PLANT CELL CULTURES FOR THE PRODUCTION OF PHYTOCHEMICALS - ANTHOCYANIN AND CAPSAICIN IN BIOREACTORS

A Thesis submitted to the

UNIVERSITY OF MYSORE

for the degree of

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

by

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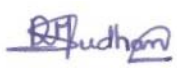
1998

Dedicated to my Parents

DECLARATION

I hereby declare that this thesis, entitled **PLANT CELL CULTURES FOR THE PRODUCTION OF PHYTOCHEMICALS -ANTHOCYANIN AND CAPSAICIN IN BIOREACTORS,** submitted herewith to the University of Mysore for the award of **DOCTOR OF PHILOSOPHY,** is the result of work done by me at Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore, under the guidance of Dr. G.A. Ravishankar, during the period 1993-1998.

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



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Date: February 19, 1998

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CERTIFICATE

I hereby certify that the thesis, entitled **PLANT CELL CULTURES FOR THE PRODUCTION OF PHYTOCHEMICALS - ANTHOCYANIN AND CAPSAICIN IN BIOREACTORS,** which is submitted by Mr. R. Madhusudhan to the University of Mysore for the award of **DOCTOR OF PHILOSOPHY,** is the result of work done by him at Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore, under my guidance during the period 1993-1998.

G.A. Ravishankar

(**G.A. Ravishankar**)
Research Guide

Date: February 19, 1998

ACKNOWLEDGEMENTS

I am extremely grateful to Dr. G.A. Ravishankar for his constant inspiration and guidance. The long pedagogic discussions I had with him taught me the intricacies of scientific research, and influenced me a lot. I am also grateful to him for the freedom of thought, experimentation, and interaction with other people.

My sincere thanks are due to Dr. L.V. Venkataraman who offered me an opportunity to work on plant cell cultures.

Due to the interdisciplinary nature of my work, I had an opportunity to interact with several people of different departments who spared their valuable time for educative discussions, and extended their instrumental facilities. I wish to record my sincere thanks to all of them. In particular, I thank Mr. N.P. Ghildyal for his valuable suggestions on studies on optimization of process parameters. Dr. S. Bhattacharya helped me greatly in rheological studies. I constantly interacted with Dr. K.S.M.S. Raghava Rao. I thank him for evaluating my research publications critically and offering valuable suggestions.

I acknowledge the help and cooperation received from the staff of Central Instrumentation and Laboratory Services, Library, Administration, and other support services departments.

I am in debt to Dr. N. Bhagyalakshmi for all kinds of helps including the counselling for personal problems. My thanks are due to Dr. G. Suvarnalatha and Dr. L. Rajendran for their help in cell line improvement and characterization of anthocyanins. I thank Dr. M.S. Narayan for his constructive criticisms, and Dr. T. Rajasekaran for his moral support.

I thank Mr. S. Ramachandra Rao for his involvement in the work on different methods for estimation ofbiomass. Without his initiativeness and help, I would have not completed the work. My thanks are due to Mr. Jacob George for going through the manuscript of this Thesis and offering his valuable suggestions.

All the colleagues in the Department always helped me. I express my sincere thanks to all the staff of this Department. In particular, I thank Mr. Harsh Pal Bais, Mr. C. Hemakumar, Mr. V. Ravi, Miss. G. Sudha, and Mr. Md. Yahya Khan for helping in preparation of this thesis.

I would like to place on record my special gratitude to all the members of my family for their love, encouragement and support. I should thank my wife for bearing with all my occupational agonies.

I thank Dr. V. Prakash, the Director, for giving me an opportunity to work in this Institute. I gratefully acknowledge the Council of Scientific and Industrial Research, New Delhi for providing me Junior, and Senior Research Fellowships.

(R. Madhusudhan)

CONTENTS

LIST OF ABBREVIATIONS

A&R	Anderson (1978)
B5	Gamborg *et al.* (1968)
BAP	6-benzylaminopurine
2,4-D	2,4-dichlorophenoxyacetic acid
DBM2	Gresshoff and Doy (1974)
DW	Dry weight
ER	Eriksson (1965)
FW	Fresh weight
H&A	Hoagland and Arnon (1950)
HTM	Hildebrandt (1977)
Kn	6-furfurylaminopurine (kinetin)
L&M	Litvay *et al.* (1985)
MS	Murashige and Skoog (1962)
NAA	a-napthaleneacetic acid
N&N	Nitsch and Nitsch (1969)
PAL	Phenylalanine ammonia lyase
S&H	Schenk and Hildebrandt (1972)
μ	Specific growth rate (day^{-1})
VVM	Volumes per volume per minute
WPM	Lloyd and McCown (1981)
WS	White (1963)

INTRODUCTION

Plants have been the source of many compounds that are indispensable to food, medicine, and industry. Over 80% of the approximately 30,000 known natural products are of plant origin (Balandrin and Klocke, 1988). It has been estimated that over 25% of the drugs prescribed in the USA are either of plant origin or derived from plants (Deus and Zenk, 1982). The world market for the plant products is very high reaching US $ 200 million and the demand is increasing at a greater percentage.

Most of the phytochemicals of commercial importance are the products of secondary metabolism in plants. These compounds are mainly synthesized as a defense against pathogens, and ecological variations (Harborne, 1982; Wink, 1988). Most of the high value phytochemicals have limited taxonomic distribution and the plants grow in strict agro-climatic conditions. The supply of chemicals from plants is subject to seasonal variations in the agro-climatic conditions and diseases. Moreover, the extraction of chemicals from plants is labour intensive.

In spite of the advancements in synthetic organic chemistry, many natural compounds still could not be synthesized economically because of the complexity of structures. Synthetic substitutes may not have all the functional attributes comparable to the natural ones. The consumer preference is increasing for 'natural' products. Genetic engineering of microorganisms to produce the phytochemicals is currently not feasible in view of the multi enzyme biosynthetic pathways for these compounds.

Plant cell culture is an attractive alternate technology for the production of high value phytochemicals (Berlin, 1984; Stafford *et ah,* 1986; Fowler and Scragg, 1988; Havkin-Frenkel *et al.,* 1997). Plant cells are biosynthetically totipotent and can produce the range of chemicals that are found in the whole plant. It should, in principle, be possible to produce any compound that is found in the intact plant by exploiting the chemical totipotency of plant cell. Table 1.1 lists some selected examples of secondary metabolites formed in plant cell cultures in levels comparable or higher than that in the whole plants. A detailed list of the prospective compounds to be produced by plant cell cultures is given by Endress (1994).

Table 1.1. Products accumulated in high yield by plant cell cultures (Compiled and updated from Stafford *etal.*, 1986, and Banthorpe, 1994).

Compound	Species	Yield (%, w/w)	Reference
Rosmarinic acids	*Anchusa officianalis*	12.0	De-Eknamkul and Ellis, 1985
	Coleus blumei	12.0	Zenk *et al.*, 1977a
Gingenosides	*Panax ginseng*	21.0	Furuya and Ishii, 1972
Anthraquinones	*Morinda citrifolia*	18.0	Zenk *et al.*, 1985
	Galium mollugo	15.0	Bauch and Leistner, 1978
Shikonin	*L. erythrorhizon*	12.4	Fujita *et al.*, 1981
Isoquinones	*Coptis japonica*	15.0	Fukui *et al.*, 1983
Shikimic acid	*Galium mollugo*	10.0	Amrhein *et al.*, 1980
Trigonelline	*Trigonella foenumgraecum*	5.0	Radwan and Kokate, 1980
Nicotine	*Nicotiana tabacum*	2.1	Ohta and Yamazawa, 1982
Serpentine	*Catharanthus roseus*	2.0	Deus-Neumann and Zenk, 1984
Jatrorhizine	*Berberis wilsonae*	10.0	Breuling *et al.*, 1985
Acteoside	*Syringa vulgaris*	17.5	Ellis, 1980
Diosgenin	*Dioscorea deltoidea*	8.0	Tal *et al.*, 1983
Quassin	*Picrasma* spp.	0.3	Scragg *et al.*, 1990
Sanguinarine	*Papaver somniferum*	12.0	Songstad *et al.*, 1990
Berberine	*Coptis japonica*	7.5	Matsubara *et al.*, 1989
Anthocyanins	*Daucus carota*	22.0	Rajendran *et al.*, 1994
Taxol	*Taxus baccata*	0.02	Fett-Neto *et al.*, 1994

Production of phytochemicals, *in vitro,* from plant cell cultures offer several advantages: (a) it is independent from geographical and seasonal constraints, and various environmental factors such as climate, diseases etc.; (b) it is a defined production system under controlled conditions which ensures uniform quality and yield, and constant supply; (c) it is possible to produce novel compounds that are normally not found in the parent plant (Table 1.2); (d) in addition, plant cells can perform stereo- and regio- specific bioconversions (Alfermann *et ah,* 1983); (e) efficient downstream recovery and product purification.

1.1 History of plant cell culture technology

At the beginning of the century, Haberlandt (1902) attempted to cultivate isolated plant cells, but cell division was never observed in these cultures. In the 1930s the first *in vitro* cultures were established (White, 1934; Gautheret, 1939), and this was followed by a period of development of culture media and of cultivation methods (Street, 1977). Twenty five years ago, the prospect of the use of plant cell cultures for the production of chemicals was not imaginable. The earliest detailed reference to plant cell cultures as an industrial route to natural product synthesis is probably the patent application of Routier and Nickell (1956). Zenk *et al.* (1975) demonstrated, for the first time, that completely dedifferentiated cell suspensions of a higher plant *(Morinda citrifolia)* can produce secondary metabolites (anthraquinones). However, the low yield of secondary metabolites in suspension cultures clearly was a bottleneck for commercialization. In those early efforts, plant cells in culture were treated in direct analogy to microbial systems, with little knowledge of plant cell physiology and biochemistry, or the influence of bioreactor operation on the physiological state of such systems. In 1982, at least 30 compounds were known to accumulate in plant culture systems in concentrations equal to or higher than that in the whole plant (Staba, 1982). A survey of the historical milestones in plant cell cultures is given by Schmauder and Doebel (1990).

Table 1.2. Examples of novel substances reported in plant cell cultures (Compiled from Berlin, 1983; Kreis and Reinhard, 1989; Ruyter and Stockigt, 1989).

Chemical class	Compound	Species
Alkaloid	Armorine	*Stephania cepharantha*
Alkaloid	Norcepharadione	*Stephania cepharantha*
Alkaloid	Rauglucin	*Rauwolfia serpentina*
Alkaloid	Epchrosin	*Ochrosia elliptica*
Terpenoid	Dihomovalerate	*Valeriana wallichii*
Terpenoid	Paniculide A	*Andrographis paniculata*
Terpenoid	Honokiol	*Thuja occidentalis*
Terpenoid	Tarennoside	*Gardenia jasminoides*
Anthraquinone	Lucidin	*Morinda citrifolia*
Phenylpropanoid	Rutacultin	*Ruta graveolens*
Phenylpropanoid	Podoverin	*Podophyllum versipella*

Strategies to optimize growth and product formation began to develop separately during the period between 1975 and 1985. A combination of various strategies for yield improvement resulted in the first commercial plant cell culture process for the production of shikonin from *Lithospermum erythrorhízon* by Mitsui Petrochemical Industries in Japan (Curtin, 1983) (Fig. 1.1). Shikonin, which is used as a dye and a medicinal (anti-inflammatory) compound, valued in 1983 at approximately $ 4,000 per kg. The expectations of this two-stage process were high and inspired the development of three more large scale plant cell culture processes in Japan, hi the United States the development of two processes for commercialization was attempted - the production of vanilla flavour from plant cell cultures by Escagenetics (Knuth and Sahai, 1991) and the production of sanguinarine by *Papaver somniferum* (Park *et al.,* 1990). A detailed list of the prospective compounds identified by Japanese industries for production through plant cell cultures is given by Komamine *et al.* (1991). Phyton Gesellschaft fur Biotechnik mbH began producing taxol, an anticancer drug, using several species of *Taxus* cultures, as well as other plant products in facilities with reactor capacities of up to 75,000 L. Cell cultures of *Taxus* may represent a viable alternative to extraction from stem bark for taxol and related taxanes. Presently, the taxol production from plant cell cultures is the prime target for a large number of centers all over the world. The recent advances in taxol production from various *Taxus* spp. have been reviewed by Zhong (1995).

1.2 Outlines of the plant cell culture processes

The techniques of plant cell culture have been extensively reviewed (Dodds and Roberts, 1985; Charlwood and Rhodes, 1990; Payne *et al,,* 1992). Typically, first callus is raised from an explant of young tissue aseptically. Cell suspensions are initiated by dispersing friable callus in liquid nutrient medium. The resulting suspension cultures are maintained by periodic subculturing. Suspensions are usually run as batch cultures but immobilized cell cultures can be continuously operated over extended periods. Various

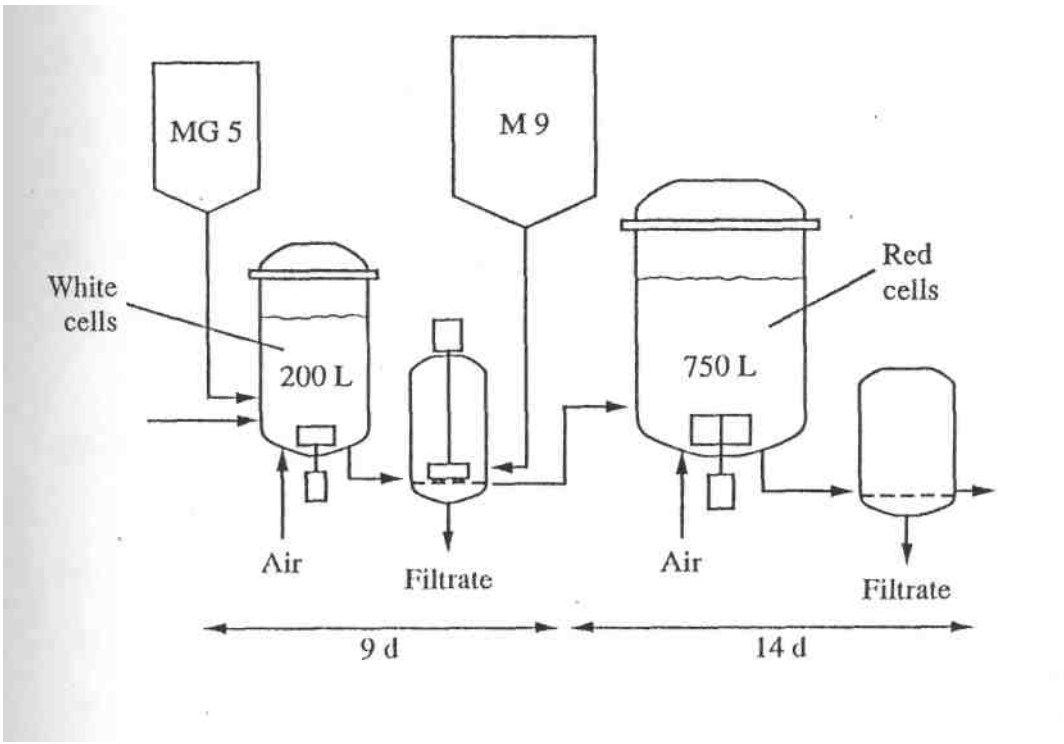

Figure 1.1. Outline of the process for production of shikonin from *Líthospermum erythrorhízon* cell culture, developed by Mitsui Petrochemical Industries Ltd., Tokyo. (Curtin, 1983. Reproduced with permission from the Publisher.)

culture parameters such as nutritional requirements, temperature, pH, and dissolved oxygen are studied and optimized. Table 1.3 summarizes the various factors that influence the secondary metabolite production. Various strategies used for improvement of secondary metabolite production are reviewed by several authors (Petersen and Alfermann, 1993; Dornenburg and Knorr, 1995). Figure 1.2 shows the various steps involved in plant cell culture processes.

1.3 Challenges in plant cell culture technology

In spite of the tremendous scope, the plant cell culture technology could not be extended to a wider range of chemicals. This is partially due to some inherent limitations associated with the plant cells. Unlike microbial cultures, plant cells pose several problems in their large scale cultivation. Table 1.4 summarizes the differences between microbial and plant cell cultures. The present status, and the challenges in this technology have been recently reviewed by Dornenburg and Knorr (1997).

Size of plant cells

Plant cells are large compared with most microorganisms, being in general 40 - 200 μm long and 10-40 μm in width. Further, the plant cells in suspension cultures tend to aggregate and form clumps, resulting in a heterogeneous population of cell clusters, ranging from a few cells to several thousands of cells, often measuring a few centimetres in diameter. Whether the formation of aggregates is due to non-separation upon cell division or true aggregation after division is not known. The aggregate structure can be loose in nature (Scragg, 1990) or may be held together by extracellular polysaccharides, which plant cells are known to produce in culture (Hale *et al,* 1987).

