedure was repeated three times. The supernatants were pooled. The betalain determination was done in the supernatant.

## 3.14. Measurements

The measurements were made every three days for 18 days.

### 3.14.1. Content of betalain

The determination of betaxanthins and betacyanins was done spectrophotometrically according to the method of Nilsson (1970) in the following way. Almost 95% of the betacyanins is composed of betanin, isobetanin and vulgaxanthin-1 comprises 95% of betaxanthins (Nilsson 1970).

Spectrophotometric measurements were taken at 476, 538 and 600nm (HITACHI, U-2000 spectrophotometer). 50 %( v/v) ethanol in distilled water was used as the blank. The absorption of different pigments was calculated using the following equation:

x =1.095(a-c)

y =b-z-x/3.1

z =a-x

where a, b and c are the absorption at 538, 476 and 600nm, respectively. Betanin absorption is *x, y* is that of vulgaxanthin and *z* is the absorption of impurities. From these, the concentration of betanin and vulgaxanthin can be calculated using the equation Epsilon $E^{cm1}_{1\%}$=750 for vulgaxanthin-1 and for betanin, $E^{1cm}_{1\%}$=1120.

For example if a = 0.079, b= 0.102 and c= 0.015

Then x = 1.095 (0 .079 - 0.015)

= 1.095 * 0.064 = 0.0700

z = a-x

= 0.079-0.0700 = 0.009

y =b-z-x/3.1

=0.102-0.009- 0.0700/3.1

=0.102-0.009-0.022

= 0.071

% Concentration of betanin = Absorption of betanin (x) / 1120

= 0.0700/1120

= 0.0000625 g /100 ml =0.000625 g/l

% Concentration of vulgaxanthin = Absorption of vulgaxanthin (y)/ 750

= 0.071/ 750

= 0.0000946 g/ 100ml = 0.000946 g/ l

The values were then multiplied by the dilution factor if the samples were diluted.

## 3.14.2. Measurement of fresh and dry weight

The growth of the hairy roots was estimated by determining the fresh and dry weights. The roots were filtered from the medium using a 90mm GFC Whatman no: 1822090 filter paper. The media was washed off with distilled water (2´ 100ml). The excessive water was wiped off using a tissue paper and the fresh weight was measured. For the dry weight measurement, an aluminium foil container was dried for 2 days in an oven (WATVIC, Catalogue number: IM24, Clayson laboratories, New Zealand) at $70^0$C and weighed. Then the hairy roots were dried on the pre-weighed foil container in the oven at $70^0$C for 48 hours. Then the dry weight was measured.

## 3.14.3. Specific growth rate

Specific growth rate was calculated using the following equation,

Specific growth rate, $\mu = Ln (N_2 / N_1) / (t_2 - t_1)$ where $N_1$ and $N_2$ are biomass concentration (dry weight) at time 1 ($t_1$) and 2 ($t_2$) respectively (Levasseur et al., 1993).

## 3.14.4. Measurement of sucrose

After the hairy roots were removed, the media were filtered through sterile 0.22µm syringe driven filter unit (Miller- GS, catalogue no. SLGS033SS). Then the samples were stored at -70º C in a freezer (Forma Scientific, model 8338, S/N 81899-538) till analysed for sucrose, phosphate and nitrate. They were then thawed for the analysis after every run. The sucrose content was measured by High Performance Liquid Chromatography (HPLC) (Dionex Corporation) fitted with P680 HPLC pump, ASI-100 automated sample injector and thermostatted column compartment. About 10µl of samples were injected into the HPLC machine. The system was equipped with Sugar Pack 1 column (Waters, Milford, Mass). The temperature of the column was set at 80ºC. The mobile phase was 0.47µm sterile membrane filtered (White, Gridded, Catalogue number: HAWG047S1, Millipore Corporation, Bedford) calcium EDTA (ethylene diamine tetraacetic acid) with a concentration 500milligram per litre of deionised water. The flow rate was 600µl/minute.

## 3.14.5. Measurement of phosphate and nitrate

Ion exchange chromatography was used to measure the concentrations of phosphate and nitrate ions in the medium. The machine was fitted with a conductivity detector; anions (nitrate and phosphate) were separated on ICS 2000 Anion column, Ionpa CAS11HC (Dionex) (4×250mm). The eluent was 32 mM KOH. The temperature of the column was 30ºC and the pump pressure was set at 2300 psi. The flow rate of the sample was 1 ml/ minute.

## 3.14.6. pH measurement

The pH of the culture medium was determined using an electronic pH meter (Hanna, Hi 991302, USA) after the hairy roots had been filtered off.

# CHAPTER 4

# RESULTS AND DISCUSSION

## Subchapter 1

### 4.1. Callus cultures of Lavandula

The hypocotyl fragments of Lavandula were cultivated in two different hormone combinations namely 2ppm IAA+ 1ppm kinetin and 1ppm IAA+2ppm kinetin in solid media. The friable calli and increase in callus size without much browning of the callus were observed in MS medium containing the hormone combination 2 mg/ l IAA + 1 mg/l kinetin. This might be because more auxin concentration led to fast growth and more cytokinin caused browning of the callus. So the same was used for further subcultures. A typical callus culture of Lavandula grown in agar medium (MS+2 IAA + 1 kinetin) is shown in Figure 4.1.

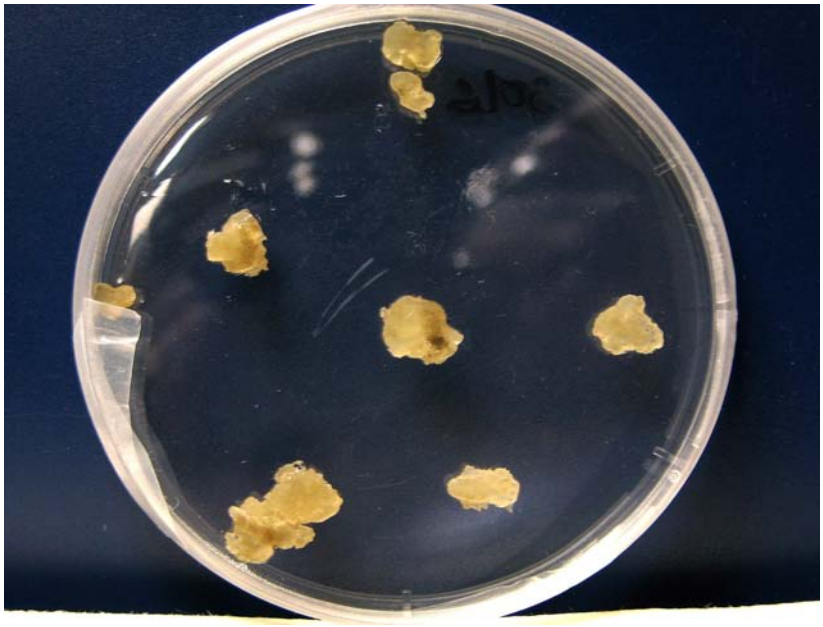

**Figure 4.1.** Callus culture of Lavandula in MS+2 mg/l IAA+1 mg/l kinetin

## 4.2. Suspension culture

The suspension culture was obtained in MS media containing both the above mentioned hormone combinations. But the cultures were maintained in liquid MS

medium containing hormones 1 mg/l IAA and 1mg/l kinetin. This medium supported the longer maintenance of the cultures without much browning of the callus. Figure 4.2 shows the suspension culture of Lavandula before the blue pigment was produced. The callus started producing pigment in 3 days.