The size of cell aggregates has several implications in the design and operation of bioreactors, and in the downstream processing (Panda *et al,* 1989; Doran, 1993). While a smaller size of cell aggregates is preferred from the standpoint of process engineering, a certain degree of cell-cell contact (Lindsey and Yeoman, 1983) and cell differentiation

Table 1.3 Factors influencing the secondary metabolite production in plant cell cultures. (Dornenburg and Knorr, 1995)

Strain improvement
Selection
Screening
Genetic engineering
Medium variation
Nutrients
Phytohormones
Precursors
Antimetabolites
Culture conditions
Inoculum size
pH
Dissolved oxygen level
Temperature
Light
Agitation
Specialized techniques
Elicitation
Immobilization
Permeabilization
Two-stage culture
Three-phase systems
Reactor

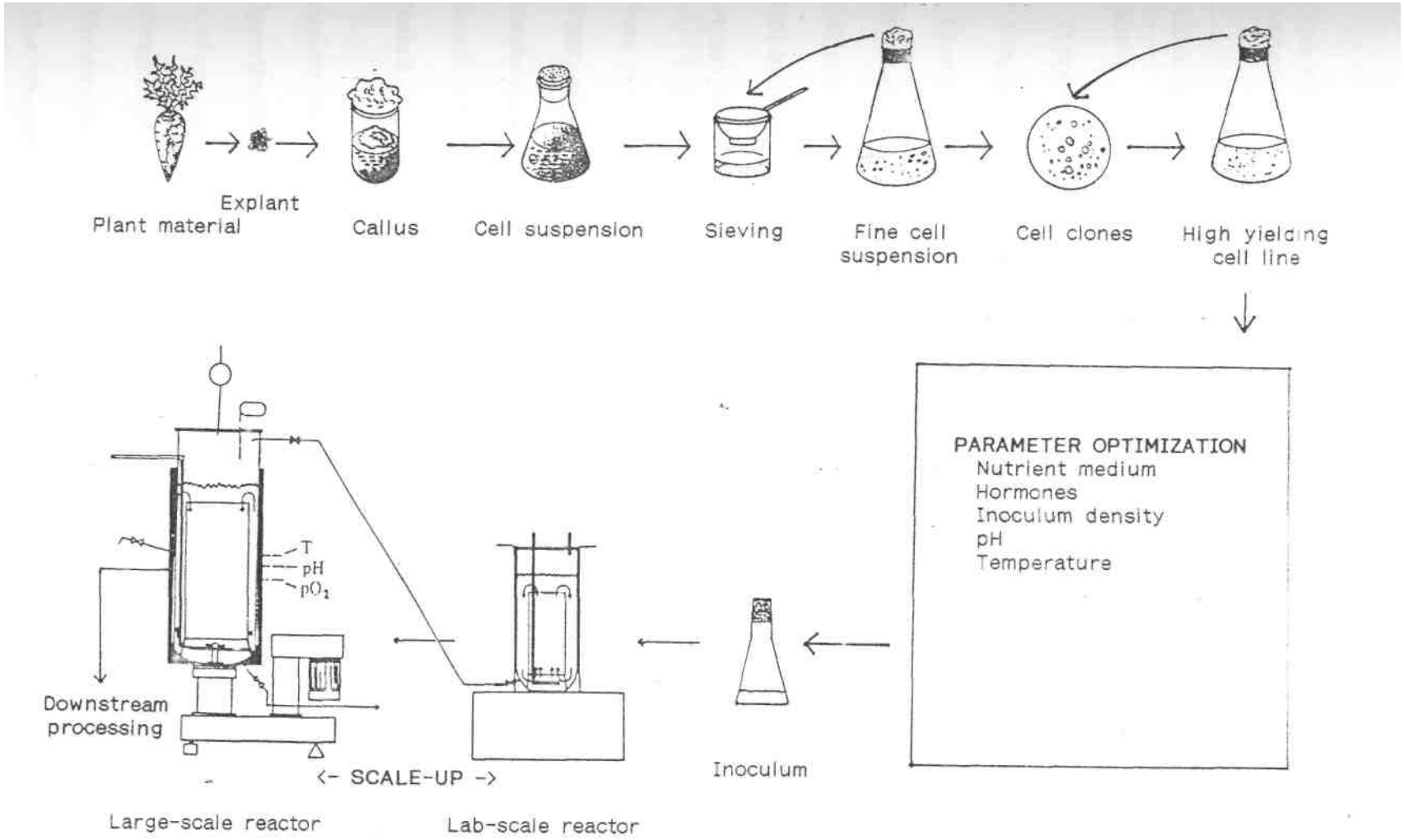

Figure 1.2. Different stages in development of a plant cell culture process.

Table 1.4 Differences between plant cell and microbial cultures. (Compiled from Dornenburg and Knorr, 1997, and Singh, 1997)

Characteristic	Plant cells	Microbial cells
Size		
Diameter (μm)	40-200	1-10
Volume (μm^3)	$>10^5$	1-50
Growth	Aggregates	Single cells, pellet, mycel
Inoculum (%)	5-10	<1
Cultivation time	2-3 weeks	2-10 days
Cell doubling time (h)	15-120	0.3-6
Oxygen consumption rate ($mM\ h^{-1}\ g^{-1}$ dry wt.)	<5	50
Shear sensitivity	High	Low (higher fungi: high)
Water content (%)	>90	~ 80
Metabolic regulation	Highly complex	Complex
Genetic stability	Often variable	Stable
Product accumulation	Mostly intracellular (vacuole)	Often extracellular (Medium)
Medium costs	Approx. 8-fold	1
Operating conditions in Bioreactors		
Cell concentration (g DW l^{-1})	10-50	10-50
Power input (W l^{-1})	0.1-0.5	1-10
Variation in viscosity (cP)	1-50	1-1,000
Mass transfer coefficient (h^{-1})	20	100

(Steward *et al.,* 1958) is often required for the synthesis of secondary metabolites. Hoekstra *et al.* (1988) showed the relationship between the degree of differentiation and the level of quinoline alkaloid accumulation in *Cinchona* cultures. Immobilization of cells and the hairy root cultures provide some solution for this problem.

Shear sensitivity

Plant cells have high tensile strength but low shear tolerance. This is because of their large size, rigid cell wall and extensive vacuole. It is suspected that the difficulties faced in the early efforts to cultivate plant cells in large reactors were due to the sensitivity of plant cells to shear stress (Mandels, 1972; Dalton, 1978). The shear stress generated by the impeller in the stirred tank bioreactors is thought to be responsible for the difficulties (Smart, 1984). To reduce these problems, a number of impeller designs have been studied. Furuya *et al.* (1984) used three impellers, a disc Rushton turbine, an anchor and angled disc turbine, and concluded that the angled disc turbine gave the best growth rate and dry weight increase from *Panax ginseng* cultures, in a 30 L bioreactor. A spiral impeller was found to be the most effective for the culture of *Coleus* cells at high densities (Ulbrich *et al.,* 1985) when compared with turbine and anchor impellers. Cultures of *Glycine max* and *Pinus elliottii* have been grown in 1.25 and 2.5 L working volumes in bioreactors with flat bladed, marine, and cell-lift impellers run at 30 - 80 rpm (Treat *et al,* 1989). The cell lift impeller yielded a similar biomass as with the flat bladed impeller, but improved cell viability. However the cell aggregate size increased. In another study using *N. tabacum* suspension cultures, a large flat bladed impeller, and a sail impeller were tried in 5 L bioreactors (Hooker *et al.,* 1990). The large flat bladed impeller was a considerable improvement over the normal flat bladed impeller in terms of growth and biomass yield.

Growth rates

Plant cells in suspension have a very slow growth rate with doubling times of 2-6

d compared with 2-6 h of microorganisms. The low growth rate appears not to be due to the low activities of the enzymes present in plant cells, but is rather a reflection of the cell's growth cycle, where the cytoplasm represents less than 10% of the total cell volume. The most rapidly growing culture that has been reported is *Nicotiana tabacum* (Noguchi *et al.,* 1982) with a doubling time of 18 h, but a typical doubling time would be 2-3 d. The growth rate is further reduced, in some cases, when the cultures are grown in large bioreactors. However, there are reports where the growth rate of cells is little affected in bioreactors. (Scragg *et ah,* 1987a). The major practical consequence of this slow growth is that the bioreactor runs are to be of 2-4 weeks. This requires considerable attention to the maintenance of sterility, and other problems such as water loss also can occur. The long bioreactor runs also mean that the number of runs that an individual bioreactor can accommodate in a year is limited to perhaps one per month. On a production scale, the development of an inoculum will take a considerable time and the overall productivity per bioreactor will be reduced. As many products of interest are accumulated after growth has ceased, the possibility of reducing the bioreactor run time is limited.

Plant cell reactors

The large scale growth of plant cells in bioreactors is required in order to develop any form of biotechnological process. The first use of a bioreactor was the report of culture of *Daucus carota* cells in a 15 L stirred tank reactor (Byrne and Koch, 1962). The early 1970s saw a number of reports on the cultivation of tobacco cells in stirred tank bioreactors (Kato *et al.* 1975) of up to 1500 L volume, culminating in the growth of tobacco cells at 15,000 L (Noguchi *et al.,* 1977). At this stage, apart from one report by Hahlbrock *et al.* (1974) on the growth of *Glycine max* and *Petroselinum,* mass cultivation was restricted to tobacco cells. However, when other cultures were investigated, it was soon recognized that plant cell suspensions had a number of problems associated with their cultivation in bioreactors. It had been observed that plant

cells are sensitive to shear stress (Mandels, 1972). This led to a general view that the conventional stirred tank reactors are unsuitable for growth of plant cells because of the high levels of shear produced from the mechanical agitation. Studies by Wagner and coworkers (Wagner and Vogelman, 1977) with cells of *Morínda citrífolia* provided strong support for this view. With this impression, plant cells were grown in a surprisingly wide variety of bioreactor types and conformations (Spier and Fowler, 1983). Much of the early work on large scale growth was carried out with air driven reactors, typically of draught tube format (Fig 1.3), which develop comparatively low levels of shear. A variety of cell cultures have been successfully grown in airlift bioreactors (Breuling *et ah,* 1985; Scragg *et ah,* 1987a). Table 1.5 lists the different types of bioreactors and volumes used for plant cell cultures.

In recent years, however, there has been a return to stirred tank reactors, and numerous examples of large scale growth in this type of reactor now exist. Indeed, the process formats used in those processes which have been commercialized have involved the use of stirred tank vessels. What has brought about this change? Fowler and Stafford (1992) remarked that 'no verified explanation exists.' It is possible that during the cell selection for other criteria, such as improved productivity, unwitting selection has also been made for cells which exhibit higher and higher levels of shear tolerance (Fowler, 1987). Tanaka (1982) has shown that the airlift bioreactor may have problems at high cell densities, and it has been shown that plant cells may not be as sensitive to shear stress as it was first thought (Scragg *et al,* 1988a).

However, there are still a good number of examples of shear intolerant plant cell lines (Leckie *et al,* 1990) and new types of bioreactor are being developed (Bohme *et aí,* 1997). Different types of bioreactors and their advantages and disadvantages used for plant cell cultures have been reviewed exhaustively by several authors (Kargi and Rosenberg, 1987; Panda *et al,* 1989; Sing and Curtis, 1994).

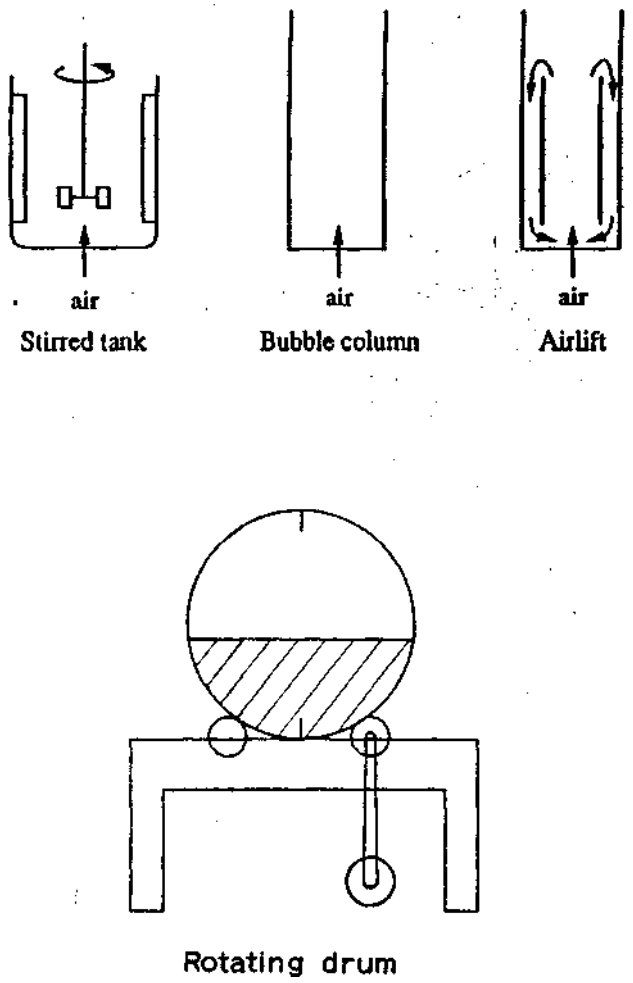

Figure 1.3. Designs of basic reactors used for plant cell cultures.

Table 1.5. Various types of bioreactors used to cultivate plant cells (updated from Scragg, 1992)

Bioreactor type	Volume(L)	Species	Year
STR	600	*Nicotiana tabacum*	1972
	10, 30, 300	*Glycine max*	1974
	15, 500	*N. tabacum*	1977
	10	*Morinda citrifolia*	1977
	7.5	*Catharanthus roseus*	1981
	20 000	*N. tabacum*	1982
	500, 750	*Lithospermum erythrorhizon*	1983
	30	*Panax ginseng*	1984
	32	*Coleus blumei*	1985
	14	*C. roseus*	1986
	70, 750, 800	*Solanum demissum*	1986
	2000, 20 000	*Panax ginseng*	1986
	5000	*C. roseus*	1986
	1000	*L. erythrorhizon*	1986
	80	*Helianthus annus*	1990
Airlift	10	*Morinda citrifola*	1977
	30	*C. roseus*	1977
	10, 100	*C. roseus*	1981
	20	*Trisptergium*	1981
	200	*Digitalis lanata*	1983
	5, 10	*C. roseus*	1984
	20	*Berberis wilsonae*	1985
	80	*C. roseus*	1987
	80	*Helianthus annuus*	1989
Rotating-drum	2.5 - 1000	*L. erythrorhizon*	1983
	2.5	*Beta vulgaris*	1987
	2	*Thalictrum rugosum*	1988
Fluidized-bed	4.2	*Coffea arabica*	1995
Membrane	2	*Aesculus hippocastanum*	1997

Scale-up of plant cell cultures in bioreactors

The goal of scale-up is to reproduce on a large scale those conditions observed to be optimal at the small scale. The reproduction of the chemical environment on various scales is relatively easy with respect to the soluble macronutrients. The sparingly soluble gaseous nutrients are more difficult to supply in a reproducible manner on different scales. This proves to be quite difficult for plant cell cultures because of the potential requirements for the supply or removal of volatile components in addition to oxygen (Rajasekhar *et al.,* 1971; Maurel and Pareilleux, 1985). The complexity of fluid flow in typical bioreactors makes the analysis of the physical environment quite difficult. Bioreactors for microbial cells are often characterized by the overall oxygen transfer coefficient, K_La, and scale-up is often based on achieving the same K_La on different scales. Although oxygen is required for plant cells, shear may prove to be the overriding consideration for large scale reactor design (Payne *et al,* 1987).

Unlike microbial cultures, plant cell suspensions have some special demands for their large scale cultivation. Though the oxygen consumption rates of plant cells are low, they require adequate bulk mixing and at the same time the cells are sensitive to dissolved oxygen level. Rajasekhar *et al.* (1971), from a study on the effect of agitation rate on the growth of *Atropa* and *Acer* cultures, concluded that reduced growth at suboptimal shaking speeds was neither due to oxygen deficiency nor accumulation of carbon dioxide, but rather due to an unknown volatile toxic factor, or a restricted nutrient uptake resulting from a stationary liquid-phase boundary surrounding the cells. Fujino *et al.* (1992) studied the effect of DO level on cell growth and β-carotene productivity of carrot cultures by changing the gas flow rate and gas composition under PID controlled manner. They found that at a DO level of 3.0 ppm the specific growth rate of cells was 0.46 day^{-1} while the β-carotene content was only 0.47 μg 10^{-8} cells. On the other hand, at the DO level of 0.05 ppm the specific growth rate was only 0.09 day^{-1} while the β-carotene content boosted up to 2.6 μg 10^{-8} cells. They then tried a two stage culture with respect to the DO level for carrot cell cultures. In the first stage of

logarithmic cell growth, DO level was kept at 2.0 ppm and in the second stage of β-carotene production the DO level was reduced to 0.15 ppm. This scheme of DO-stat culture resulted in the maximum productivity of β-carotene. This study illustrates the complexity in large scale culture of plant cells.

Though the technique animal cell culture is equally young compared to plant cell culture, the engineering aspects of plant cell cultures received scarce attention while the same were studied extensively for animal cell cultures (Birch *et al,* 1987). The high cost of the mammalian cell products is probably the reason for this unfair treatment.

Immobilized cell cultures

Certain products synthesized inside the cells diffuse out into the surrounding medium. Physical containment of such cells allows the phase separation resulting in a continuous production over extended periods. Several secondary metabolites which are not stored in vacuoles are thus ideal candidates to be produced from immobilized cells. Parr (1988) gave a comprehensive list of such compounds, which includes several alkaloids, anthraquinones, phenolics and terpenoids. Interestingly, the microenvironment of immobilized plant cells has several additional advantages for secondary metabolite formation. Mainly, the cell-cell contact is enhanced, and, as a consequence of the physical stress, the primary metabolism is slowed down allowing the diversion of intermediates into secondary metabolism. Table 1.6 summarizes the advantages of immobilization of plant cells. Various aspects of immobilized plant cell cultures have been reviewed elaborately by several authors (Rhodes, 1985; Brodelius, 1988; Hulst and Tramper, 1989; Williams and Mavituna, 1992).

1.4 Research on plant cell culture in India

Inspired by the success of plant cell culture processes in Japan, Germany, and U.S.A., a number of Indian companies have now seriously embarked on the research on production of high value phytochemicals from plant cell and hairy root cultures. India

Table 1.6. Advantages of immobilized plant cells (Shuler, 1981).

* Continuous operation at high dilution rates.
* Better cell-cell contact.
* Due to the physical constraint on the cell growth, diversion of intermediates into secondary metabolism.
* Due to the chemo-stat operation, the feed-back inhibition of product is overcome.
* Problems of genetic instability are reduced since the replication of cells is repressed.
* Less contamination.
* The cells are protected from the hydrodynamic shear forces.
* Downstream process can be integrated with the reactor system, and recycling of the nutrient medium is possible.

has a vast flora of medicinal and aromatic plants and rich traditions of using herbal medicines for treatment of various ailments. However, the scarce availability of plant material made the *Ayurvedic* practices difficult. Realizing the untapped resource of the germ plasm, several industrial, R&D and academic institutions in India launched a number of projects on plant cell cultures. Dabur India Ltd., New Delhi projected the commencement of a plant for the production of taxol, an anti-cancer drug, from the cell cultures of *Taxus* spp. by the year 2000. Cipla Pharmaceuticals Pvt. Ltd., Bangalore is striving hard to produce diosgenin and other steroids using the plant cell culture technology. Dr. Reddy's Research Foundation, Hyderabad is similarly interested in taxol and other medicinal compounds such as camptothesin. Central Institute of Medicinal and Aromatic Plants, Lucknow has reported the successful culturing of hairy roots for obtaining commercially important medicinal compounds (Banerjee *et al.,* 1995; Kukreja, 1996). The group at Regional Research Laboratory, Trivandrum has been working on development of *Catharanthus roseus* to obtain anti-cancer drugs. Table 1.7 lists various institutions in India and their activities.

While the private sector mainly concentrated on medicinal compounds, Central Food Technological Research Institute, Mysore pioneered the research on production of food value metabolites from plant cell and hairy root cultures (Ravishankar and Venkataraman, 1990). Saffron ingredients from *Crocus sativus* cell cultures, anthocyanin from *Daucus carota* cell cultures, betalaines from hairy root cultures of *Beta vulgarís* and capsaicin from immobilized *Capsicum* spp cells are the few processes at different levels of scale-up.

Plant cell cultures and the micropropagation of economically important plants assume more importance in the wake of the new intellectual property regime.

1.5 Food additives from plant cell cultures

The technical feasibility of plant cell cultures has been demonstrated up to quite large scales of operation. However, the focus has been mainly on the production of

Table 1.7. Research on **plant cell culture in India** for the **production of secondary metabolites.**

Company/University	Products
Dabur Res. Foundation, Sahibabad	anticancer compounds, camptothecin, taxol
Dr. Reddy's Res. Foundation, Hyderabad	anticancer compounds, camptothecin, taxol
Bhabha Atomic Research Centre, Trombay	ajmalicine, camptothecin, reserpine, tylophorine
Central Food Tech. Res. Inst., Mysore	anthocyanin, capsaicin, betalaines, saffron metabolites, vanilla flavour
Central Institute of Medicinal and Aromatic Plants, Lucknow	scopoletine, artemisinin, essential oils
Regional Res. Lab., Jammu	Scopoletine, atropine, morphinane alkaloids, diosgenin, hyosyamine
Regional Res. Lab., Trivandrum Indian Inst.	azadiractin
of Chem. Tech., Hyderabad MS Univ.,	berberine
Vadodara	nicotine, tropane alkaloids, thymol, phenolic compounds
Hamdard Univ., Delhi	Withania somnifera alkaloids
Sukhadia Univ., Udaipur	anthraquinones
Dunger College, Bikaner	tropane alkaloids
Tropical Botanic Garden and Res. Inst., Trivandrum	micropropagation of medicinal plants
Univ. of Jammu, Jammu	-do-

medicinal compounds. In view of the recent advancements in the bioreactor design, bioprocess engineering, and the better understanding of *in vitro* physiology of plant secondary metabolism, the cell culture technology can also be extended to compounds of food applications (Ravishankar and Venkataraman, 1990). Table 1.8 lists some of the potential food additives which can be produced from plant cell cultures. *In vitro* production of food additives has been reviewed by many workers (Stafford, 1991; Knorr et al., 1993; Singh, 1997). Plant cell cultures for food additives has received greater attention in the recent years with the increasing concern about the safety of synthetic food additives. Several synthetic food additives are being phased out. With the growing population, to meet the demand, foods are processed for convenience into a variety of products and an increasing number of additives are being used. About 2500 different additives are added intentionally to foods to produce the desired effect (Branen, 1990). Food additives produced by fermentation processes are generally considered safe.

Among the natural food colours, anthocyanins are the most extensively studied. In view of the limited supply of these pigments, cell culture technique has been explored by several workers for *in vitro* production (Yamamoto, 1984; Seitz and Hiderer, 1988; Zhong *et al,* 1991; Rajendran *et al,* 1994). Similarly, capsaicin, a food additive for pungency, is an another food related compound which has been studied extensively, primarily because of its extracellular nature (Lindsey and Yeoman, 1984; Hau and Yeoman, 1991; Sudhakar Johnson *et al,* 1990, 1991; Suvarnalatha *et al,* 1993; Sudhakar Johnson and Ravishankar, 1996). A brief account of these two compounds is given below.

Anthocyanin

After chlorophyll, anthocyanins are the most ubiquitous pigments seen nature. Widely distributed in the pericarps of several fruits, flowers, and vegetables, these are glycosylated polyhydroxy and polymethoxy derivatives of flavylium (2-phenylbenzopyrylium) salts and belong to a group of compounds generically called

Table 1.8. Potential food additives for production from plant cell cultures (Updated from Ravishankar and Venkataraman, 1990).

Product	Species	Reference
COLOURS		
Anthocyanin	*Vitis vinifera*	
	Perilla frutescens	Zhong and Yoshida, 1995
	Daucus carota	Rajendran *et al.*, 1994
Betalaines	*Beta vulgaris*	Hamill *et al.*, 1987
Crocin	*Crocus sativus*	Sujatha *et al.*, 1990
Crocetins	*Gardenia jasminoides*	George and Ravishankar, 1995
FLAVOURS		
Angelica	*Angelica sylvestus*	
Garlic	*Allium sativum*	Madhavi *et al.*, 1991
Vanilla	*Vanilla planifolia*	Hevkin-Frankel *et al.*, 1991
Cocoa	*Theobroma cocoa*	Jalal and Collin, 1979
Basmathi	*Oryza sativa*	Suvarnalatha *et al.*, 1994
SWEETENERS		
Stevioside	*Stevia rabaudiana*	Swanson *et al.*, 1992
Thaumatin	*Thaumatococcus danielli*	Gabelman *et al.*, 1994
Glycyrrhizin	*Glycyrrhiza glabra*	Ko *et al.*, 1989
FOOD ADDITIVE		
Capsaicin	*Capsicum frutescens*	Sudhakar Johnson and Ravishankar, 1996
TONICS		
Ginseng biomass	*Panax ginseng*	Furuya and Ishii, 1972

flavonoids. Structurally, they are made up of two or three portions, the aglycone base, a group of sugars, and a group of acyl acids. The aglycone moiety is referred to as anthocyanindin. There are more than 15 anthocyanidins, (Francis, 1989) of which only 6 are more common (Fig. 1.4). The biosynthesis of anthocyanins follows the general phenylpropanoid pathway (Ebel *et ah,* 1974) (Fig. 1.5).

While the anthocyanins are thought to have a limited physiological role in pollination and seed dispersal, they are commercially very important pigments. They are used for colouring a variety of food products including beverages. Anthocyanins are known to have several pharmacological attributes such as anti-inflammatory, anti-ulcer and wound healing properties (Vega *et ah,* 1987; Kochi and Hisashi, 1990).

The commercial source of these pigments is the grape peel. In view of the quoted price of US $ 1200 - 1500 kg-^{1}, and the estimated market of US $ 135 million (Ilker, 1987), plant cell culture as an alternative source of supply of these pigments has been investigated all over the world.

Capsaicin

Capsaicin is the principle of pungency of the spice *Capsicum* and is possibly the earliest food additive used, as reported in the early cultures of Mexico and northern South America. The alkaloid, $C_{18}H_{29}O_3N$, is the acid amide of vanillylamine and C_9 –C_{11} isotype fatty acids. It is a colourless crystalline substance with severe burning pungency. The proposed pathway for biosynthesis of capsaicin is by phenylpropanoid metabolism via various hydroxylated cinnamic acids (Fig. 1.6).

Commercial capsaicin is often a mixture of capsaicin, dihydrocapsaicin and norhydrocapsaicin. Capsaicin contents of various chilli varieties have been reported to range from 0.2- 1.0% (Thirumalachar, 1967). Capsaicin, in the form of oleoresin or the dried powder of red pepper, is used to for its pungency and colour in a variety of fresh and processed foods. Besides, it is used as a counter irritant in lumbago, neuralgia and rheumatic disorders. Capsaicin has been reported to have antibacterial (Gal, 1965) and

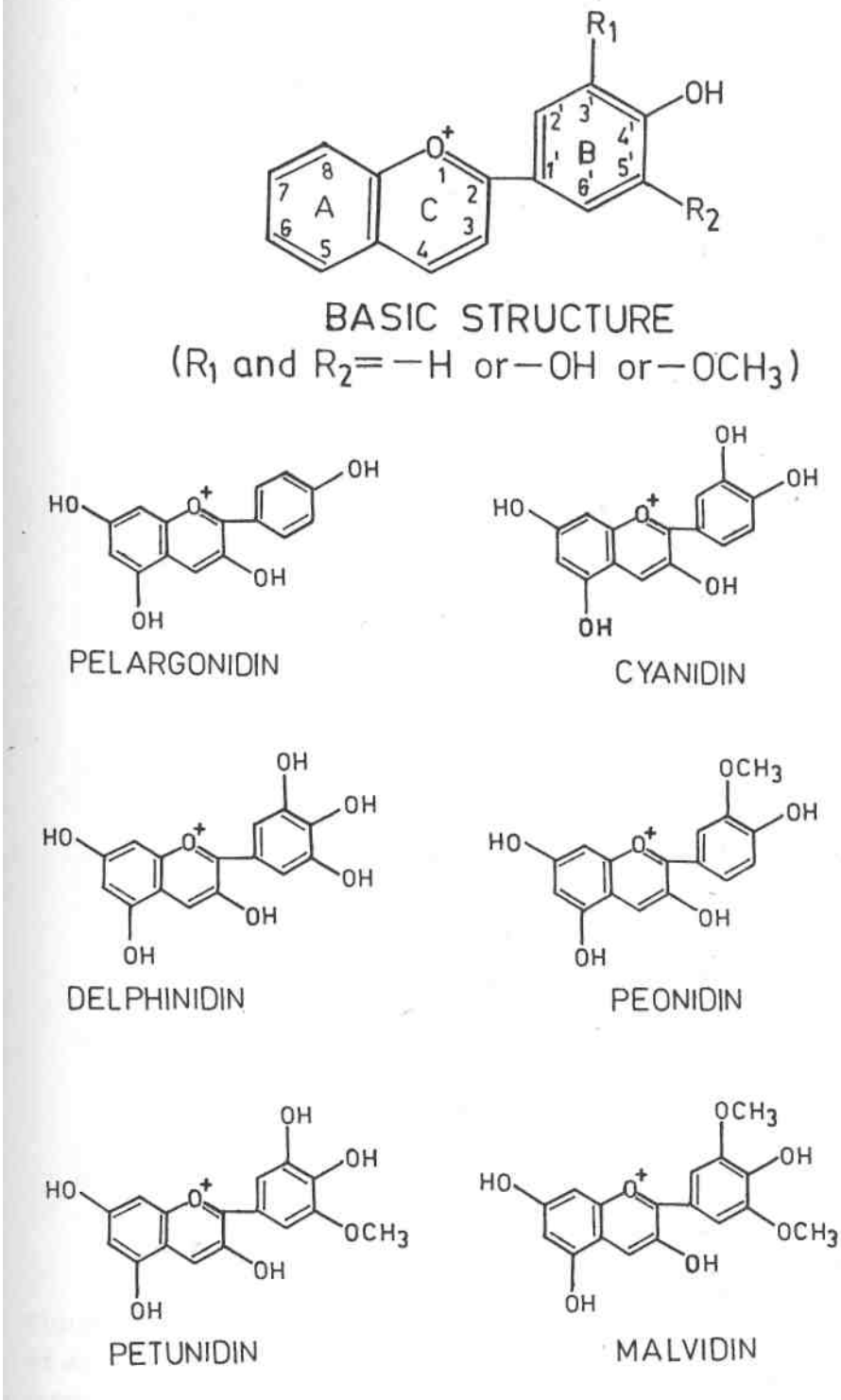

Figure 1.4. Common anthocyanidins.

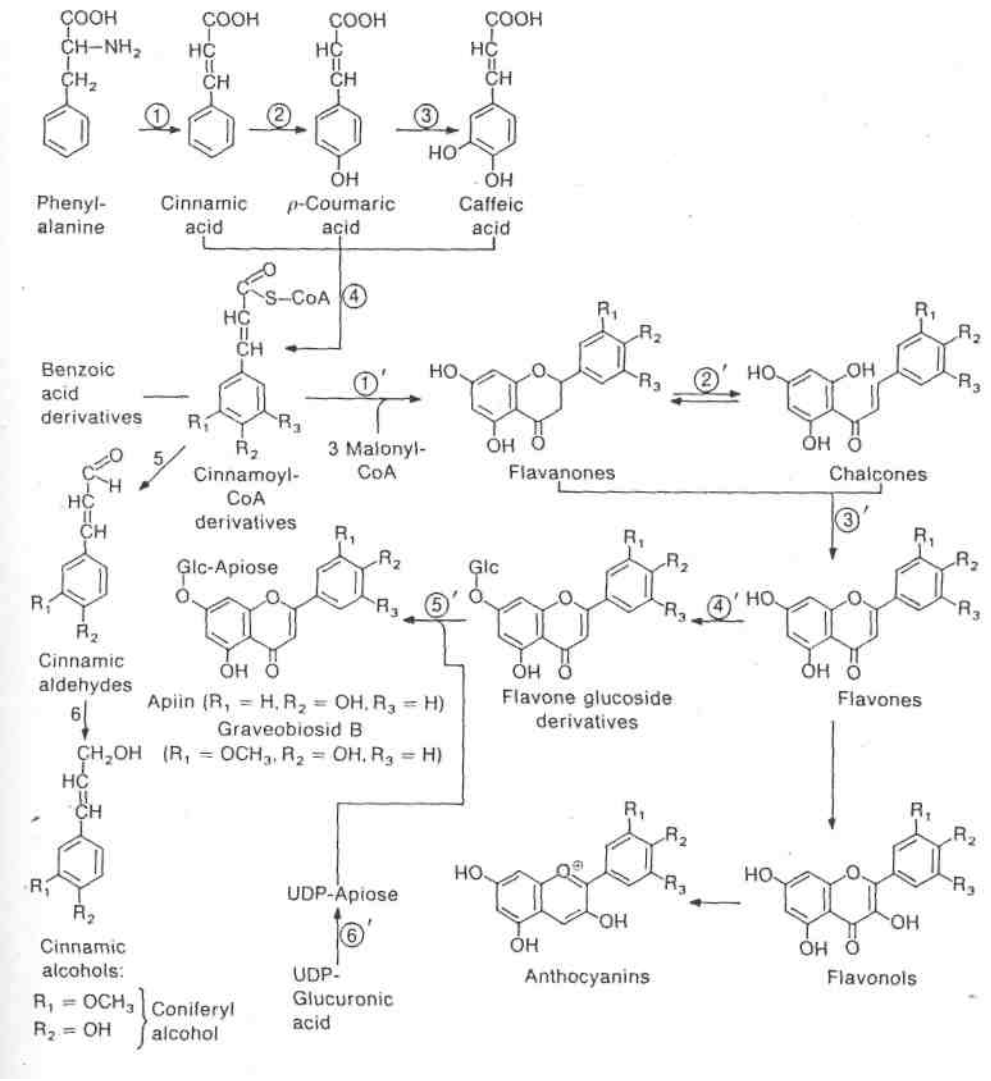

Figure 1.5. Cinnamic acid metabolism and flavonoid biosynthesis (Luckner *etal.* 1977). **Phenylpropanoid pathway:** (Group I). **1.** phenylalanine ammonia lyase 2. cinnamate 4-hydroxylase 3. 4-hydroxycinnamate 3-hydroxylase 4. cínnamate-CoA ligase. **Flavonoid pathway** (Group II). 1*. flavanone synthase 2' chalcone-flavanone isomerase 3'. chalcone-flavanone oxidase 4*. glucosyltransferase 5'. apiosyltransferase 6'. UDP-apíose synthetase. **Lignin pathway** (Group III). 5. cinnamoyl-CoA reductase 6. aromatic aldehyde reductase