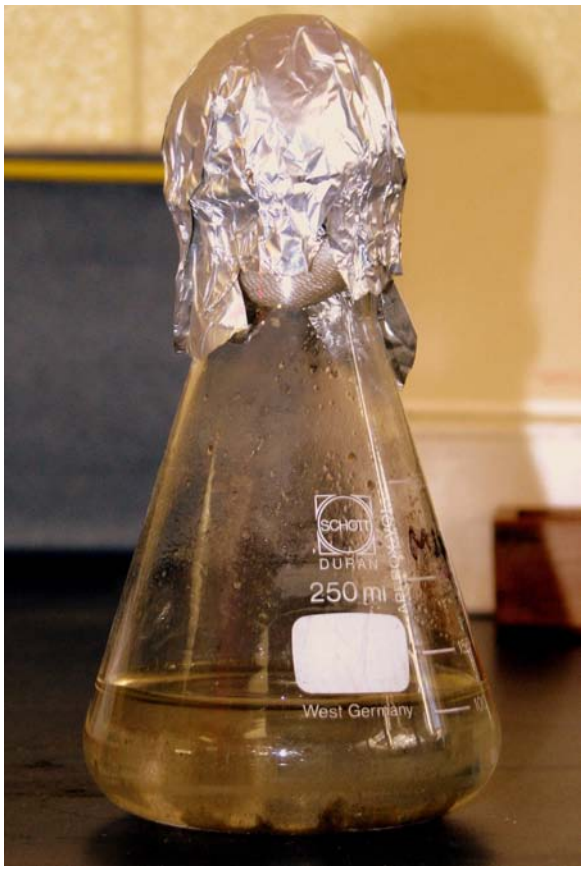


**Figure 4.2.** A suspension culture of Lavandula in full strength MS medium with 1 mg/l IAA and 1 mg/l kinetin

## 4.3. Effects of different growth hormones on callus growth and blue pigment production

To study the effects of different plant hormones on the growth of Lavandula callus and blue pigment production, the callus was cultivated in 30 ml sterile liquid MS medium with various hormone combinations as given below:

1. MS +1mg/l indole 3 acetic acid (IAA) +1 mg/l kinetin.
2. MS+2 mg/l IAA+1mg/l kinetin.

3. MS+2 mg/l IAA+1mg/l benzyl amino purine (BAP).
4. MS+2 mg/l IAA+2 mg/l BAP

The culture was started using 0.3gram fresh weight of callus. The flasks were agitated at 100 rpm at a temperature of 25ºC. The cells were cultivated at an illumination 11µmolm $^{-2}s^{-2}$.

## 4.3.1. The study of cell growth and pigment production in MS medium containing the above mentioned hormone combinations

### 4.3.1.1. Cell growth

Growth hormones are essential for growth and development of plant cells. Auxins help in the elongation of cells and cytokinins facilitate cell division. A good suspension culture can be obtained by a balanced concentration of auxins and cytokinins.

The results in Figure 4.3 show that cells grew in the first 3 days. There was no further growth until day 9. This might be because the cells are in a lag phase in which they are adjusting with the new environment. The cell growth was maximum on 15$^{th}$ day for callus culture in 2 mg/l IAA+1mg/l kinetin and 2 mg/l IAA+1mg/l BAP. The maximum growth values were 11.7 and 16.8 g/l dry weight, respectively. For the callus cultures in MS+1mg/l IAA+1 mg/l kinetin and 2mg/l IAA+2 mg/l BAP, the cells attained the highest growth on 18$^{th}$ day which were 8.23 g/l and 15.83 g/l dry weight, respectively. The cell growth is very less in the culture with 1 mg/l IAA as compared to the other cultures that used 2 mg/l IAA. This suggests that the auxin concentration is an important factor affecting the callus growth and less concentration of auxin itself leads to less growth.

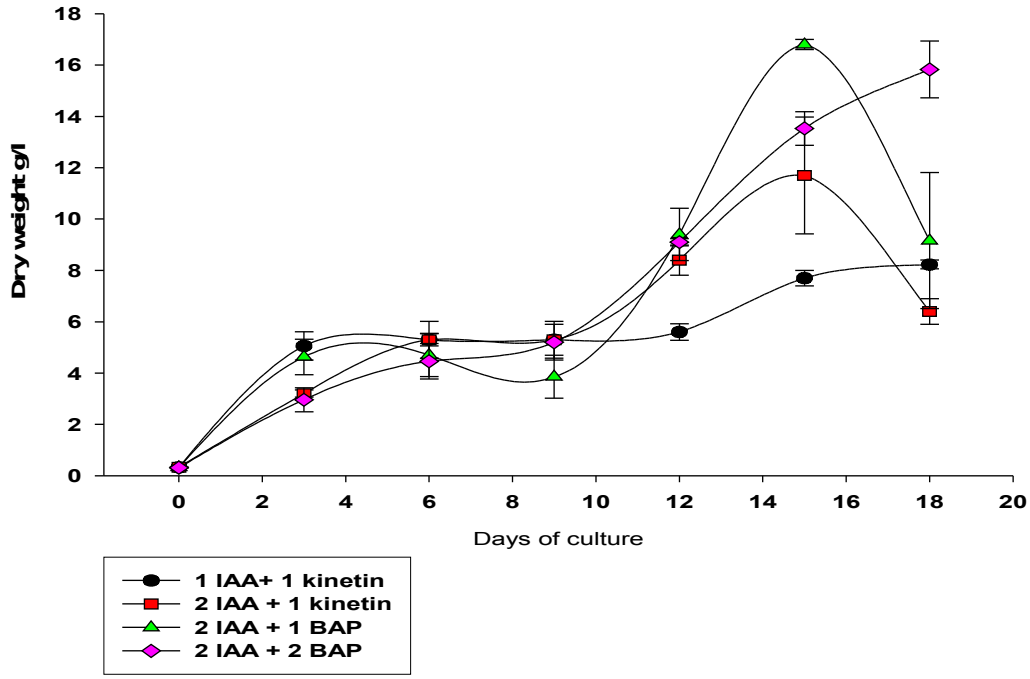


**Figure 4.3.** Dry cell weight profile of Lavandula callus cultures

#### 4.3.1.2. Blue pigment production

The specific absorbance profile of the callus cultures are plotted in the Figure 4.4 .The blue pigment was produced in all callus cultures. The callus cultures of Lavandula secrete yellow compounds, the (Z, E)-2-( 3-4-dihydroxy phenyl) ethenyl ester of 3-(3,4-dihydroxyphenyl)-2-propenoid acid and its (E,E)-isomer (≡ caffeic acid) which combines with $Fe^{2+}$ in the media to form blue pigment (Trejo-Tapia et al., 2003). In most of the experiments the maximum blue pigment production was seen in between $9^{th}$ and $15^{th}$ day. Then a decrease in the curve was seen. This can be due to low production rate, commencement of the degradation process and depletion of $Fe^{2+}$ in the media (Prakash 2006). The highest specific absorbance was found in the callus culture in MS medium containing 2 mg/l IAA and 1 mg/l kinetin (specific absorbance 8.43 $A_{600}$ / gram DW) on $9^{th}$ day and the lowest value was seen in the culture with the hormone combination 1 mg/l IAA+ I mg/l kinetin. The blue pigment productions as in different media are shown in Figure 4.5, 4.6, 4.7 and 4.8.