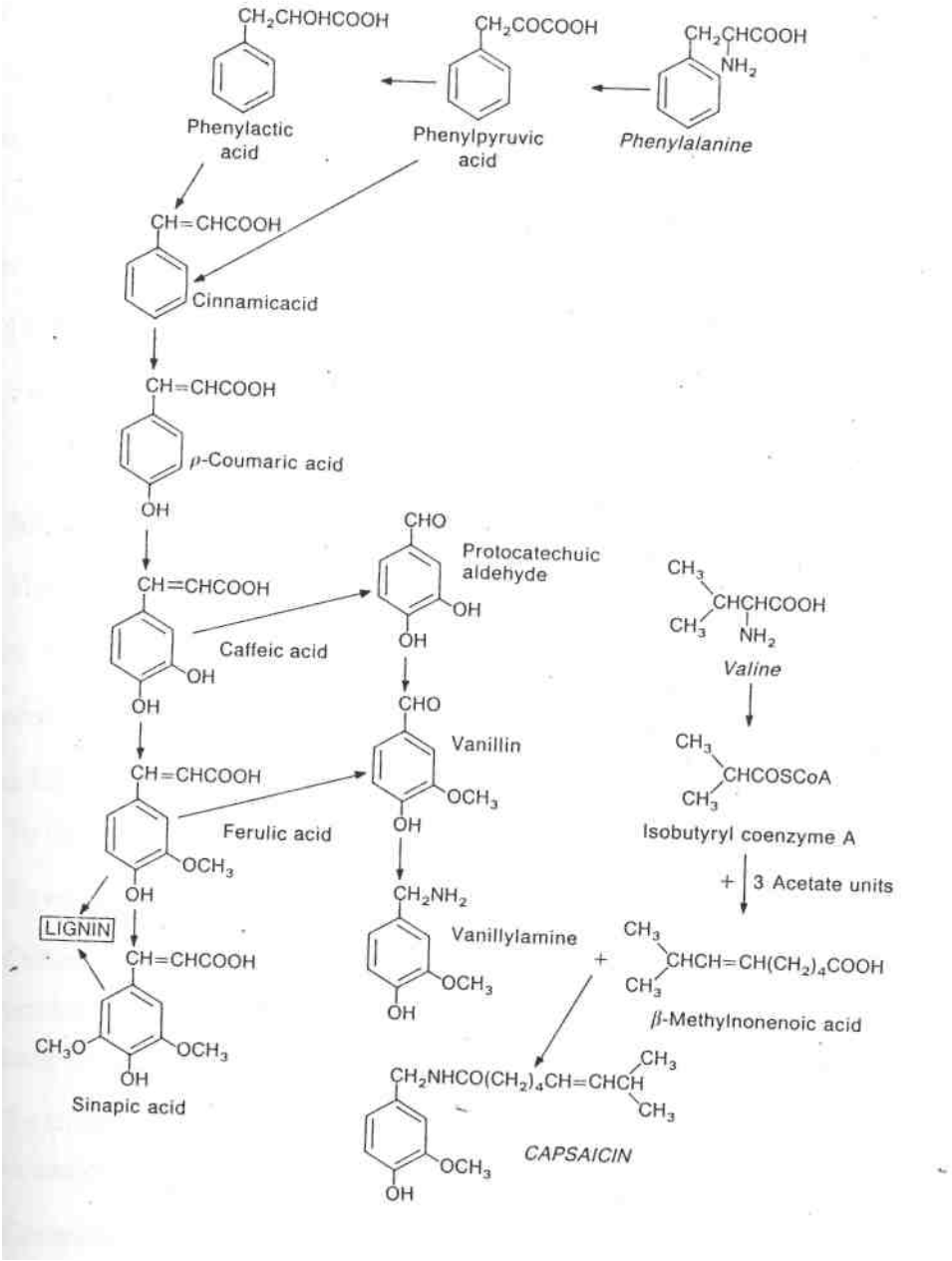

Figure 1.6. Biosynthetic pathway for capsaicin from phenylalanine and valine (Yeoman *et al.,* 1980).

antifungal (Sim, 1964) activity. Gutsu *et al.* (1982) suggested that capsaicinoids may function as immunity factors.

Commercial production of capsaicin by separation from the oleoresin and other carotenoids involves several steps and the cost of production is high. A few groups in the U.K. and Mexico have bee working on the possibilities of its production *in vitro.* Due to its high demand in food and pharmaceutical industries and high price (approx. US $ 1000 kg^{-1}), the continuous production from immobilized cell cultures has been the subject of intensive research.

1.6 Aim and objectives of the present study

The present investigation dealt with the studies on production of anthocyanin from *Daucus carota* cell cultures, and capsaicin from immobilized cells of *Capsicum frutescens* at shake flask, and lab scale reactor level. The specific objectives of the study were as follows:

* To develop a high yielding cell line of *Daucus carota.*
* To establish and maintain the cell suspension cultures of *D. carota.*

  Optimization of process parameters of the cell culture process for anthocyanin production. The parameters included nutrient medium, hormonal supplementation, temp, pH, light, dissolved oxygen.

  To study the kinetics of growth and production of anthocyanin by *D. carota* cells in suspension cultures.

  Comparative evaluation of various methods for estimation of biomass in plant cell suspension cultures.

  Growth, and production of anthocyanin from *D. carota* cells in bioreactor at 2 L level.
* Rheological characterization of the culture broth in bioreactor.
* Immobilization of *Capsicum frutescens* cells in alginate, and polyurethane foam matrices.

  Studies on continuous production of capsaicin from immobilized *C. frutescens* cells using a suitable column reactor.

MATERIALS & METHODS

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2.1 Initiation of callus cultures

Seeds of *Daucus carota* (carrot) and *Capsicum frutescens* Mill. IHR 1203 were obtained from the Indian Institute of Horticultural Research, Bangalore. Selected seeds were surface sterilized with mercuric chloride solution (0.1%, w/v) for 4 min and washed several times with sterile distilled water to remove the traces of mercuric chloride. Seeds were then aseptically transferred to sterile Petri plates containing moistened Whatman No.l filter paper and allowed to germinate in dark. The one-week-old seedlings were then transferred aseptically onto semi solid nutrient medium containing Murashige and Skoog's (1962) salts supplemented with sucrose (3%, w/v), myo-inositol (100 mg l^{-1}), indole-3-acetic acid (IAA) (2 mg l^{-1}) and kinetin (0.2 $mg1^{-1}$), in case of *D. carota,* and 2,4-dichlorophenoxyacetic acid (2,4-D) (2 $mg1^{-1}$) and kinetin (Kn) (0.5 $mg1^{-1}$), in case of *C. frutescens,* solidified with agar (1%, w/w). The pH of the medium was adjusted to 5.8 before autoclaving at 1.3 kg cm^{-2} for 20 min. The cultures were incubated at 25 ± 2 °C under continuous light of 3000 lux using 40 W tubular fluorescent lamps (Philips India Ltd., Calcutta) as the source of light. Callus which appeared after one week of transferring to the above medium was allowed to grow for 3 weeks. The callus thus obtained was maintained by regular subculturing at 3-week, and 2-week intervals for *D. carota,* and *C. frutescens,* respectively.

2.2 Initiation and maintenance of cell suspension cultures

Callus tissues (approx. 4 g) were transferred to 40 ml of liquid medium of the same composition described in section 2.1.1. but without the gelling agent in 150 ml Erlenmeyer flasks. They were incubated on a rotary shaker (New Brunswick Scientific Co., Inc., New Jersey) at 90 rpm under continuous light (3000 lux). The cell suspensions were filtered through appropriate sieves to obtain single cells and few-celled aggregates which were used as inoculum for subculture. Fine cell suspension cultures were obtained by repetitive selection for small cell aggregates at

every stage of subculturing. The cell suspension cultures were maintained by periodic subculturing at two-week intervals.

2.3 Sterility test

The sterility of the cell cultures was tested by using the Bacto-Fluid Thioglycollate Medium (Difco Laboratories Inc., Michigan). The composition of the medium is given in Table 2.1. The purpose of a small amount of agar in the liquid medium was to support the growth of both aerobes and anaerobes without any seal or other special precautions. Bacto-Fluid Thioglycollate Medium was rehydrated by suspending 29.5 grams in 1 litre of distilled water and heated to boiling to dissolve the medium completely. 10 ml of medium was distributed into screw cap tubes (20 x 45 mm) and sterilized by autoclaving at 1.3 kg cm^{-2} for 20 min. Cooled quickly to room temperature. Culture suspension was inoculated with the help of a loop into the screw cap tubes and incubated at 37 °C for 48 hours using temperature controlled shaking water bath. Any turbidity of the medium was indicative of contamination. Every culture was tested for sterility before subculturing.

2.4 Viability assay

A rapid method developed by Sudhakar *et al* (1996) was used to assess the viability of cells. The method uses a mixture of calcofluor white and fluorescene diacetate (FDA). Calcofluor, commercially available as Ranipal, was dissolved in MS basal medium to get a concentration of 0.00625% and FDA stock solution was prepared by dissolving 0.5 g in 100 ml of acetone. This stock solution was stored at -4 °C. Before use, 0.1 ml of stock was diluted with 5 ml of culture medium and stored in ice. Best results were obtained when used within an hour. The dye mixture was prepared by adding one drop of diluted FDA solution to one drop of calcofluor white. Approximately equal volumes of cell suspension and dye mixture were taken on a microscope slide and observed after 2 min, under a Leitz Diaplan fluorescence

Table 2.1. Composition of the Bacto-Fluid Thioglycollate Medium.

Yeast extract	5.0 g
Casitonel	5.0 g
Dextrose	5.0 g
Sodium chloride	2.5 g
1-cystine	0.75 g
Thioglycollic acid	0.30 ml
Agar	0.75 g
Resazurine	0.001 g

Ref.: Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical Laboratory Procedures. 1953. Difco Laboratories Inc., Michigan.

microscope fitted with two exciter filters, BP 340-380 and BP 450-490.

2.5 Few commonly used plant cell culture media

To investigate the physico-chemical properties of plant cell culture media, the following twelve most commonly used nutrient media were selected for the study: Hoagland and Arnon (1950) (H&A), Murashige and Skoog (1962) (MS), Eriksson (1965) (ER), Gamborg *et al.* (1968) (B5), White (1963) (WS), Nitsch and Nitsch (1969) (N&N), Schenk and Hildebrandt (1972) (S&H), Gresshoff and Doy (1974) (DBM2), Hildebrandt (1977) (HTM), Anderson (1978) (A&R), Lloyd and McCown (1981) (WPM), Litvay *et al.* (1985) (L&M). Liquid media were prepared as per the composition given by the authors (Table 2.2). However, no phytohormones were added. For uniformity sucrose was added at 3% (w/v) as the sole carbon source in all the media.

2.6 Estimation of buffer capacity

Acid-base titration curves of few commonly used plant cell culture media were plotted by measuring the changes in pH upon addition of small amount of H^+ or OH^-. Buffer capacity was estimated from polynomial regression analysis of pH and H^+ data (Segel, 1976).

2.7 Cell aggregate separation

Cell aggregates were separated into six different size groups using test sieves of mesh sizes 355, 500, 850, 1000, and 1500 µm.

2.8 Estimation of biomass

Biomass was measured as dry weight, after filtering out the cells from the liquid medium, and drying at 60 °C for 24 hours in a hot air oven. In all the subsequent Sections, unless otherwise specifically mentioned, 'biomass' refers to the dry cell

Table 2.2. Composition of few commonly used plant cell culture media.

Constituent	MS	B5	WS	N&N	L&M	Eriksson	SH	HTM	DBM2	H&A	WPM	A&R
NH_4NO_3	1650.0			720.0	1650.0	1200.0			1000.0		400.0	400.0
$(NH_4)_2SO_4$		134.0					300.0			115.03		
$NH_4H_2PO_4$												
H_3BO_3	6.2	3.0	1.5	10.0	31.0	0.53	5.0	0.375	0.3	2.86	6.2	6.2
$CaCl_2.\ 2H_2O$	440.0	150.0		219.83[a]	22.0	440.0	200.0				96.01[b]	439.92[c]
$Ca(NO_3)_2.\ 4H_2O$			208.4					400.0	241.2	656.4	386.0	
$CoCl_2.\ 6H_2O$	0.025	0.025			0.125	0.0025	0.1		0.025			0.025
$CuSO_4.\ 5H_2O$	0.025	0.025	0.01	0.025	0.50	0.0025	0.2	3.0	0.025	0.08	0.25	0.025
$EDTA.Na_2.\ 2H_2O$	37.26	37.3		37.3	37.3	37.3	20.0		37.3		37.3	74.5
$EDTA.ZnNa_2$						15.0						
$FeSO_4.\ 7H_2O$	27.8	27.8		27.8	27.8	27.8	15.0		27.85		27.8	55.7
$Fe_2(SO_4)_3$			2.5									
$Fe(C_4H_4O_6)_3$												
$MgSO_4.\ 7H_2O$	370.0	250.0	750.0	184.89[d]	1850.0	369.69[e]	400.0	180.0	34.98[f]	5.32	369.69[h]	369.69i
$MnSO_4.\ H_2O$	16.9[j]	10.0[k]	3.788	18.9	21.0	1.69	10.0	3.41	1.0	492.57[g]	22.3	16.9
$MnCl_2.\ 4H_2O$										1.81		
MoO_3			0.001							0.016		
$Na_2MoO_4.\ 2H_2O$	0.25	0.25		0.25	1.25	0.025	0.1		0.25		0.25	0.25
KI	0.83	0.75	0.75		4.15		1.0		0.8			0.3
KNO_3	1900.0	2500.0	80.0	950.0	1900.0	1900.0	2500.0	65.0	1000.0	606.6		480.0
KCl			65.0					37.30	65.0			
K_2SO_4											990.0	
KH_2PO_4	170.0			68.0	340.0	340.0		6.06	300.0	0.22	170.0	
NaH_2PO_4		130.5	16.5					800.0				330.6
Na_2SO_4			200.0									
$ZnSO_4.\ 7H_2O$	8.6	2.0	3.0	10.0	43.0		1.0		0.3		8.6	8.6
Glycine	2.0		3.0	2.0		2.0			4.0	2.0	2.02	
Myo-inositol	100.0	100.0		100.0	100.0		100.0			100.0	100.0	100.0
Nicotinic acid	0.5	1.0	0.5	5.0	0.5	0.5	5.0		10.0	0.5	0.1	
Pyridoxine-HCl	0.5	1.0	0.1	0.5	0.1	0.5	0.5		0.1	0.5	0.1	
Thiamine-HCl	0.1	10.0	0.1	0.5	0.1	0.5	5.0		0.1	0.5	0.5	0.4
Folic acid				0.5					1.0	0.1		
Biotin				0.05					0.2			

[a]Original $CaCl_2$ 166.0mg/l. [b]Original $CaCl_2$ 72.5mg/l. [c]Original $CaCl_2$ 332.2mg/l. [d]Original $MgSO_4$ 90.37mg/l.
[e]Original $MgSO_4$ 180.78mg/l. [f]Original $MgSO_4$ 17.099mg/l. [g]Original $MgSO_4$ 240.76mg/l. [h,i]Original $MgSO_4$ 180.7mg/l.
[j]Original $MnSO_4.\ 4H_2O$ 22.3mg/l. [k]Original $MnSO_4.\ 4H_2O$ 13.2mg/l.

weight. The medium filtrate was used for the estimation of glucose, fructose, sucrose, conductivity, and osmolarity.

2.9 Measurement of surface colour intensity

Surface colour intensity of cell aggregates of *D. carota* was measured in terms of the red-green coordinate value of CIELAB Color Space (Nassau, 1993), using a Shimadzu LISR-2100 Colour Measuring System. Barium sulphate was used a standard for white.

2.10 Estimation of glucose

Glucose was estimated by the glucose oxidase and peroxidase method (Trinder, 1969). Glucose oxidase converts glucose to gluconic acid and hydrogen peroxide. The peroxide in the presence of peroxidase forms a coloured complex of hydroxybenzoate and 4-aminophenazone. The intensity of the colour formed is proportional to the glucose level.

A kit of the enzymes, reagents, and the glucose standard obtained from Span Diagnostics Pvt. Ltd., Surat, was used following the procedure prescribed by the supplier.

2.11 Estimation of fructose

Fructose was estimated by measuring the reducing sugars using dinitrosalicylic acid reagent (DNS method) (Miller, 1959) and subtracting the amount of glucose. The DNS reagent contained 3,5-dinitrosalicylic acid (1%, w/v), phenol (0.2%, v/v), sodium sulphite (1%, v/v), and sodium hydroxide (1%, w/v). The reagent was prepared by placing all the solid components in a container and dissolving them simultaneously by stirring with the required volume of sodium hydroxide solution. The reagent was stable for about two weeks.

The colour tests were made with 3 ml aliquots of the reagent added to 3 ml of

appropriately diluted sample in 15 ml test tubes. The reagent blank received 3 ml distilled water. The mixtures were heated for 5 min in a boiling water bath and then cooled under running tap water to adjust to ambient temperature. The colour intensity was measured at 575 nm. Glucose was used as standard for preparing calibration curve.

2.12 Estimation of sucrose

Unutilized sucrose in the culture media was estimated by using the DNS method after inversion by overnight incubation with 6N HC1 at room temperature. The hydrolysed sample was neutralized with cone. NaOH solution and the volume was made up to a known quantity (Herlich, 1990)

2.13 Estimation of nitrate

The nitrate concentration was determined as described by Greenberg *et al.* (1992). 1 ml of NaCl (30%, w/v), and 5 ml of H_2S0_4 (80%, v/v) were added to 5 ml of appropriately diluted cell-free medium samples. The absorbance at 410 nm was measured. 0.25 ml of brucine reagent (mixture of 1 g brucine and 0.1 g of sulphanilic acid in 100 ml of dist. water) was added to this mixture. The resulting solution was boiled for 20 min and the absorbance at 410 nm was measured again. The difference in absorbances was used to estimate the concentration of nitrate by comparison with solutions of known nitrate concentration treated identically. Potassium nitrate was used as the standard.

2.14 Estimation of ammonia

The ammonia concentration in the liquid nutrient medium was determined using the phenol-hypochlorite reaction following the method given by Weatherburn (1967). Two reagents were prepared: A. 5 g of phenol with 25 mg of sodium nitroprusside (sodium pentacyanonitrosyloferrite III) in 500 ml of dist. water. The reagent was

stored in amber bottle in refrigerator. B. 2.5 g of sodium hydroxide, and 4.2 ml of sodium hypochlorite in 500 ml of dist. water.

5 ml of reagent A was added to 20 µl of appropriately diluted sample solution. The test tubes were shaken vigorously and allowed to stand for 20 min covered with parafilm. The absorbance was read at 625 nm against identically treated water blank. The sensitivity of the method was about 0.5 µg of ammonia nitrogen. Ammonium sulphate was used as standard.

2.15 Estimation of phosphate

Phosphate was measured as phosphorous by using molybdate reagent according to the procedure given by Chen *et al.* (1956). The molybdate reagent was prepared as follows: One volume of H_2S0_4 (6N) was mixed with two volumes of distilled water and one volume of ammonium molybdate solution (2.5%, w/v). Then one volume of ascorbic acid (10%) was added and mixed well. The reagent was prepared fresh every day.

Procedure: 4 ml of the molybdate reagent was added to 4 ml of appropriately diluted sample (to contain up to 8 µg of phosphorous). The reagent blank contained 4 ml of distilled water. The test tubes containing the reaction mixture were capped with parafilm and incubated at 37 °C in a temperature controlled shaking water bath for 2 hours. After the incubation, the test tubes were allowed to cool and the absorbance was read at 820 nm against the blank. The amount of phosphate in the sample was calculated from a calibration graph plotted from the absorbances of solutions of known phosphorous concentration. Dihydrogen potassium phosphate was used as the standard.

2.16 Measurement of conductivity

The electrical conductivity was measured using a conductivity cell with cell constant of 1.0 connected to a direct current conductivity metre model - LF54 (Wiss-

Techn-Werstatten, Weilheim). The conductivity was expressed in the units of mS (milli Siemen).

2.17 Measurement of osmolarity

Osmolarity was measured by using an automatic cryoscopic osmometer:* Osmomat 030-D (Gonotech, GmbH, Germany). The instrument was calibrated between 0, and 300 mOsmol kg^{-1} using the standard solutions supplied by the manufacturer. The osmolarity was expressed in the units of mOsmol kg^{-1} .

2.18 Measurement of turbidity

Cell suspension cultures of *D. carota* and *C. frutescens* were used in the studies on estimation of biomass based on the turbidity of cell free medium. The cells were separated from the culture medium by passing through a sieve of mesh size 37 µm. The filtrate, referred to as 'cell free medium' was used for the estimation of turbidity, extracellular polysaccharides, and proteins.

Turbidity was measured as optical density at 630 nm against distilled water.

2.19 Estimation of extracellular polysaccharides

Extracellular polysaccharides present in the cell free culture medium were precipitated by adding three volumes of iso-propyl alcohol. The precipitate was redissolved in water and estimated using phenol-sulphuric acid method (Dubois *et al,*

*The principle used in the measurement of osmolarity is a colligative property of solutions, namely the freezing point of a solution decreases with a decrease in the molal fraction of the solvent. The depression in the freezing point is given by the expression:

$T_F = K_F m$

where, T_F = freezing-point depression
K_F = molal freezing point depression constant (for water, K= 1.855)
m = molal fraction of the solvent

1956).

2 ml of sugar solution containing between 10 and 70 µg of sugar pipetted into a colorimetric tube, and 1 ml of phenol (5%, v/v, in water) was added. Then, 5 ml of cone. H_2S0_4 was added rapidly, the stream of acid being directed against the liquid surface rather than against the side of the test tube in order to obtain good mixing. The tubes were allowed to stand for 10 min, then they were shaken and placed for 10 to 20 min at 30 °C in a temperature controlled shaking water bath for 20 min. The absorbance of the characteristic yellow-orange colour was measured at 490 nm. The colour was stable for several hours. Blanks were prepared by substituting the distilled water for the sugar solution. Sucrose was used as standard.

2.20 Estimation of extracellular proteins

Extracellular proteins were precipitated with trichloroacetic acid (Peterson, 1983). The Peterson's precipitation step was as follows:

a. 0.1 ml of deoxycholate (0.15%) was added to 1.0 ml of protein sample.
b. Vortexed and allowed to stand at room temperature for 10 min.
c. 0.1 ml of trichloroacetic acid (72%) was added, vortexed, and centrifuged at 2000 g for 30 min.

The pellet obtained after decanting the supernatant was redissolved in water and hydrolysed with equal volume of NaOH (2N) at 100 °C for 10 min in boiling water bath. The hydrolysate was estimated for protein according to Lowry *et al.* (1951).

2.21 Assay of phenylalanine ammonia lyase (E.C. 4.3.1.5)

500 mg of cell biomass was homogenized in a mortar and pestle with 0.1 M borate buffer (pH 8.8), 250 mg of activated charcoal, and acid washed neutralized sand. The extract was centrifuged at 30,000 g for 15 min'. The supernatant was used as enzyme extract.

Estimation of PAL activity was carried out according to Hahlbrock *et al.* (1971)

with a slight modification. The assay mixture contained 30 µ mole of borate buffer (pH 8.8), 30 µ mole of 1-phenylalanine and 1 ml of appropriately diluted enzyme extract in a total volume of 3 ml. Blank contained 1 ml of borate buffer instead of the enzyme extract. The reaction mixture was incubated at 37 °C in a temperature controlled shaking water bath for one hour. The concentration of t-cinnamic acid formed was measured at 290 nm using a uv-visible spectrophotometer. The amount of t-cinnamic acid formed was calculated using a standard calibration curve prepared from the optical densities of different concentrations of t-cinnamic acid in borate buffer. One unit of the enzyme activity was defined as 1 µg of t-cinnamic acid formed per hour.

Protein was estimated according to Lowry *et al.* (1951) using bovine serum albumin as the reference standard.

2.22 Stirred tank bioreactor

2.5 L stirred tank bioreactor (New Brunswick Scientific Co., Inc., New Jersey) with flat blade (disc) turbine type impeller was used for culture of high yielding *D. carota* cells. The reactor was of the standard configuration with direct coupled top driven impeller (Fig. 3.31), and equipped with a polarographic dissolved oxygen probe, pH electrode, and a foam level sensor. Different measurement and control units of the bioreactor provided accurate control of the various process parameters such as pH, DO, temp, and the foam level at their set points.

An electrically driven air compressor (Kirloskar Pneumatic Co. Ltd., Poona) with a tank capacity of 100 L was used to supply air into the bioreactor. The pressure inside the tank was maintained between a narrow range of 80 - 100 psi to reduce the cyclic fluctuation in the air flow rate.

Sterile air was provided through polytetraflouroethylene (PTFE) membrane filters (Sartorius Biotech (India) Pvt. Ltd., Bangalore). The pressure drop across the outlet filter was negligible at air flow rates employed and consequently the culture broth

inside the bioreactor was at the atmospheric pressure.

The bioreactor was operated in batch mode with a culture period of 14 days. 2 1 of medium was inoculated with 35 g of cell from shake flasks. pH was maintained at 5.5 and the temperature was maintained at 25 ± 2 °C. The dissolved oxygen level was set at 40% of air saturation with a fixed air flow rate of 0.5 VVM. During the culture, the foam was controlled by adding silicon anti-foam emulsion.

2.23 Measurement of oxygen consumption rate

The oxygen consumption rate (OCR) was measured as described by Srinivasan *et al.* (1995). 500 mg of cells were placed in a 25 ml sample holder with 20 ml of nutrient medium. The cells were kept in suspension by stirring with a magnetic bar. Air was fed into the flask through a stainless steel needle until a constant dissolved oxygen (DO) concentration was measured. The needle was removed and the unit was quickly closed using a rubber stopper with a polarographic DO electrode (Ingold Electrodes Inc., Masachussets) inserted through it. This procedure resulted in displacing any remaining air in the sample holder. The decrease in DO concentration due to uptake by the cells was monitored using the $p0_2$ measurement and control module of 10 litre bioreactor (Bioengineering AG, Switzerland). The decrease in the DO concentration was plotted against the time, and the oxygen uptake rate was calculated from the slope of the curve. The measurement was carried out at room temperature (22-25 °C).

2.24 Rheological studies

Samples were drawn from the bioreactor at intervals and analyzed for rheological properties using the methodology followed by Bhattacharya and Bhat (1997).

The coaxial cylinder principle was adopted to obtain shear-rate and shear-stress data. The ratio of the external diameter of the rotating bob to the internal diameter of the stationary cylinder was 0.954. A Rheometer (Model No RT10, Haake GmbH,

Karlsruhe, Germany) was used to determine the rheological behaviour of the culture broth at an increasing shear-rates (up to 200 s^{-1}). All rheological measurements were carried out at 25 ± 0.1 °C and the experiments were conducted in duplicate.

The rheological parameters measured were the shear-rate and shear-stress. The shear-rate and shear-stress data were subjected to fitting to the common rheological models, viz. the Newtonian model (Eq. 1), and the power law model (Eq. 2) using the Haake software (Version 4.1, 1995). The extent of fitting to the either of these models was judged by finding the chi-square ($\chi 2$) value. The flow behaviour index and consistency index (for power law model) were estimated from non-linear regression analysis of the shear-rate and shear-stress data. The apparent viscosity of the culture broth was determined at a shear-rate of 100 s^{-1}.

$$\sigma = \eta \gamma \quad (1)$$

$$\sigma = k\gamma^n \quad (2)$$

where σ = shear-stress (mPa)
η = apparent viscosity (mPa s)
γ = shear-rate (s^{-1})
k = consistency index (mPa s^n)
n = flow behaviour index (dimensionless)

2.25 Estimation of anthocyanin

Anthocyanin was extracted from the cells by using acidified methanol (HC1 1 %, v/v) and estimated spectrophotometrically at 535 nm, using the molar extinction coefficient of 29,500 M^{-1} cm^{-1} (Stickland and Sunderland, 1972) for cyanidin-3-monoglucoside (Molecular weight 445.2 g mol^{-1}).

2.26 Identification of anthocyanidins

Five different anthocyanidins were prepared from different sources. They were, concentrated extracts of (a) *Vitis vinifera* fruit skin, (b) banana bract, (c) *Hibiscus*

flowers, (d) *Solanum melangena,* (e) *Ipomea tricolour.* The anthocyanidins of these species are well documented (Ribereau-Gayon, 1959; Simmonds, 1954; Lowry, 1976; Harborne, 1967a; Asen *et al,* 1977). Malvidin-3-glucoside, in a partially purified form, was obtained as a gift sample from Dr. Geza Hrazdina, Cornell University, PA. The extraction and concentration of pigments from these standards were done as described for cells.

Sample preparation

The identification of anthocyanidins in cell extract and standards were performed after acid hydrolysis (Francis, 1982). 4 ml of HC1 (2N) was added to 1 ml of the above extracts. The mixture was boiled at 100 °C in a water bath for 35 minutes for complete hydrolysis. After cooling, the mixture was extracted with ether to remove any carboxylic acids. To the aqueous solution, 2 ml of amyl alcohol was added and mixed thoroughly to extract the pigment. The upper amyl alcohol layer which contained the anthocyanidins was removed and dried on a watch glass. One ml of HPLC grade methanol was added to this and centrifuged at 5000 x g to remove any undissolved particles. These samples were then used for identification.

Identification

Identification of anthocyanidins was performed based on the retention times in high performance liquid chromatography (HPLC) adopting the procedure described by Wilkinson *et al.* (1977) with a minor modification. Shimadzu high performance liquid chromatograph (CR-7A) was used with C_{18} column (3.9 x 300 mm; pore size 10 μ) (μ-Bondapak, Waters). Methanol-acetic acid-water (70:10:20) was used as the mobile phase at a flow rate of 1.5 ml min^{-1}. Detection was done using a SPD-10A UV-visible detector at 530 nm. The peaks were recorded in CR7A chromatopac. Retention times of anthocyanidins, obtained from various sources as given in Section 2.26, were compared with those of cell extract.

2.27 Immobilization of cells using alginate

Sodium alginate (Sigma Chemical Co., USA) was dissolved in hot distilled water (1.5%, w/v) and sterilized at 1.3 kg cm^{-2} for 20 min. The cells at the end of log growth phase in suspension cultures were separated from the medium by filtration and suspended in the sterile sodium alginate solution, and mixed well to get a homogeneous suspension. The suspension was then extruded into $CaCl_2$. $2H_20$ (1.35 g in 150 ml water) through sterilized glass tubes of 4 mm diameter. After 30 min of incubation, the resulting beads were washed thoroughly with sterile distilled water to remove the traces of calcium chloride before packing into the column reactor.

2.28 Solubilization of alginate beads

The alginate beads immobilizing the cells were solubilized by the method described by Jones and Veliky (1981). The beads were suspended in a suitable volume of potassium phosphate buffer (0.1 M; pH 7.5) and agitated at 90 rpm for 1 h on a rotary shaker. Cells were sedimented by centrifugation for 5 min at 100 rpm. A higher centrifugation resulted in sedimentation of insoluble calcium phosphate. The cloudy supernatant was removed with a Pasteur pipette and the cells were recovered for estimation of dry weight.

2.29 Immobilization of cells in polyurethane foam

Polyurethane foam (PUF) of 2 cm thickness was cut in to discs of 5.5 cm diameter. The PUF discs were washed thoroughly with Teepol to remove the traces of monomers. The discs were incubated with the culture medium to load the cells into the foam. After two weeks of incubation, the PUF discs loaded with the cells were packed aseptically into the glass column reactor. The cell loading was estimated as follows:

Few discs loaded with the cells were taken out and the excessive cell biomass grown on the surface of the PUF disc was scrapped off. The PUF discs along with the

cells were dried at 60 °C for 24 hours in a hot air oven. The increase in the weight of the PUF disc was a measure of the dry weight of the cells loaded into the PUF disc. Fresh weight of the cells loaded into the PUF disc was calculated using the dry weight to fresh weight ratio.

2.30 Packed-bed column reactor

PUF discs loaded with *C. frutescens* cells were aseptically packed into a glass column reactor of 1 1 volume (Fig. 3.41). The liquid nutrient medium was sprayed onto the PUF bed through a nozzle at a constant flow rate of 25 ml $hour^{-1}$ using a peristaltic pump. Sterile air was bubbled from a port located at the bottom of the glass column.

The effluent medium was collected at the downstream and analyzed for capsaicin.

2.31 Estimation of capsaicin

Capsaicin from culture medium was extracted with two volumes of ethylacetate in a separating funnel and the upper portion was collected and dried using a flash evaporator. The resultant residue was redissolved in ethylacetate to give an appropriate concentration for subsequent estimation.

Capsaicin was estimated by paper chromatography as described by Govindarajan and Anantha Krishna (1974). Extracts were spotted on Whatman No. 1 chromatographic strips (4.5 x 20 cm). The strips were developed by ascending chromatography in methanol: buffer (60 : 40) solvent system. The buffer solution was prepared by mixing 3.1 g of boric acid and 3.7 g of potassium chloride in 750 distilled water and the pH was adjusted to 9.6 using 0.1N NaOH and the final volume was made up to 1000 ml. After the solvent moved up to 17 cm, the strips were taken out and allowed to dry at room temperature. The dried strips were passed uniformly and quickly through 0.1% (w/v) Gibb's reagent (2,6-dichloro-p-benzoquinone-4-

chlorimine) in ethyl acetate. Capsaicin became visible as a blue spot near the solvent front (Rf 0.8 - 0.9). The colour was further developed by mild spray with buffer on both sides of the paper. The strips were dried in the dark for 30 min for full colour development.

The blue spots of were cut and eluted in 10 ml of ethanol in the dark and the intensity of colour was measured at 615 nm against a reagent blank from the corresponding area of the strip run simultaneously without sample. The amount of capsaicin in the sample was calculated from a calibration curve prepared by using the standard capsaicin (Sigma Chemical Co., USA).

2.32 Preparation of elicïtors

Aspergillus niger cultures, obtained from the Food Microbiology Department of C.F.T.R.I., Mysore, were maintained on potato dextrose agar (PDA) medium and were grown in Bacto Czapek Dox liquid broth (Difco Laboratories, Inc., Michigan). The PDA medium was prepared by boiling sliced potatoes (200 g) in water till they were soft. Filtrate was diluted to 1000 ml with water. Dextrose was added (2%, w/v) prior to the adjustment of pH to 5.5 using tartaric acid.

Ten-day-old cultures of *A. niger* from the liquid medium were harvested. The mycelium was separated from the medium. Twenty grams of mycelium was ground at room temperature and extracted in distilled water. The extract was centrifuged at 400 x g for 15 min. The supernatant fraction was made up to 200 ml and used as the elicitor. The elicitor concentration was expressed as g equivalent of mycelium. The extract was stored at 0 °C till use.