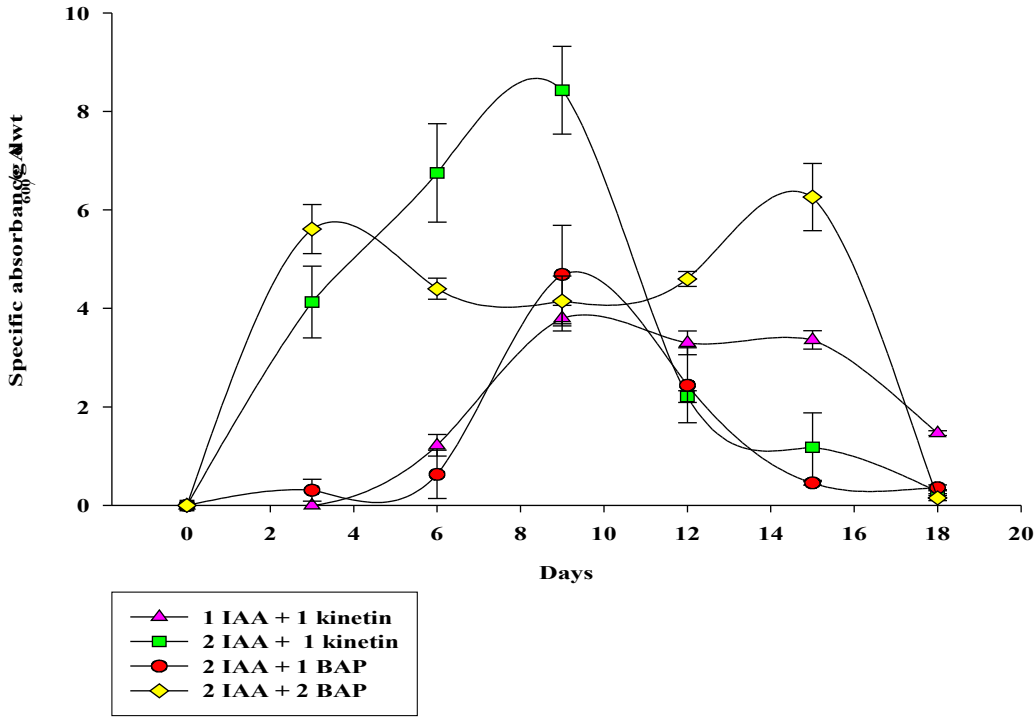


**Figure 4.4.** Blue pigment production profile of Lavandula


**Figure 4.5.** The callus culture in MS+2 mg/l IAA+ 1 mg/l kinetin on 9$^{th}$ day

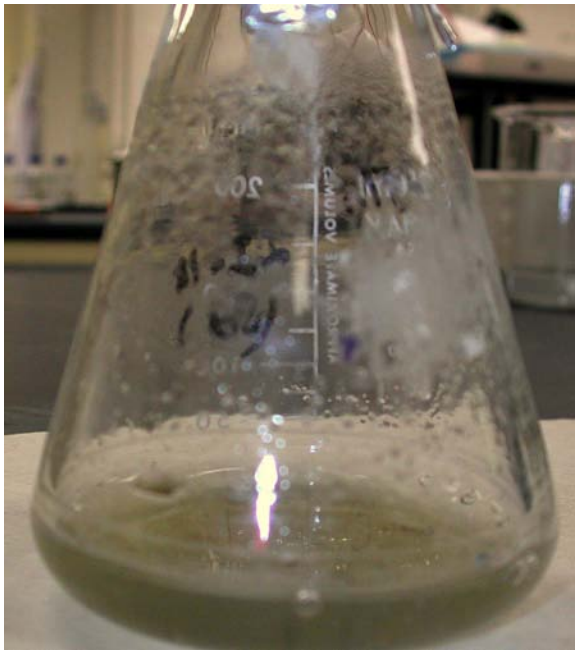

**Figure 4.6.** Blue pigment in MS+2 mg/l IAA+1mg/l BAP on 12th day


**Figure 4.7.** Callus culture in 1mg/l IAA+1 mg/l kinetin on 9th day

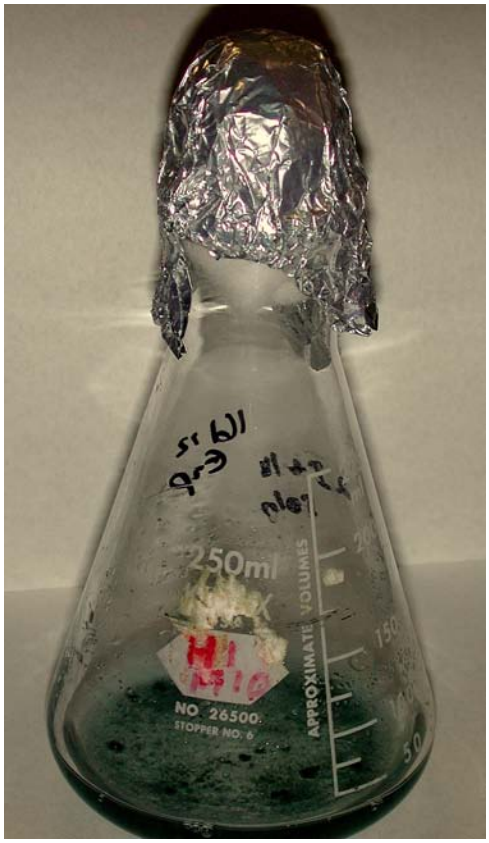


**Figure 4.8.** Callus culture in 2 mg/l IAA +2 mg/l BAP on 15th day

## 4.4. Batch cultivation of callus cells in 2L stirred tank bioreactor

### 4.4.1. Cell growth

Batch cultivation of Lavandula callus was carried out in 2L stirred tank bioreactor (Figure 4.9). A volume of 1.5 l of the media in which the maximum specific absorbance was obtained in shake flask culture (MS + 2 mg/l IAA + 1 mg/ l kinetin) was used for the culture. The growth curve (Figure 4.10) showed almost the same pattern as the shake flask culture but the biomass production was very low compared to the shake flask culture as the maximum cell growth was 2.815g/l dry weight on 9th day. This could be because the cells were damaged by the agitation as a Ruston turbine at 100 rpm agitation speed was used. Plant cells are sensitive to shear stress. Air flow and impeller generate shear stress in a bioreactor (Scragg et al., 1988). Specially designed blades for the impeller can be a better option to improve the growth and productivity.

Slow growth rate, low content of metabolites, low product secretion and genetic instability are the major problems found when plant cells were transferred from shake flasks to bioreactor (Zhong 2001).

**Figure 4.9.** Cultivation of Lavandula cells in 2L stirred tank bioreactor

## 4.4.2. Pigment production

The pigment production was also less compared to the shake flask culture. A maximum absorbance value of 0.058/ 3 ml at 590 nm and a specific absorbance of 7.6/ g dry weight were obtained on 9$^{th}$ day (Figure 4.10).

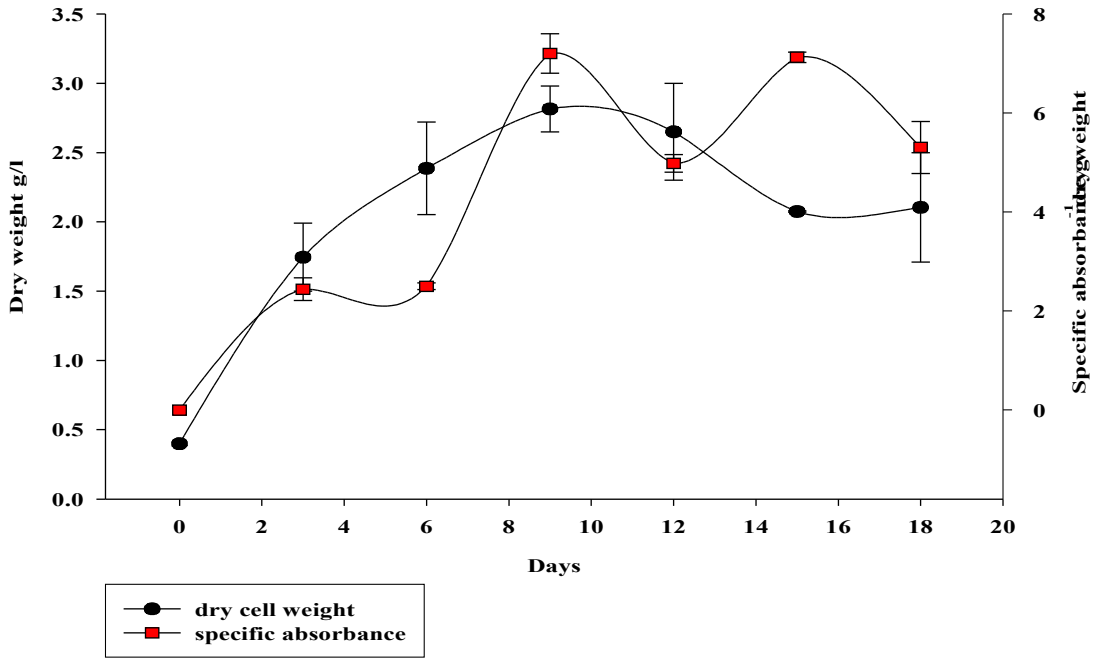


**Figure 4.10.** Dry cell weight and pigment production profile of Lavandula callus in 2L bioreactor

## 4.4.3. Nutrient consumption

30 g/l sucrose was used in the experiment. Most of the sugar molecules had been consumed by the callus cells during the first days of culture which was the preparatory period of cell division and the value reached zero by 12$^{th}$ day itself (Figure 4.11).

The nitrate and phosphate profile also show that the growth and pigment production were dependent on the consumption of these nutrients (Figure 4.11). The nitrate concentration reached a final value of 1500 mg/l from an initial concentration of 2500 mg/l and there was only half amount of phosphate detected at the end of the cultivation (60 mg/l).