RESULTS

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3.26 Production of anthocyanin in stirred tank bioreactor

3.27 Oxygen consumption rate of *D. carota* and C. *frutescens* cells

3.28 Rheological characteristics of *D. carota* culture broth

3.29 Characterization of anthocyanins

3.30 Production of capsaicin using bubble column reactor

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3.32 Production of capsaicin using packed-bed reactor

3.33 Enhancement of capsaicin production with fungal elicitor

3.1 Initiation of cell cultures

Seeds of *Daucus carota* and *Capsicum frutescens* germinated within a week upon implanting on moistened filter paper. The excised seedlings gave rise to callus upon transferring onto semi-solid medium containing MS salts (Murashige and Skoog, 1969) with sucrose (3%, w/v) supplemented with indole-3-acetic acid (2 mg 1^{-1}) and kinetin (0.2 mg 1^{-1}) for *D. carota;* 2,4-dichlorophenoxyacetic acid (2 mg 1^{-1}) and kinetin (0.5 mg 1^{-1}) for *C. frutescens.* Callus of *D. carota* was friable and green in colour with zones of pigmented cells, while the callus of *C. frutescens* was friable and white (Fig. 3.1). Callus cultures of *D. carota* with intense colour were established by repeated selection and subculturing of highly pigmented cell clusters (Fig. 3.2).

Suspensions of cells were obtained by transferring the calli of *C. frutescens* and *D. carota* into the liquid medium of the same composition as the semi-solid medium except the gelling agent. Fine cell suspension cultures were established by repeated selection and subculturing of small cell aggregates (Fig. 3.3).

3.2 Growth profile of primary cultures of *D. carota*

A time course study of the accumulation of biomass in primary cell cultures of *D. carota* showed a non-sigmoidal growth curve (Fig. 3.4). The growth cycle consisted of a lag phase of about 3 days followed by a linear increase in the biomass for over 12 days. The biomass remained stationary for the next five days. The increase in the biomass was about 11.5 fold over the initial inoculum. The average dry weight to fresh weight ratio was around 0.08 and it decreased slightly with the progress in culture age. At the end of three weeks, the cell density reached to a maximum of 8.7 g dry weight 1^{-1} . Even though the inoculum contained small cell aggregates, the cells grew into large clumps by the end of the culture period. However, the tendency to grow into large clumps gradually decreased with the repeated selective subculturing of small cell clusters.

Figure 3.1a. Primary culture of *Daucus carota* with pigmented zones of cells.

Figure 3.1b. Callus and cell suspension cultures of *Capsicum frutescens.*

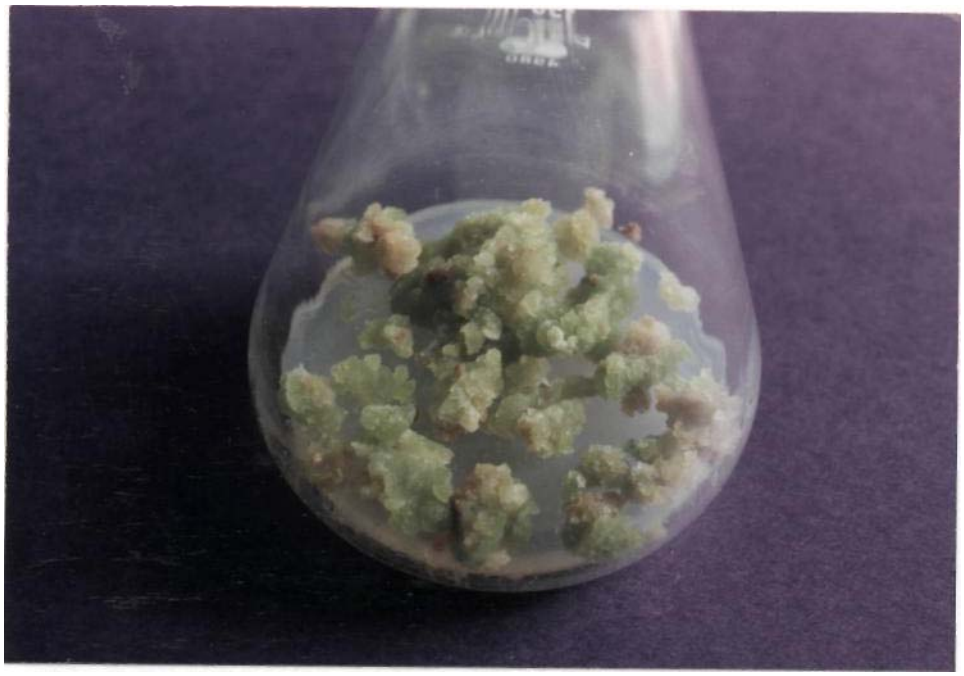


Figure 3.2. High anthocyanin yielding cell line of *Daucus carota.*

Figure 3.3. Cell suspension cultures of *Daucus carota.*

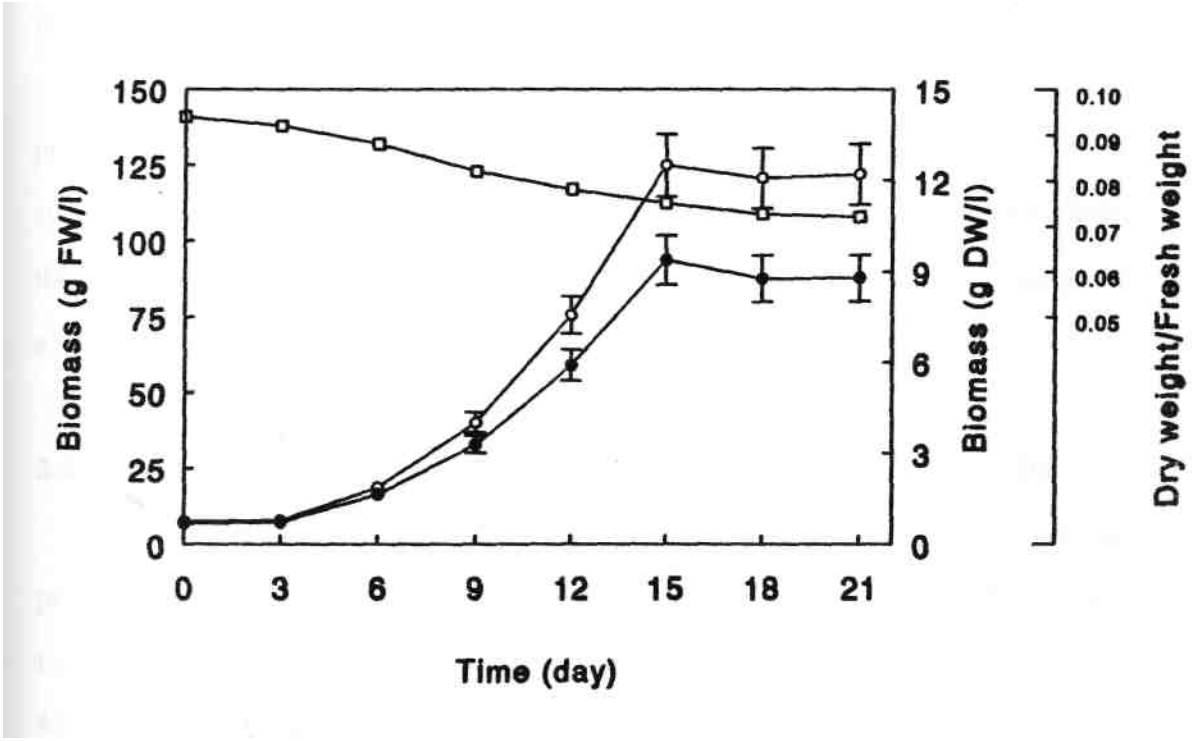


Figure 3.4. Time course of biomass accumulation in the primary cell cultures of *Daucus carotac* Fresh weight (o); Dry weight (•); and Ratio of dry weight to fresh weight (□) (mean ± s.d.; n = 3).

3.3 Formation of anthocyanin in the primary cultures

A time course study of anthocyanin formation showed a non-growth associated pattern in the primary cell cultures of *D. carota* (Fig. 3.5). The anthocyanin content was nearly constant for the initial 15 days and increased rapidly at the onset of stationary phase. A maximum anthocyanin content of 7.2% (w/w, on dry weight basis) was observed on day 18.

As a result of the near constant content, the time course of anthocyanin production paralleled the growth curve for the initial ten days. However, with the sudden increase in the anthocyanin content before the onset of the stationary phase, there was a corresponding rapid increase in the productivity of anthocyanin. There was a total production of 0.6 g 1^{-1} anthocyanin over a period of 18 days.

3.4 Some physico-chemical properties of plant cell culture media

In order to quantify and understand the biological effects of physico-chemical properties, osmolarity, conductivity, buffer capacity, and the solubility of oxygen of twelve commonly used plant cell culture media were investigated. (See section 2.5 of Materials and Methods for the list of media selected for the study).

Conductivity

Considerable differences were recorded for conductivity of various media studied (Table 3.1). While a high conductivity, in general, indicates a high ionic strength, it was not possible to estimate the absolute value of ionic strength because of the differential equivalent conductances of the compounds. It may be inferred from Table 3.1 that the ionic strength of the different nutrient media studied is as follows: ER > L&M > MS > B5 > SH > DBM2 > N&N > WPM > HTM > A&R > H&A >WS.

Osmolarity

The osmolarity of different plant cell culture media studied showed considerable

Figure 3.5. Time course of anthocyanin formation in the primary cell cultures of *Daucus carota:* Biomass (•); Anthocyanin content (o); and Anthocyanin production (□) (mean ± s.d.; n = 3).

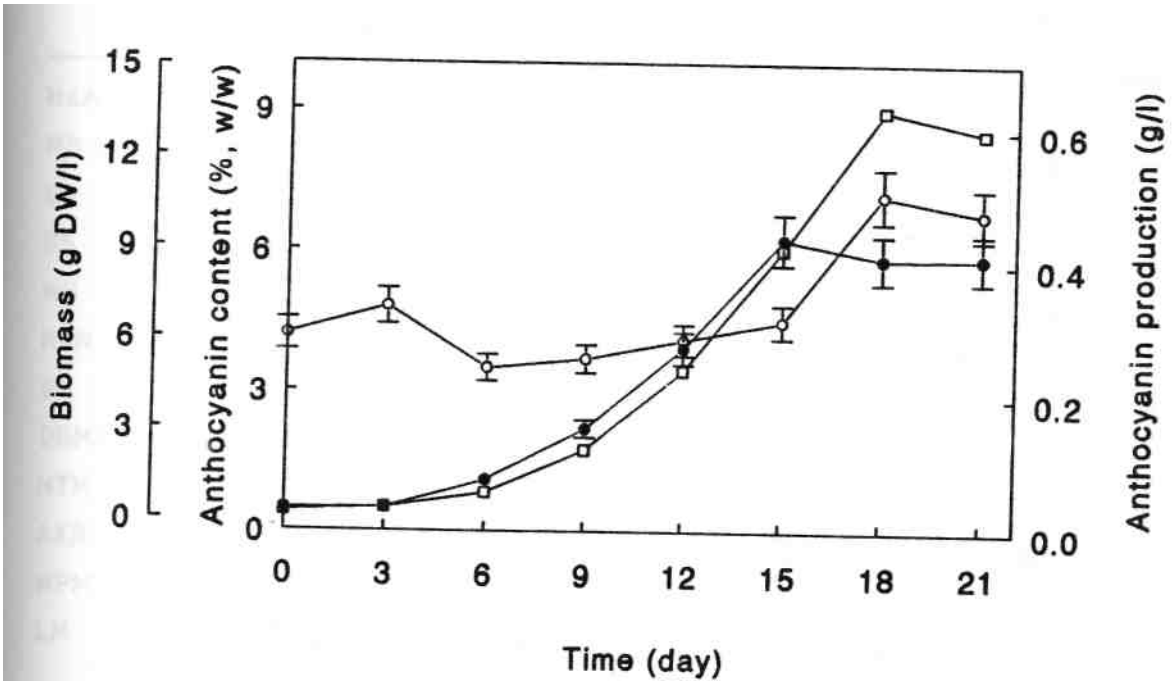


Table 3.1. Some physico-chemical properties of few commonly used plant cell culture media.

Medium[a]	Conductivity (mS)	Osmolarity (mOsmol kg^{-1})	Buffer capacity (μM)	Solubility of oxygen (mg l^{-1})
H&A	1.80	125	453	7.71
MS	5.50	196	618	7.15
ER	6.43	207	1564	7.01
B5	3.98	160	329	7.38
WS	1.30	114	82	7.79
N&N	2.95	150	247	7.54
SH	3.95	164	1071	7.39
DBM2	3.50	157	947	7.46
HTM	2.80	144	288	7.56
A&R	2.37	148	1153	7.63
WPM	2.85	140	576	7.55
LM	5.75	195	1153	7.11

"See text for abbreviations.

"Buffer capacity is the amount of alkali required to change the pH by one unit at pH 5.5 (i.e. pH 5.5 to 6.5) at 25 'C.

differences as seen with the conductivity values (Table 3.1). The values of the osmotic strengths of all the plant cell culture media deviated from those of the calculated values of George *et al.* (1987).

Buffer capacity

All the nutrient media studied showed a poor buffer capacity. Figure 3.6 shows the acid-base titration curves of plant cell culture media studied. Eriksson's medium (1965) exhibited the highest buffer capacity (156 μmole l^{-1} at pH 5.5) which was comparable to that of 11.23 mM acetate buffer. On the other hand, the buffer capacity of White's medium was lowest which was comparable only to that of 0.6 mM acetate buffer. As can be seen from Table 3.1, the buffer capacities of different media were as follows: ER > A&R > L&M > SH > DBM2 > MS > WPM > H&A > B5 > HTM > N&N > WS.

Solubility of oxygen

The solubility of oxygen in the various cell culture media studied ranged narrowly from 7.01 - 7.79 mg l^{-1} (Table 3.1), corresponding to 87 - 96% of the solubility in distilled water. Because of a phenomenon called 'salting out,' slightly less oxygen dissolved in plant cell culture media than in water due to the presence of salts and non-electrolytes such as sucrose.

The present study generated data on some physico-chemical properties of few commonly used plant cell culture media which would be useful in bioprocessing and in the selection of basal media for plant cell and tissue cultures.

3.5 Influence of basal media on growth and formation of anthocyanin

Four commonly used basal media were tested for their support of biomass accumulation and anthocyanin production in the cell cultures of *D. carota.* They were Murashige and Skoog (1962) (MS), Linsmaier and Skoog (1965) (LS) Gamborg, *et*

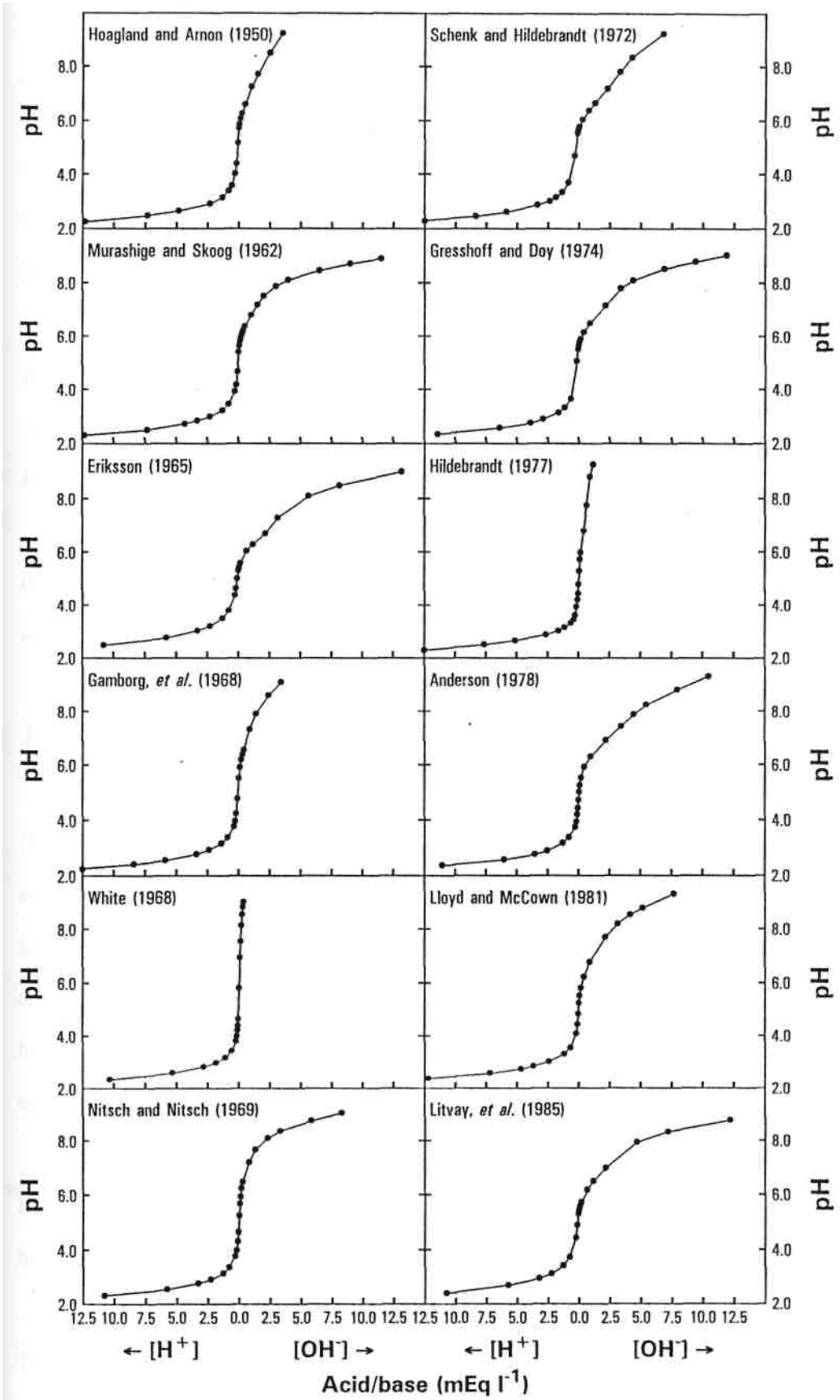


Figure 3.6 Acid-base titration curves of few commonly used plant cell culture media.

al. (1968) (B5), and Schenk and Hildebrandt (1972) (S&H). Liquid media were prepared as per the composition given by the authors with IAA (2 mg 1^{-1}) and kinetin (0.2 mg 1^{-1}). For uniformity, sucrose was added at 3% (w/v) as the source of carbon in all the media.

Of the four media studied, MS was found to be the best in terms of both the growth and anthocyanin content (Fig. 3.7). Fhe biomass accumulation was 20% more with MS salts compared to that with SH salts, while LS and B5 salts supported still less growth.

Anthocyanin content was considerably high (7.2%, w/w) in MS medium compared to that in all the other three media studied which ranged form 5.6 to 6.2% (w/w).

3.6 Influence of hormones on growth and formation of anthocyanin

Four auxins, *viz.,* 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA), and indole-3-butyric acid (IBA), and two cytokinins, *viz.,* kinetin (6-furfurylaminopurine) and 6-benzylaminopurine (BAP), were studied to determine the optimum level of hormones for the maximum production of anthocyanin. Prior to commencement of the experiment, the cells were maintained in hormone free MS basal medium to minimize the hormonal carry over effect. Each auxin was used singly at four concentrations: 0.1, 0.5, 1.0, and 2.0 mg 1^{-1} .

2,4-D showed dose dependant increase in the biomass in the concentration range studied (Fable 3.2). However, the presence of 2,4-D was inhibitory to anthocyanin biosynthesis as the content was lower than that in the control cultures.

On the contrary, IAA treated cultures showed increased anthocyanin production. There was a slight increase in the biomass accumulation with the increase in IAA concentration.

Both NAA and IBA did not show any significant effect ($P>0.05$) on growth as compared to the control cultures. However, the anthocyanin content of the NAA

Figure 3.7. Influence of basal media on growth and anthocyanin formation in *Daucus carota* cell suspension cultures (mean ± s.d.; n = 3).

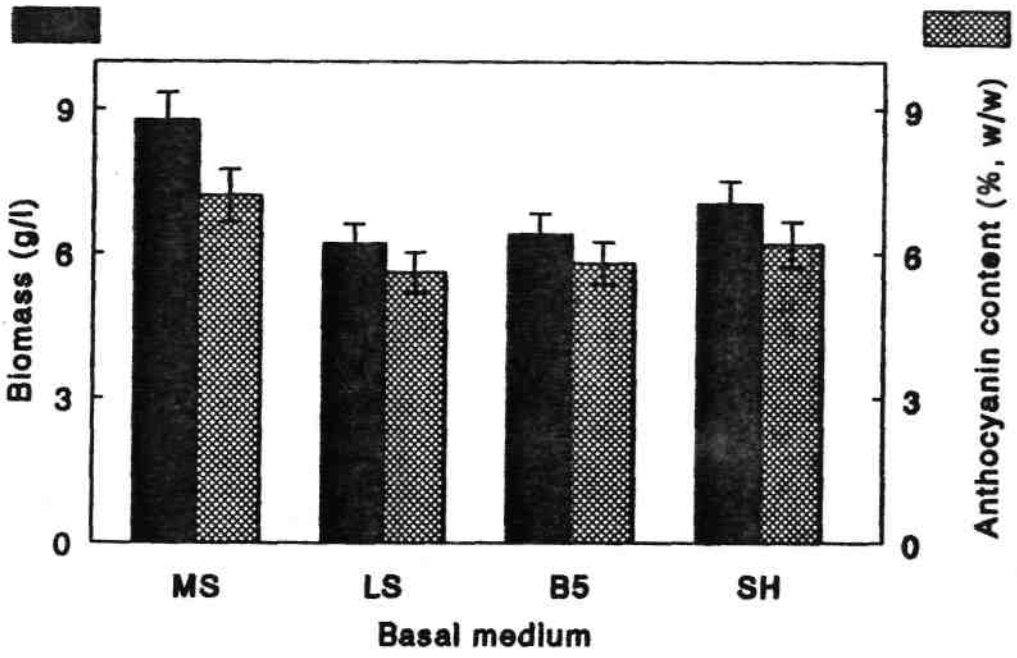


Table 3.2. Influence of auxins on growth, and formation of anthocyanin in *Daucus carota* cells in suspension cultures.

Auxin	Conc. (mg l^{-1})	Biomass (g l^{-1})*	Anthocyanin content (%, w/w)*
Control**	0.0	5.81 ± 0.54	4.24 ± 0.39
2,4-D	0.1	8.26 ± 0.92	3.61 ± 0.31
	0.5	9.17 ± 1.04	3.70 ± 0.39
	1.0	10.75 ± 1.17	3.42 ± 0.30
	2.0	11.83 ± 1.21	3.24 ± 0.31
IAA	0.1	8.01 ± 0.87	5.77 ± 0.52
	0.5	8.18 ± 0.81	5.91 ± 0.61
	1.0	8.37 ± 0.79	5.93 ± 0.61
	2.0	8.54 ± 0.86	6.12 ± 0.54
NAA	0.1	5.80 ± 0.51	5.62 ± 0.53
	0.5	5.85 ± 0.62	5.51 ± 0.50
	1.0	5.92 ± 0.54	5.65 ± 0.62
	2.0	6.05 ± 0.57	5.63 ± 0.58
IBA	0.1	5.75 ± 0.61	4.21 ± 0.38
	0.5	5.80 ± 0.53	4.32 ± 0.41
	1.0	5.72 ± 0.54	4.35 ± 0.47
	2.0	5.90 ± 0.63	4.40 ± 0.40

*Mean ± S.D.; n = 5.

"Control. MS basal medium supplemented with sucrose (3%, w/v).

treated cultures was comparable to that of IAA treated ones, while IBA did not show any significant effect (P>0.05) as compared to the control cultures.

Table 3.3 summarizes the effect of cytokinins, kinetin and BAP, at four concentrations: 0.1, 0.5, 1.0, and 2.0 mg l^{-1}. Kn at 0.1, 0.5, and 1.0 mg l^{-1} concentrations resulted in enhanced yield of anthocyanin. However, further high concentration of kinetin was inhibitory to anthocyanin biosynthesis. Kn did not show significant effect (P>0.05) on biomass accumulation. Neither biomass accumulation nor the anthocyanin content was significantly affected by the addition of BAP (P>0.05).

In another experiment with combined addition of IAA, and Kn, the combination of IAA at 2.0 mg 1^{-1} and kinetin at 0.2 mg 1^{-1} was found to be the optimum hormonal supplementation required for the maximum anthocyanin production (Fig. 3.8).

3.7 Development of a high yielding cell line of *D. carota*

A high yielding cell line of *D. carota* was developed by repetitive selection for high anthocyanin containing cell clusters (Fig. 3.9). The selection was performed for over fifty subcultures spanning about two years. The size distribution of the cell aggregates was controlled by repeated selection for small cell aggregates.

3.8 Growth profile of *D. carota* cell cultures

A time course study of biomass accumulation in the high yielding *D. carota* cells in suspension cultures recorded a typical sigmoidal growth curve (Fig. 3.10). The growth curve consisted of a lag phase of about 2 days followed by an exponential increase in the biomass. Log-linear plot of biomass (Fig. 3.10) estimated the specific growth rate to be 0.217 day which corresponds to a doubling time of 3.2 day. Accumulation of biomass came to stationary on the 15th day with a maximum cell density of 18.92 ± 1.17 g dry weight l^{-1}. The growth index was 12.2 which is slightly higher as compared to that of the primary cultures. The average dry weight to fresh

Table 3.3. Influence of cytokinins on growth, and formation of anthocyanin in *Daucus carota* cells in suspension cultures.

Cytokinin	Conc. (mg l^{-1})	Biomass (g l^{-1})*	Anthocyanin content (%, w/w)*
Control**	0.0	5.81 ± 0.54	4.24 ± 0.39
Kinetin	0.1	5.82 ± 0.87	5.71 ± 0.42
	0.5	5.91 ± 0.96	5.78 ± 0.51
	1.0	5.85 ± 1.15	5.51 ± 0.47
	2.0	5.92 ± 1.01	4.87 ± 0.32
BAP	0.1	5.87 ± 0.76	4.51 ± 0.47
	0.5	5.90 ± 0.92	4.35 ± 0.42
	1.0	5.78 ± 0.88	4.28 ± 0.51
	2.0	5.84 ± 0.93	4.32 ± 0.45

*Mean ± S.D.; $n = 5$.

"Control. MS basal medium supplemented with sucrose (3%, w/v).

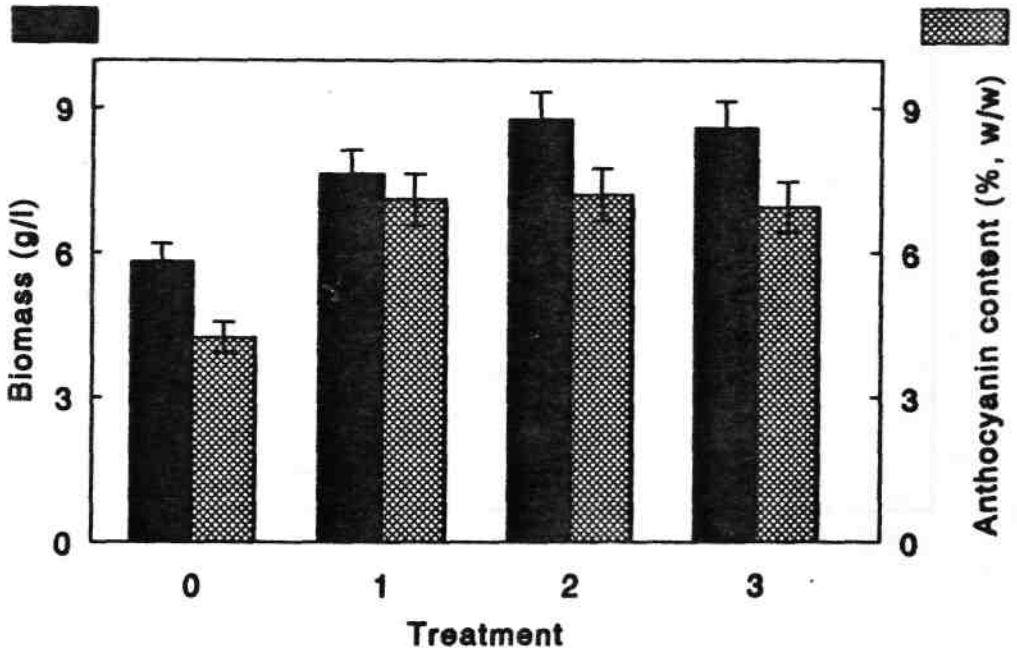


Figure 3.8. Influence of **IAA** and Kinetin on growth and anthocyanin formation in *Daucus carota cell* suspension cultures: Control (0); 1 mg l"[1] **IAA** + 0.1 mg l"[1] kn (1); 2 mg l"[1] **IAA** + 0.2 mg l"[1] kn (2); and 2 mg l"[1] IAA + 0.5 mg l"[1] kn (3) (mean ± s.d.; n = 3).

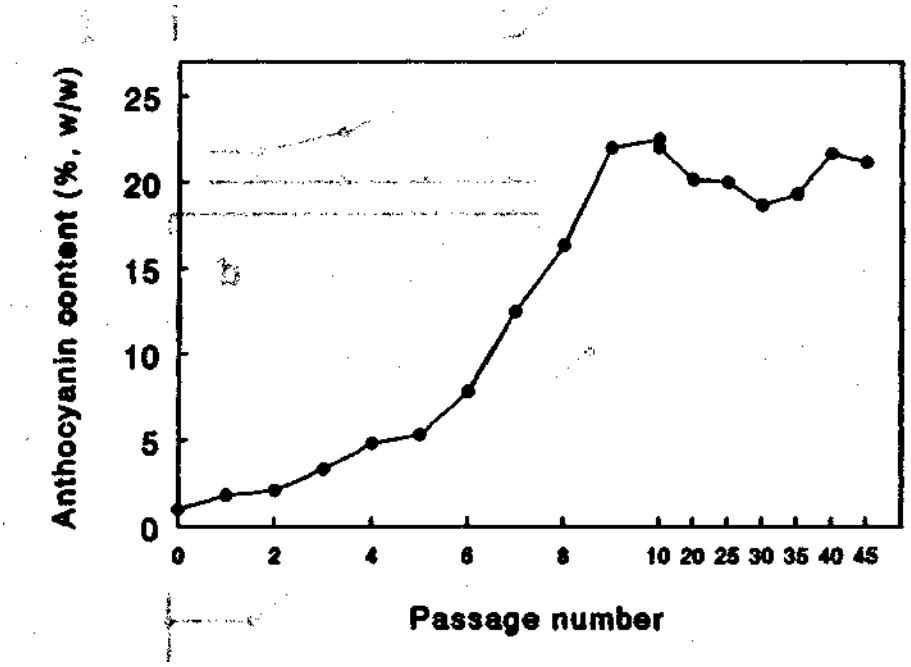


Figure 3.9. Improvement of anthocyanín content in *Daucus carota* cell cultures by repetitive selection for high anthocyanin containing cell clusters.

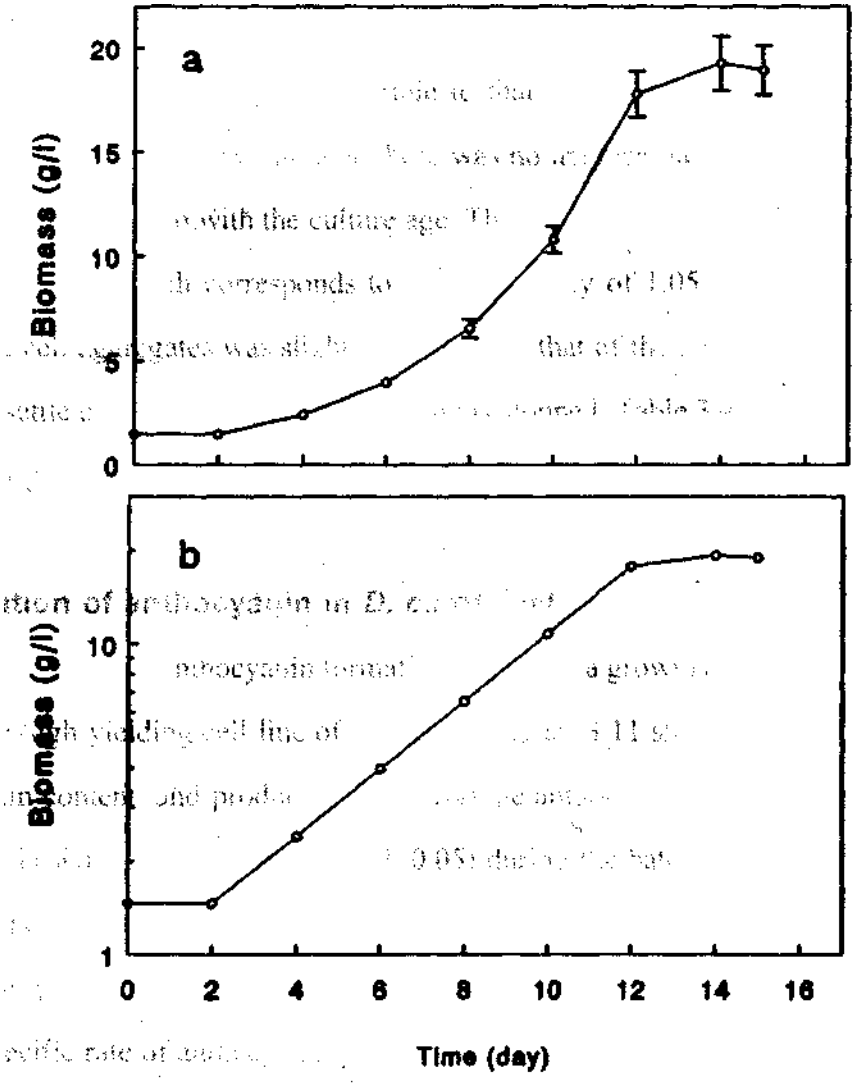


Figure 3.10. Time course of biomass accumulation in *Daucus carota* cell cultures. Linear plot (a); and Log-linear plot (b) (mean ± s.d.; n = 3).

weight ratio was 0.85 which was comparable to that in the early primary cultures. However, unlike in the primary cultures, there was no appreciable change in the dry weight to fresh weight ratio with the culture age. The packed volume of cells was 0.95 ml g^{-1} fresh weight, which corresponds to a mass density of 1.05 g ml^{-1} . Since the density of the cell aggregates was slightly higher than that of the culture medium, the cells used to settle down slowly when the mixing is stopped. Table 3.4 lists the kinetic parameters of growth.

3.9 Formation of anthocyanin in *D. carota* cell cultures

The time course of anthocyanin formation suggested a growth associated product pattern in the high yielding cell line of *D. carota.* Figure 3.11 shows the time course of anthocyanin content, and production. The average anthocyanin content of 15.88% (w/w, on dry basis) was nearly constant ($P>0.05$) during the batch culture period. As a result of the near constant content, the time course of anthocyanin production paralleled the growth curve.

The specific rate of anthocyanin production (q) was 105.2 mg g^{-1} day^{-1} . There was a total production of 3.1 g 1^{-1} in a period of 15 days which corresponds to an average productivity of 0.2 g 1^{-1} day^{-1} . Even though the anthocyanin content was nearly constant throughout the batch period, much of the anthocyanin was produced at the end of the log phase of growth (Fig. 3.12). Over 60% of the total anthocyanin production occurred between the day 12 and 15. Table 3.4 lists the kinetic parameters of anthocyanin production.