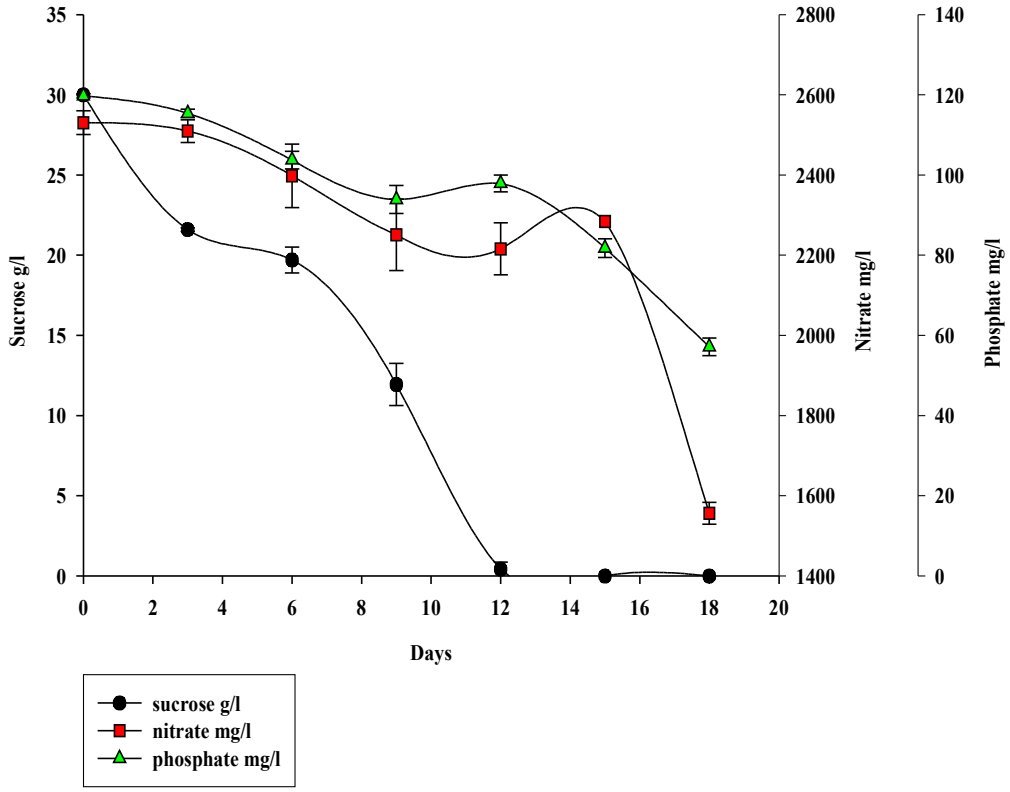


**Figure 4.11.** Nutrient consumption profile of Lavandula callus culture in 2L bioreactor

## 4.4.4. pH change

The pH was recorded every three days of the experiment. The initial pH was adjusted to 5.8. Then a decline in the value was observed in the first 8 days of the culture as the pH attained a value of 5.1. After that the pH remained almost stable during the whole period of growth. This leads to a conclusion that $CO_2$ generated by the respiration of the callus cells resulted in the decline of the pH (Figure 4.12). This might also be because of proton efflux resulted during the absorption of $NH_4^+$. The ammonium ion uptake by the root cells also might have acidified the medium (Nour and Thorpe 1994; Shin et al., 2003).

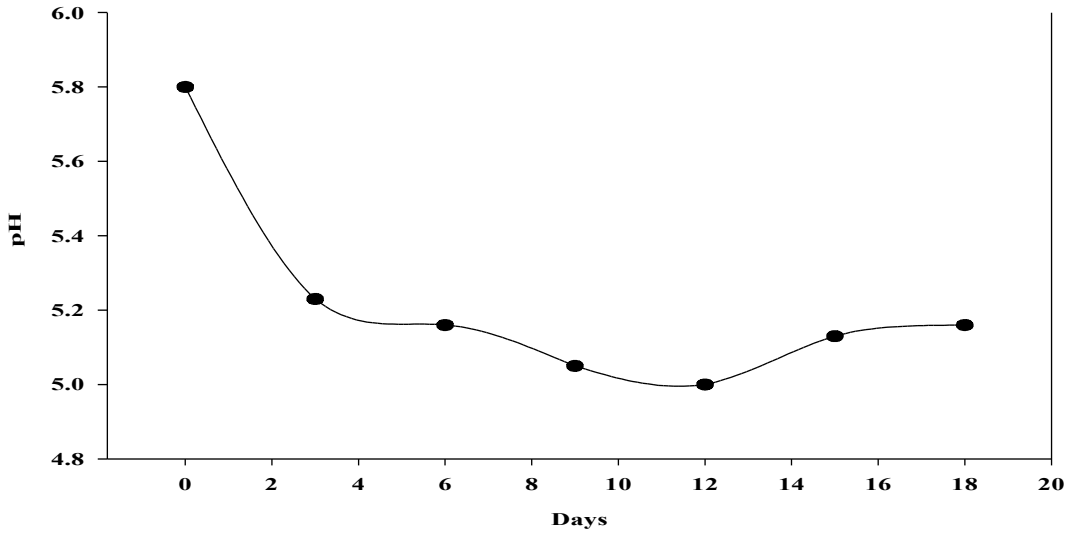


**Figure 4.12.** pH profile of Lavandula callus culture in 2L bioreactor

# Sub chapter 2

## Beet root hairy root culture

## 4.5. Beet root Seed germination

The seeds of beet root started germinating after one week on the sterile solid MS medium. A three week old beet root seedling is shown in Figure 4.13.

**Figure 4.13.** A three week old beet root seedling

## 4.6. Production of hairy roots

Two weeks after infecting the leaves with *Agrobacterium*, hairy roots began to emerge from the incision site (Figure 4.14). The roots were orange in colour with numerous branches and colourless tips. As the roots grew in size, they were firmly attached to the agar medium.

**Figure 4.14.** Three weeks old hairy roots produced from the incision site of beet root leaves grown in agar medium

## 4.7. Hairy root culture in liquid MS medium

The hairy roots when grown in liquid medium (Figure 4.15 and 4.16) were highly branched and the growth was extremely rapid when compared to growth on solid medium. This might be because in the liquid medium the nutrients could be absorbed from all surfaces of the hairy roots as the shaking enhanced uniform distribution of nutrients.

**Figure 4.15.** Hairy roots in liquid medium (15 days old)

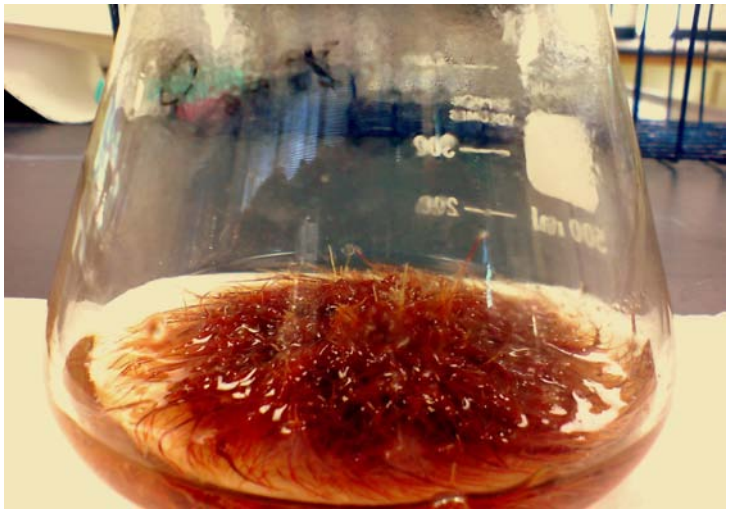

**Figure 4.16.** 15 days old hairy roots in liquid medium taken out of the flask

## 4.8. The hairy root growth in half and full MS medium

About 0.25 gram fresh weight of hairy roots was cultivated in full and half strength MS medium to analyse the cell growth and pigment production for 18 days. The roots were grown in 500ml shake flasks with 100 ml medium and agitated at 100 rpm in the dark at 25$^{o}$C. Three flasks were harvested each day for the measurement. The measured values from the three flasks were averaged.

### 4.8.1. Cell growth

The cells started growing after 3 days. The dry weight attained its maximum value on the 15$^{th}$ day of cultivation which was 11g/l and 12 g/l for half and full MS media respectively. The biomass obtained in full MS was nearly twice that of half strength MS until the 15$^{th}$ day. This shows that double concentration of nutrients resulted in the faster growth of hairy roots although the final biomass concentrations were not different. The results (Figure 4.17) show that the root growth followed phases comparable to that of callus growth, a lag phase where the cells prepare to adjust with

the new environment, a log phase in which the growth reaches the maximum level, a stationary phase and then a decline phase. A maximum specific growth rate (μ) of 0.27/day was obtained in half MS and 0.32/day was obtained in full MS medium (Figure 4.18).

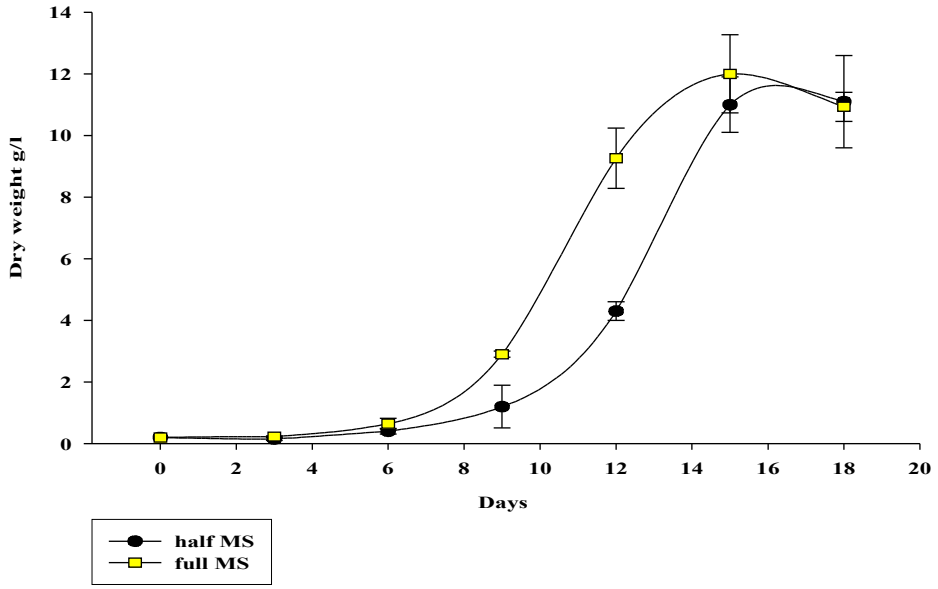

**Figure 4.17**. Cell growth profile of hairy roots in half and full MS medium

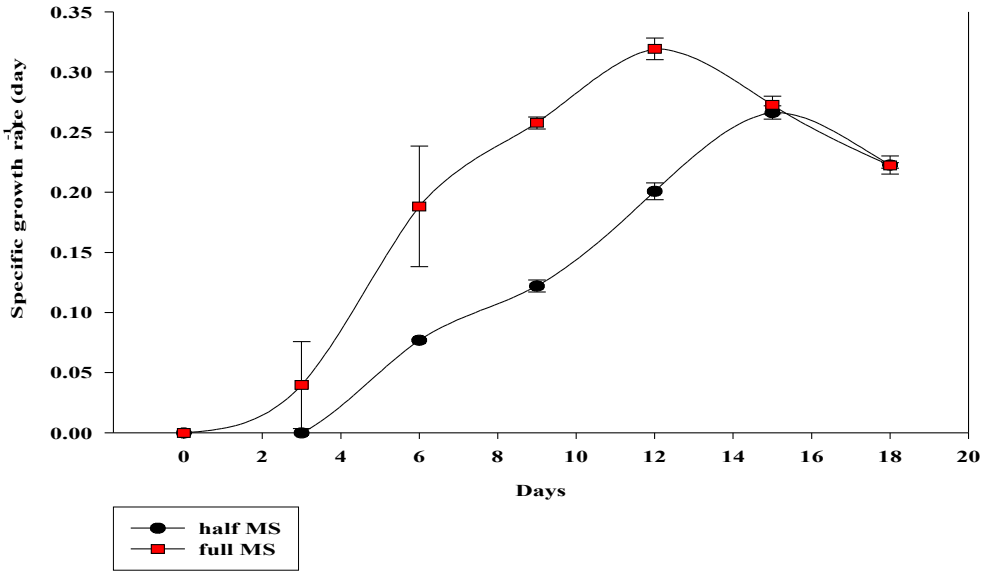


**Figure 4.18.** Specific growth rate profile of hairy roots in half and full MS medium

## 4.8.2. Betalain production

The roots synthesised betalain between 6th and 15th days (Figure 4.19). This is because betalaines production was related with the root biomass and biomass increased the most during this period. 26.69 mg of betalain was obtained on 15th day which was the maximum value for half MS and 25mg was obtained in full MS. The specific betalain production showed a maximum value of 1.8 mg/g fresh weight for half MS and 1.70 mg/g fresh weight for full MS (Figure 4.20). Therefore the specific production was not much influenced by the strength of the media. These results show that the pigment production in hairy root culture is related with the growth of the roots. The highest amount of pigment was produced when the growth was the maximum. The red pigmet extracted from the hairy root cells is shown in Figure 4.21.

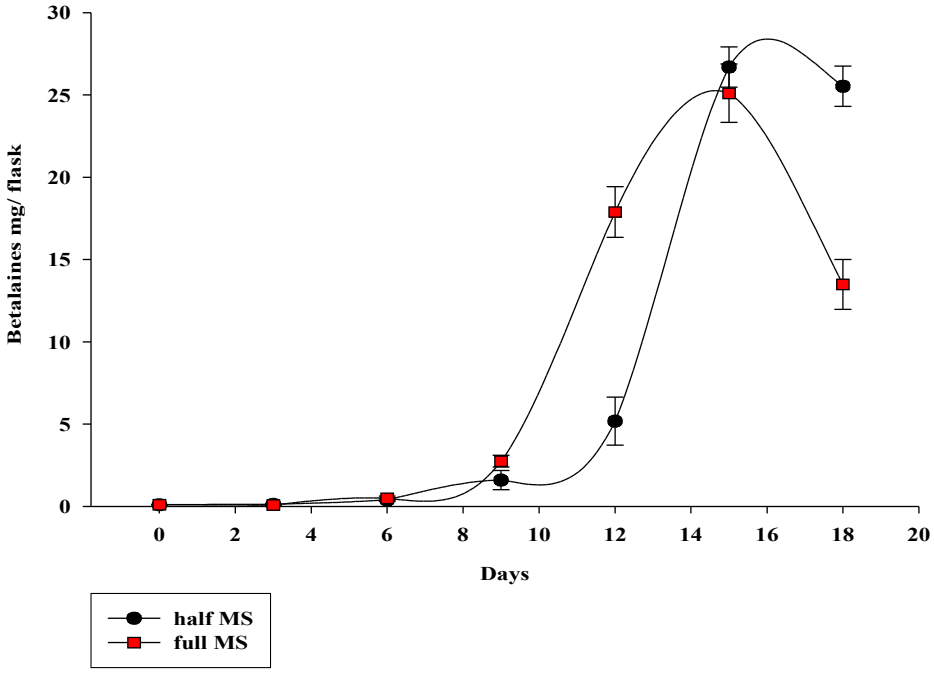


**Figure 4.19.** Pigment production profile of hairy roots in half and full MS

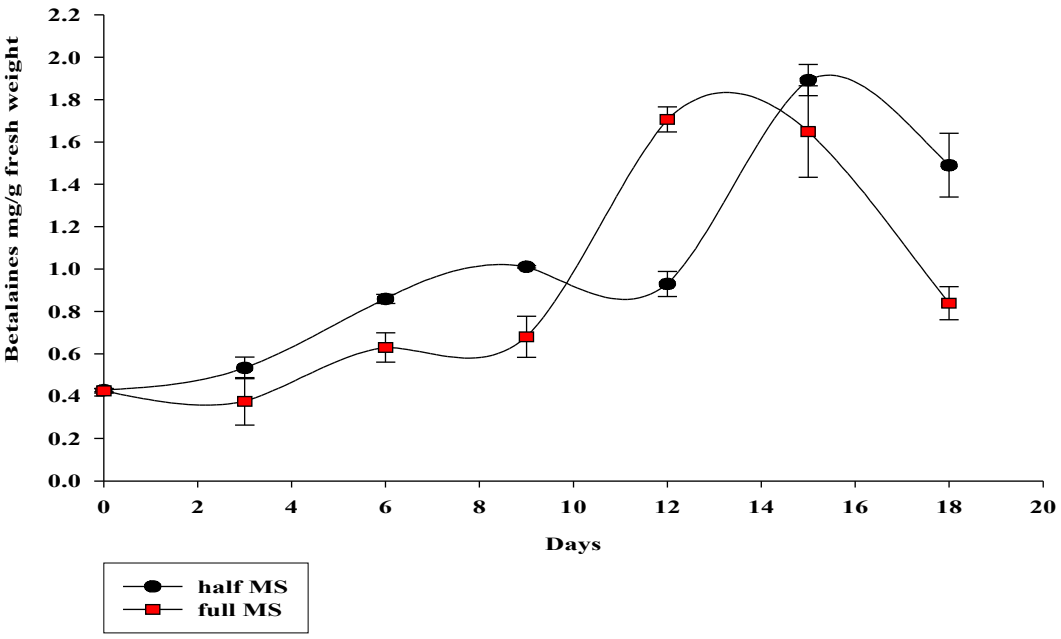


**Figure 4.20.** Specific betalaine production profile of hairy roots in half and full MS

**Figure 4.21.** Red pigment extracted from hairy root cells using 50% v/v ethanol

## 4.9. Hairy root culture in full MS with different hormone combinations

In view of its higher productivity, the further experiments were carried out using full strength MS medium. The roots were grown separately in full MS with 0.5 mg/l NAA, 0.5 mg/l BAP and 0.5 mg/l IAA to check the effects of these hormones on the cell growth and specific production. The experiments were carried out in 500 ml Erlenmeyer shake flasks with 100 ml of the medium and the flasks were agitated at a speed of 100 rpm in the dark.

### 4.9.1. Cell growth and pigment production

Cell growth and pigment production profiles in full MS with different hormones are plotted in Figures 4.22, 4.23 and 4.24. The growth and pigment production followed the similar pattern as before (Figure 4.17 and 4.19). There was less growth during the

first days that can be considered as the preparatory phase and maximum growth was reported in the exponential phase. Auxins; naphthalene acetic acid and indole 3 acetic acid did not have much effect on cell growth as the peak values were 12 g/l and 11 g/l respectively. Comparatively, specific pigment production was more in full MS with 0.5 mg/l IAA than the other cultures in which the peak value was 1.579 mg/g fresh weight. Benzyl amino purine, the cytokinin inhibited the cell growth. The maximum dry weight was 1.59 g/l and betalain produced was 1.57 mg/ flask. This might be because BAP is known to be toxic to the roots (Bunk 1997).

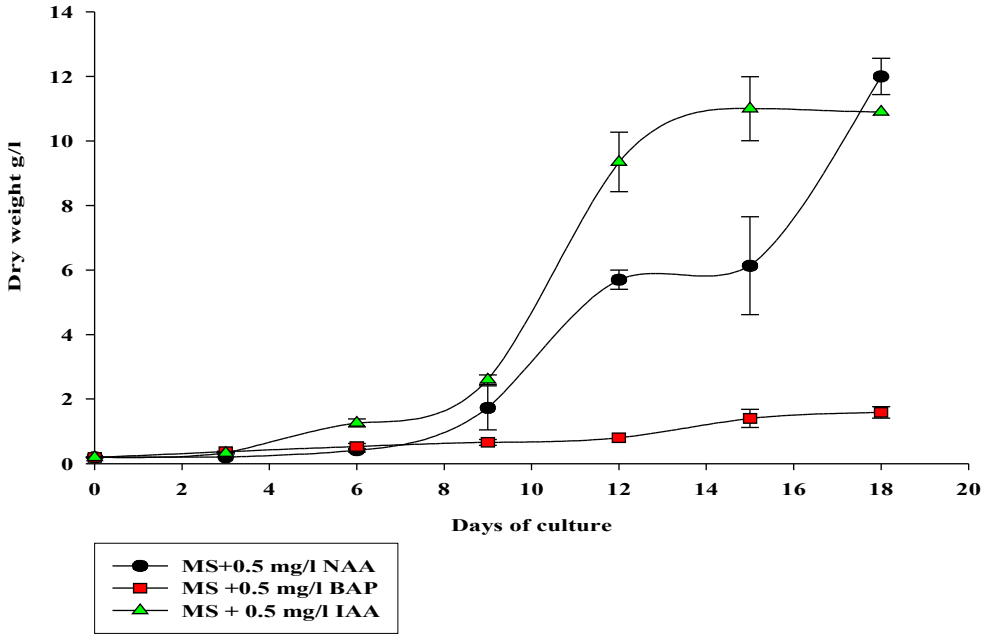


**Figure 4.22.** Dry cell weight profile of hairy roots in MS media with different hormone concentrations

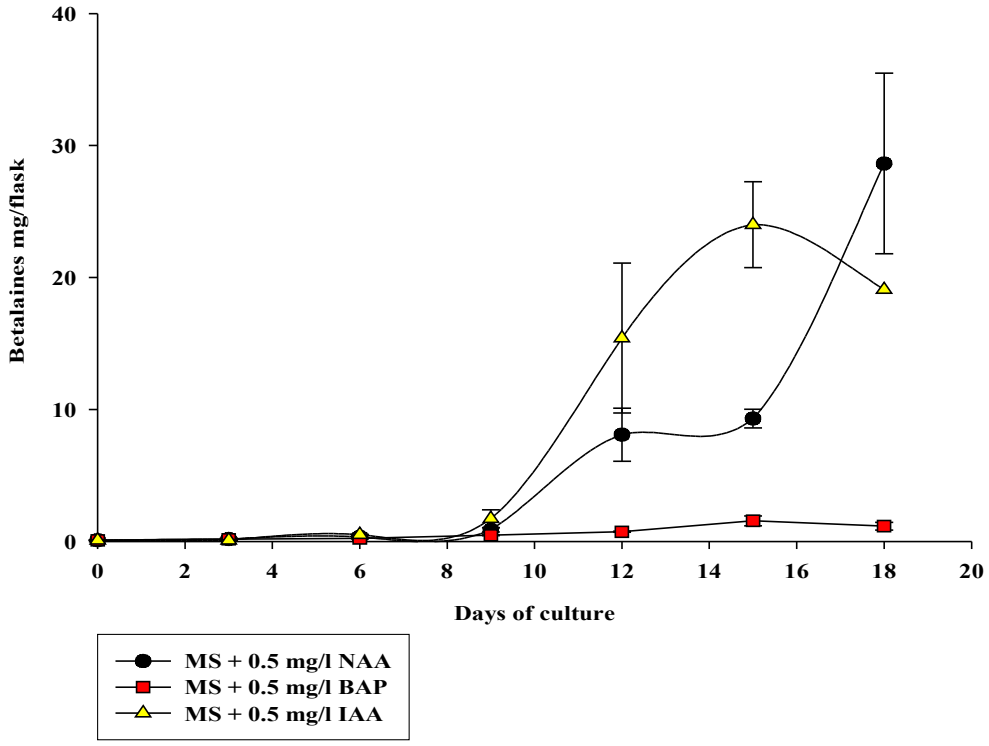


**Figure 4.23**. Betalain production profile of hairy roots in MS media with different hormone concentrations

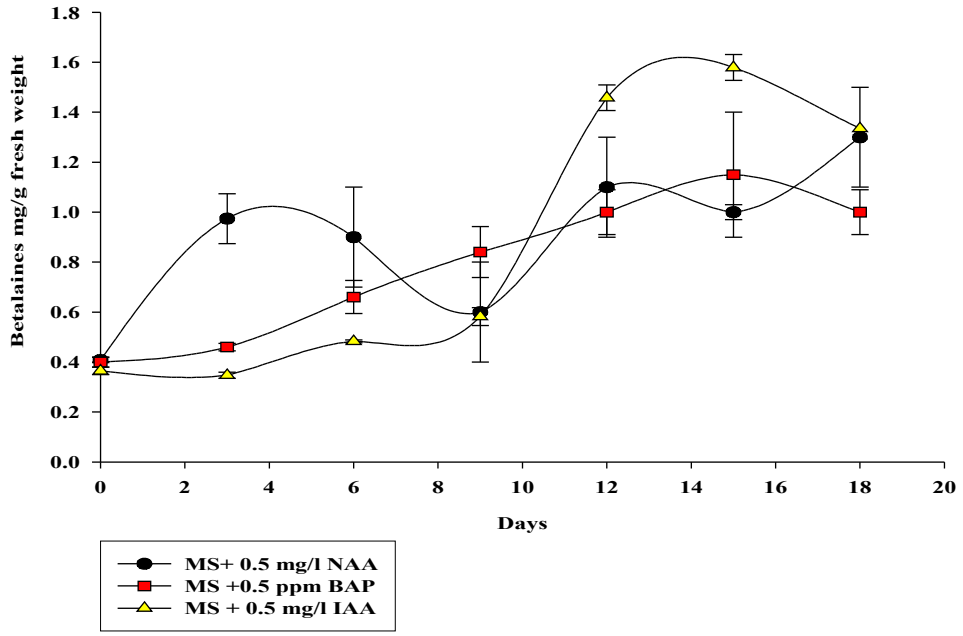


**Figure 4.24.** Specific betalain production of hairy roots in MS media with different hormone concentrations

## 4.9.2. Nutrient consumption

### 4.9.2.1. Sucrose

The sugar, nitrate and phosphate concentrations in the broth were measured during hairy root culture. Sucrose has been found to be the most suitable carbon source for culture of plant cells and hairy roots (Pavlov et al., 2005). Sucrose consumption profiles are shown in Figure 4.25. Initial sucrose concentration was 30g/l in all cases. Sucrose was consumed most rapidly in the full MS medium as no other nutrients apparently limited growth in the full strength media. There was a rapid decline during the first six days. This can be explained as the cells used the sugar for the preparatory phase and the considerable growth of the cells and pigment production were seen only after complete sugar molecules had been utilised.

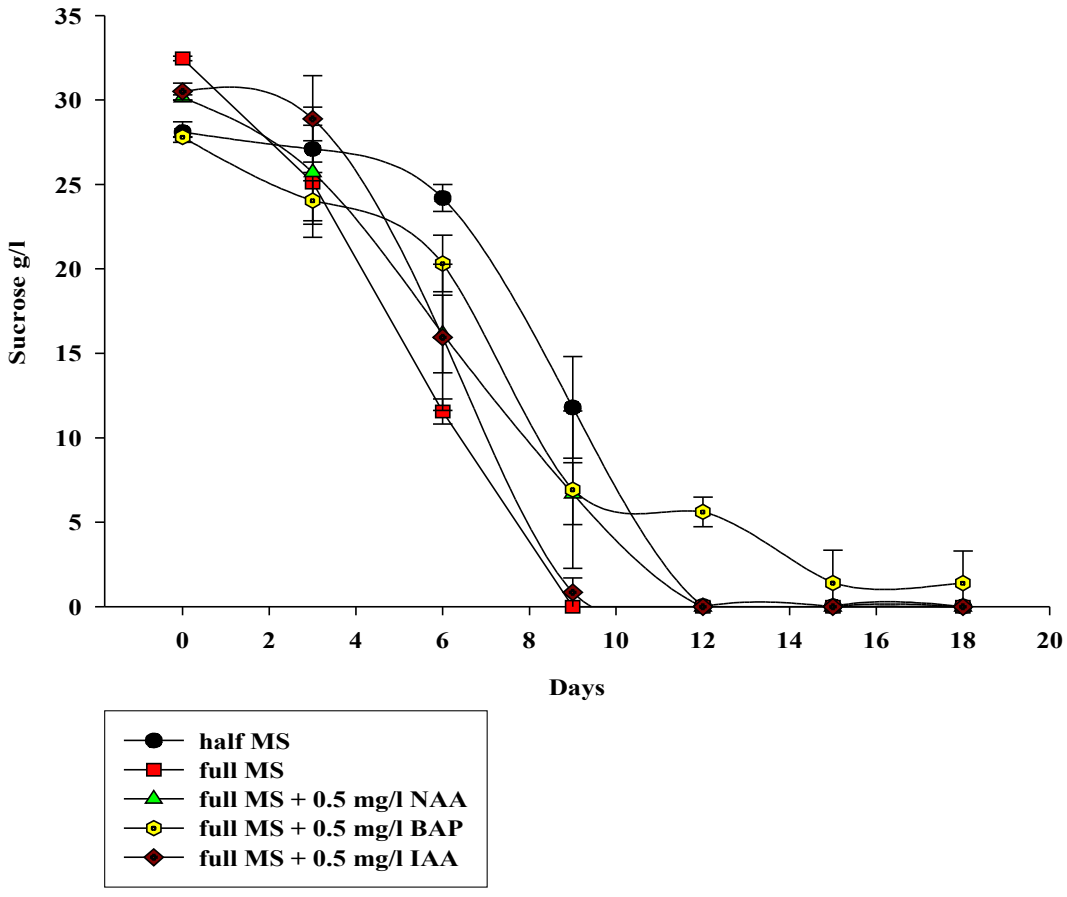


**Figure 4.25.** Sucrose consumption profile of hairy roots in different media

#### 4.9.2.2. Phosphate and nitrate

The initial concentration of phosphate was 51.1 mg/litre in half MS and 120 mg/ l in all other experiments In all media except MS+0.5 mg/l BAP, phosphate had been completely consumed by day 9 (Figure 4.26). Cell growth and pigment production (Figure 4.17 and Figure 4.20) occurred only when most of the phosphate had been consumed.

Compared to phosphate, nitrate was consumed more slowly (Figure 4.27). Nitrate consumption accelerated when the roots entered the exponential growth phase. Except in half MS medium, detectable concentration of nitrate remained on day 18, at the end of the fermentation.

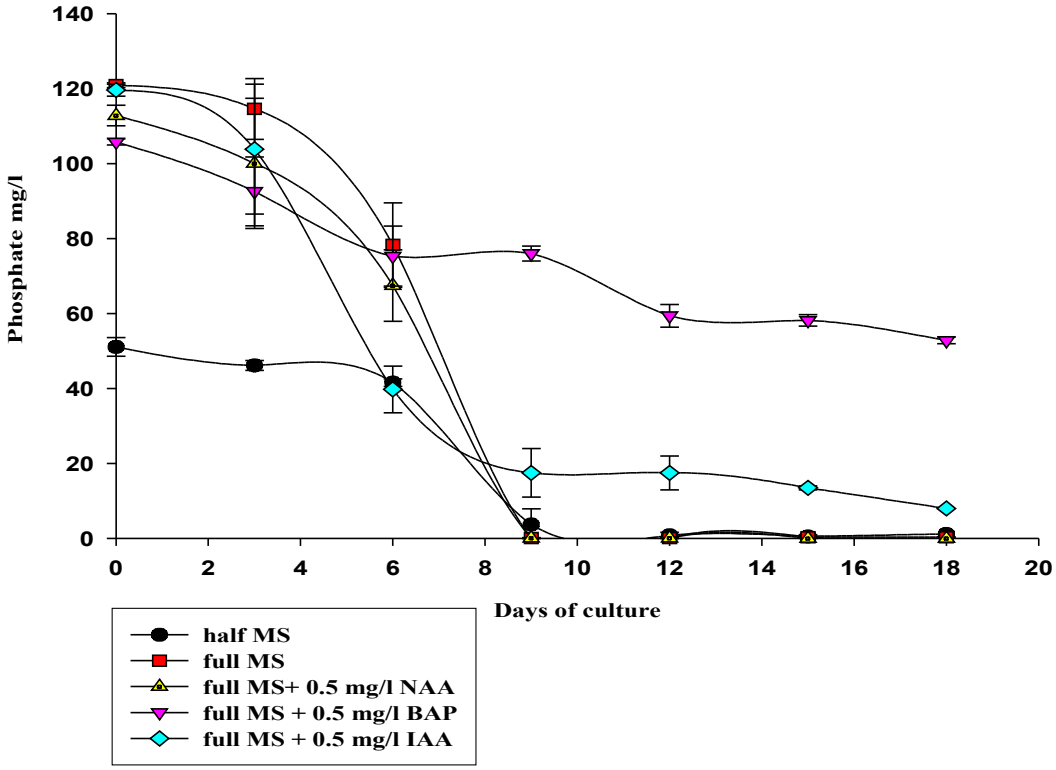


**Figure 4.26.** Phosphate consumption profile of hairy roots in different media

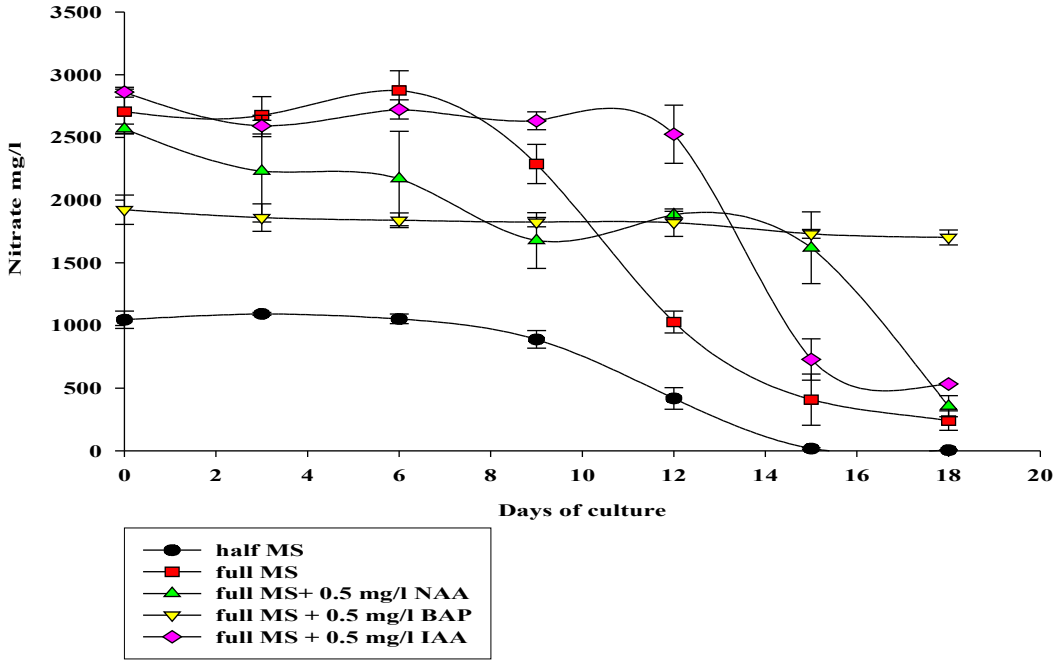


**Figure 4.27.** Nitrate consumption profile of hairy roots in different media

## 4.9.3. Measurement of pH

The pH was measured using a pH meter every three days in all experiments. A drop in the pH was seen on the first 6 days and then the pH remained almost constant (Figure 4.28). This drop might be due to the generation of carbon dioxide by the respiration of hairy roots and $NH_4$ uptake also might have acidified the medium as reported earlier (Nour and Thorpe 1994; Shin et al., 2003).

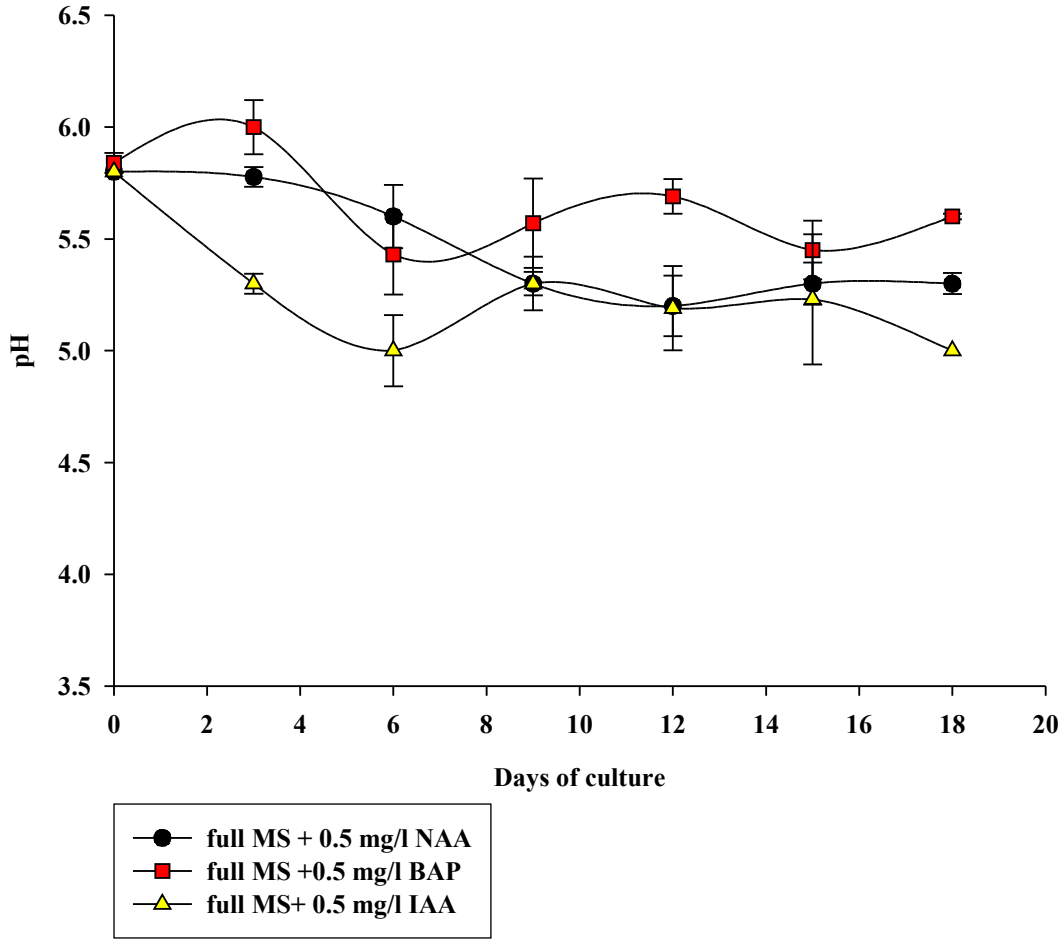


**Figure 4.28.** pH profile of hairy root cultures in different media

# CHAPTER 5

# OUTCOMES AND CONCLUSIONS

Plant cell and hairy root cultures can be potentially used to produce pigments and colorants under highly controlled conditions, for possible applications in foods and cosmetics. This work focussed on blue and red pigment production from *Lavandula augustifolia* and *Beta vulgaris*, respectively. The intention was to compare suspension cell cultures and hairy root cultures of these plants for the production of respective pigments. A substantial and prolonged experimental effort succeeded in generating callus cultures and suspension cell cultures from *L. augustifolia*, but not the hairy root cultures. In contrast, hairy root cultures were successfully generated from *B. vulgaris*,

but callus culture could not be produced and, therefore, suspension cell cultures were not produced. In view of the extensive effort invested in generating calli, suspension cell cultures and hairy root cultures, it was decided to study the two types of culture systems in parallel, as possible models of pigment production.

Blue and red pigments were successfully produced by suspension cell culture and hairy root cultures of *L. augustifolia* and *B. vulgaris*, respectively. Blue pigment production occurred in suspension culture of *L. augustifolia* in full-strength Murashige and Skoog (MS) medium with all the hormone combinations that were tested. These combinations were: (1) 1 mg/l each of indole-3-acetic acid (IAA) and kinetin; (2) 2 mg/l of IAA and 1 mg/l of kinetin; (3) 2 mg/l of IAA and 1 mg/l of benzyl amino purine (BAP); and (4) 2 mg/l each of IAA and BAP. Maximum growth of cells was obtained with the hormone combination (3). Generally, less cell mass was produced in low-IAA media suggesting that IAA promoted growth, as expected. (IAA is a well-known auxin, or growth promoter.) The hormone combination that gave the maximum specific production of the blue pigment was the combination (2). Pigments are generally secondary metabolites and, therefore, their production is generally fully dissociated from biomass growth. The medium formulation that gave the highest concentration of the blue pigment in shake flask suspension cultures was further tested in an aerated and stirred bioreactor. In the bioreactor, the production of cells mass as well as the pigment was significantly lower than in the shake flask, suggesting that the suspended cells were sensitive to aeration and agitation. This is suggested as a possible subject of further study by others. Use of stirred bioreactors with impellers designed specifically to produce good mixing under reduced shear, may prevent the observed loss in biomass and pigment productivity. Fed-batch culture is recommended for future study, as it will

prevent depletion of some of the nutrients that inevitably occurred in the batch cultures used in this work. Effects of additives such as elicitors and $Fe^{2+}$ on pigment production requires future investigation as elicitors in general have improved productivity of secondary metabolites of plant cells and $Fe^{2+}$ is directly linked with the blue pigment formation in *L. augustifolia*.

Hairy root cultures grew extremely rapidly compared to the suspension cell cultures. Hairy roots also produced large amounts of the red pigments. Growth of hairy roots was influenced by the composition of the culture medium. For example, in the first 12-days, the full strength MS medium better promoted biomass growth and

pigment production compared to the half-strength MS medium, although the final biomass and pigment concentrations were not affected. Growth and pigment production were not influenced by any of the hormones tested (i.e. naphthalene acetic acid, BAP, and IAA added individually at a concentration of 0.5 mg/l). In fact, BAP adversely affected hairy roots and reduced their growth significantly. This suggests that BAP may to toxic to hairy roots. This is consistent with the known effects of BAP on conventional roots. Further optimization of the culture medium, including investigation of fed-batch culture, are suggested for future investigations in attempts to improve the production of the red pigments.

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