3.10 Influence of pH on growth and formation of anthocyanin

To determine the optimum pH for maximum production, the growth and production of anthocyanin in *D. carota* cells was studied at three different pH (Fig. 3.13). The pH of the liquid nutrient medium tended to shift towards the acidic side by about 0.5 pH units upon sterilization by autoclaving. Accordingly, the pH of the

Table 3.4. Kinetic constants of growth and production of anthocyanin in *Daucus carota* cell cultures.

Parameter	Value
Growth	
Specific growth rate	0.217 day^{-1}
Doubling time	3.2 day
Growth index	12.2
Maximum cell density	18.92 $\pm$ 1.17 g l^{-1}
Dry wt. to fresh wt. ratio	0.085
Packed cell volume	0.95 ml g^{-1} fresh wt.
Anthocyanin production	
Average content	15.88% (w/w, on dry basis)
Specific productivity (q_p)	105.2 mg g^{-1} day^{-1}
Total production	3.1 g l^{-1} in 15 days
Average productivity	207 mg l^{-1} day^{-1}

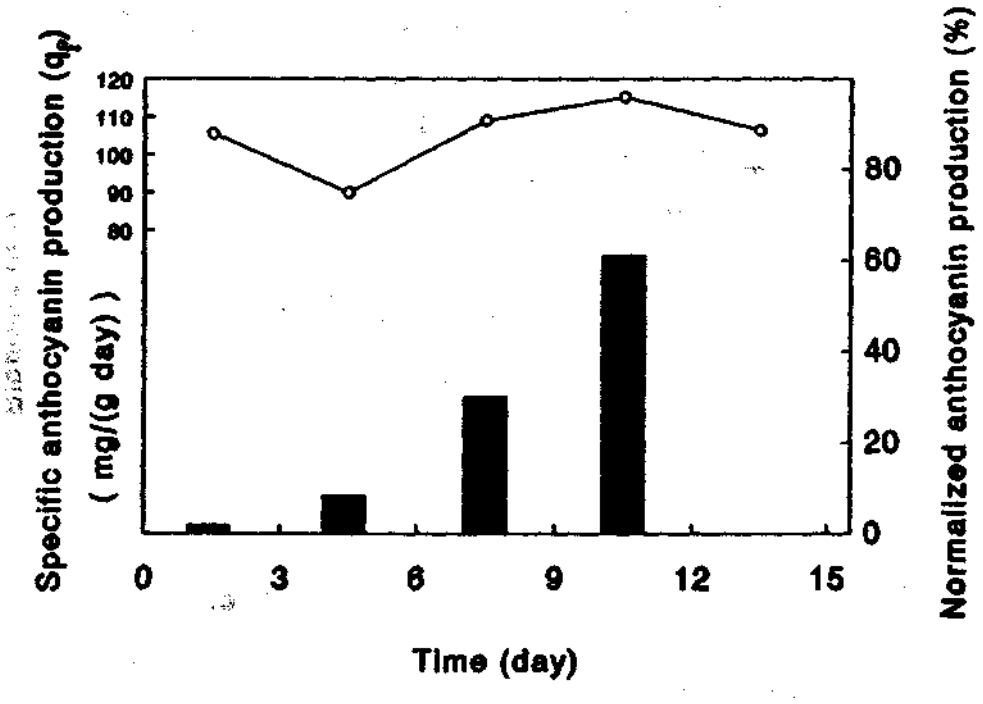
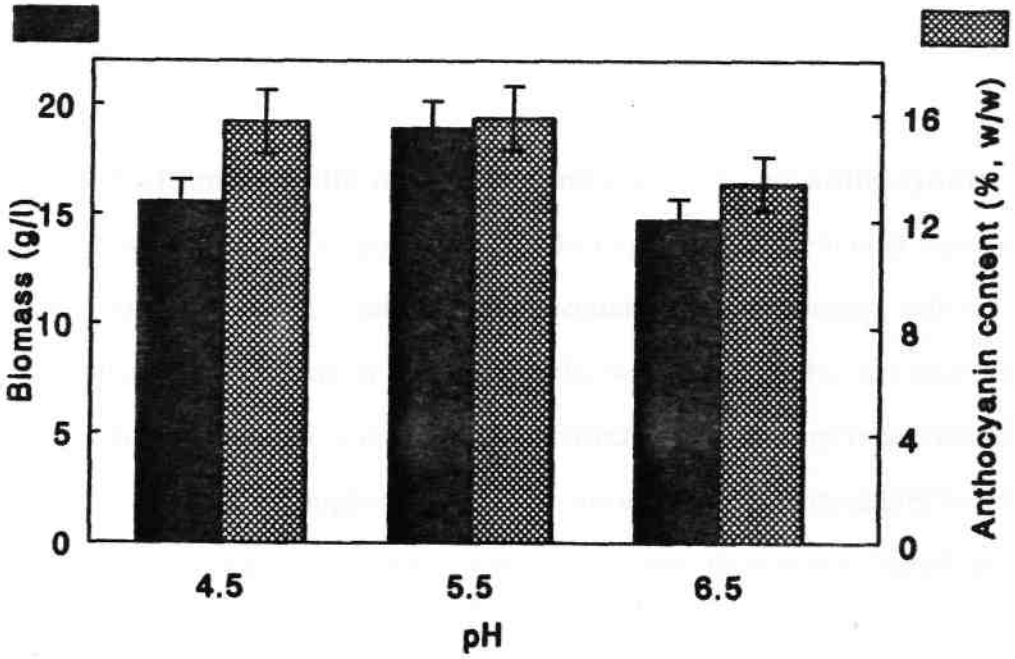


Figure 3.12. Time course of anthocyanin production in *Daucus carota* cell suspension cultures: Specific production rate (o); and Normalized production (■) (mean; n = 3).

Figure 3.13. Influence of pH on growth and formation of anthocyanin in *Daucus carota* cell suspension cultures (mean ± s.d.; n = 3).



medium was so adjusted that the final pH would come to the desired value.

Both the growth and production of anthocyanin were maximum at pH 5.5 while the same were significantly lower ($P<0.05$) at the near neutral pH (6.5). Similarly, the growth was significantly lower ($P<0.05$) at pH 4.5. However, the acidic pH did not affect the anthocyanin content ($P>0.05$).

3.11 Influence of temperature on growth and formation of anthocyanin

The effect of temperature on growth and production of anthocyanin in *D. carota* cells in suspension cultures was studied. The temperature was kept constant within ± 2 °C. The anthocyanin content in *D. carota* cells was found to be sensitive to temperature, while the growth was relatively unaffected in the temperature range studied (Fig. 3.14). Both the higher (30 °C) and lower (20 °C) temperatures were significantly inhibitory to anthocyanin biosynthesis. However, there was no significant change in growth at all the three temperatures studied.

The evaporational loss of water from the nutrient medium during the period of culture (15 days) was about 12.5% (v/v). But it did not vary considerably within the temperature range of 20 - 30 °C.

3.12 Optimum inoculum density

To determine the optimum inoculum density for the maximum production of anthocyanin, the growth, and the anthocyanin formation was studied at four different inoculum levels (Table 3.5). The specific growth rate (μ) was maximum at 0.5 g l^{-1} inoculum, and dropped appreciably with the increase in the inoculum density. However, the maximum cell density was almost same at all the four inoculum levels studied. On the other hand, the culture period required to attain the maximum cell density was considerably short with high initial inoculum compared to that required with a lower inoculum density. However, the low inoculum density gave higher folds of increase in biomass. Neither the anthocyanin content, nor the total production

Figure 3.14. Influence of temperature on growth and formation of anthocyanin in *Daucus carota* cell suspension cultures (mean ± s.d.; n = 3).

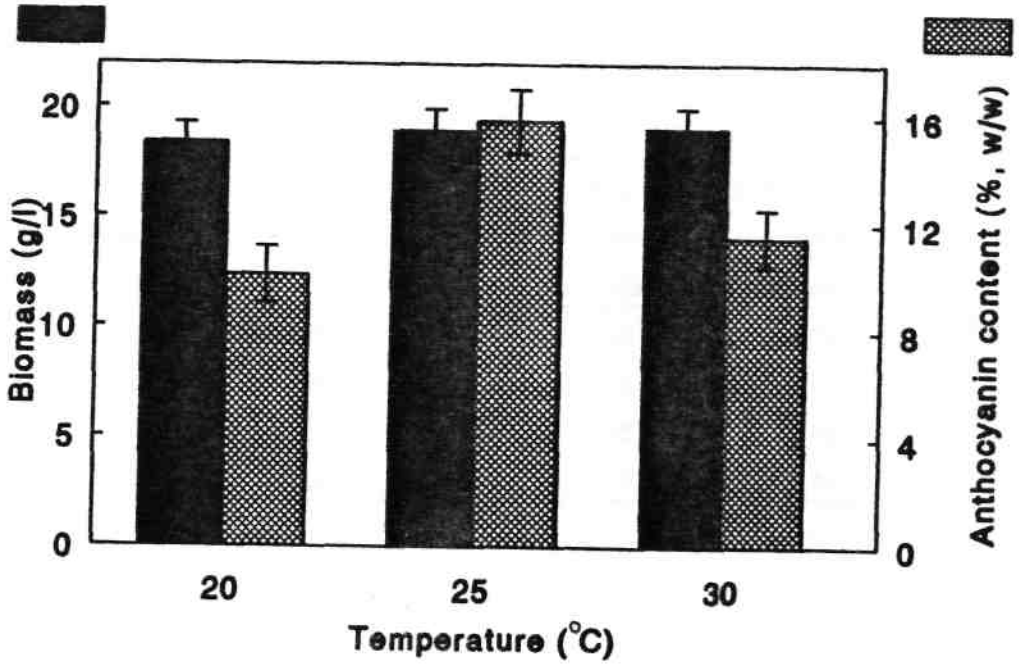


Table 3.5. Effect of inoculum density on the growth and production of anthocyanin from *Daucus carota* cell cultures.

Inoculum density ($g\ l^{-1}$)	μ (day^{-1})	Growth index	Maximum cell density ($g\ l^{-1}$)	Culture period (day)	Anthocyanin content (%, w/w)
0.5	0.30	34.06	17.53	19	16.18
1.0	0.29	16.81	17.81	18	16.02
1.5	0.27	11.61	18.92	15	15.88
2.0	0.24	9.07	20.14	13	15.43

μ = Specific growth rate

appreciably varied with change in the inoculum level. From the trade-off between the length of culture period, and the fold-wise increase in the biomass, it can be seen that an inoculum density of 1.5 g l^{-1} *(ca.* 17.5 g fresh wt. 1^{-1}) was optimum for the production of anthocyanin.

Table 3.6 lists the optimum culture conditions for the production of anthocyanin from *D. carota* cell cultures.

3.13 Size distribution of cell aggregates

The cells of *D. carota* in suspension cultures showed a tendency to aggregate into clumps of different size. The size of the cell aggregates spanned from <355 to >1500 μm with over 92% of biomass was present in the aggregates of 500 - 1500 μm in diameter (Fig. 3.15). The profile of biomass distribution in different size groups of cell aggregates revealed that the biomass distribution was centred at between 500 - 850 μm as the maximum biomass (36%) was present in that narrow size group. The size distribution of cell aggregates remained unchanged during the culture period.

3.14 Anthocyanin content in different size cell aggregates

The anthocyanin content in different size groups of cell aggregates showed an interesting profile. The content showed an increase with the increase in cell aggregate size up to a diameter of 500 - 850 μm (Fig. 3.16) with the peak content of 21.9% (w/w, on dry weight basis). However, the anthocyanin content decreased sharply, when the cell aggregate size exceeded 500 - 850 μm. The anthocyanin content in the cell aggregates of diameter > 1500 μm was only half of that in the aggregates of diameter < 355 μm.

Consequent to the differential biomass distribution, and anthocyanin content, the contribution of different size groups of cell aggregates toward the final production of anthocyanin varied (Fig. 3.17). Nearly 50% of the total production was contributed by the cell aggregates of 500 - 850 μm in diameter, followed by 24% from the cell

Table 3.6. Optimum culture conditions for the production of anthocyanin from *Daucus carota* cell cultures.

Parameter	Optimum
Nutrient medium	MS basal medium with sucrose (3%, w/v)
Hormonal supplementation	IAA (2 mg l^{-1}), and kinetin (0.2 mg l^{-1})
Inoculum density	1.5 g l^{-1}
pH	5.5
Temperature	25 ◦C
Culture period	12 - 15 days
Light	3000 - 4000 lux

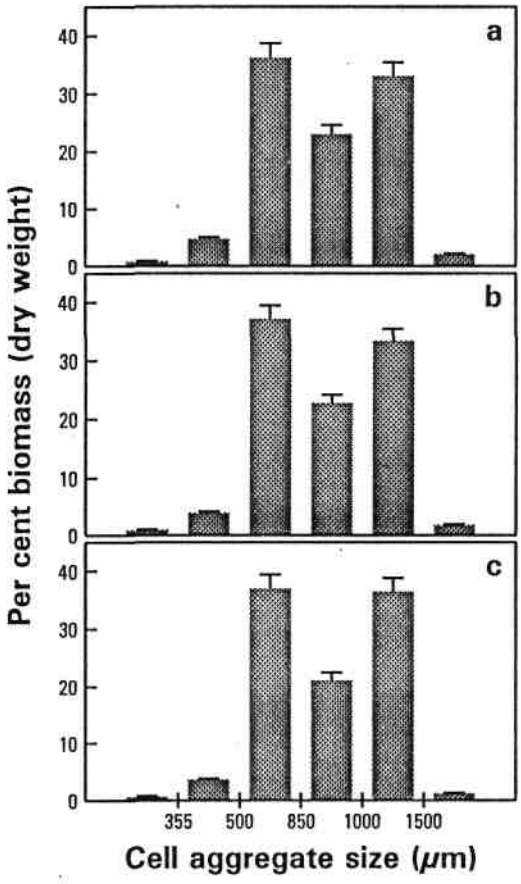


Figure 3.15. Biomass distribution in different size groups of cell aggregates of *Daucus carota* in suspension cultures: 0 day (a); 6th day (b); 12th day (c). (mean ± s.d.; n = 3).

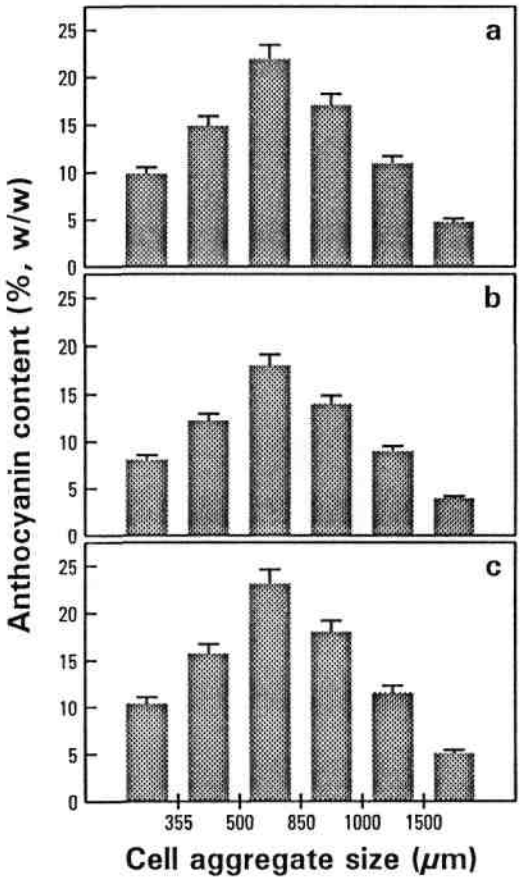


Figure 3.16. Anthocyanin content in different size groups of cell aggregates of *Daucus carota* in suspension cultures: 0 day (a); 6th day (b); 12th day (c). (mean ± s.d.; n = 3).

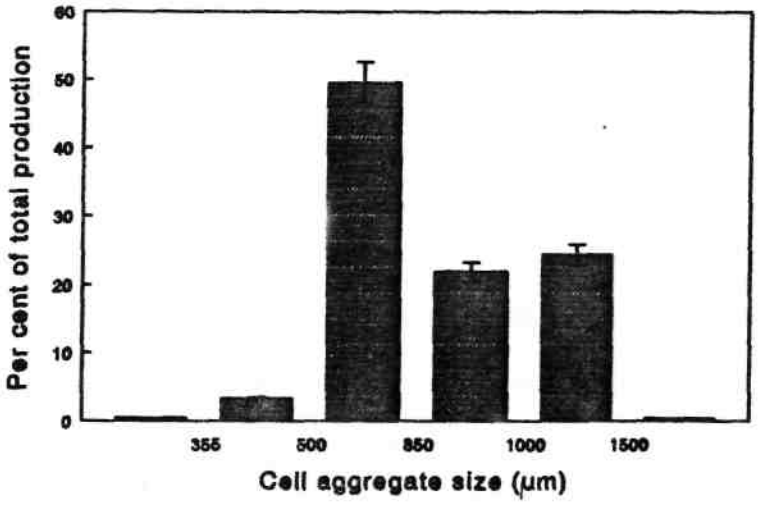


Figure 3.17. Contribution of different size groups of cell aggregates toward the production of anthocyanin in *Daucus carota* cell suspension cultures (mean ± s.d.; n = 3).

aggregates of the size of 1000 - 1500 μm. While the cell aggregates of the narrow range of 850 - 1000 μm contributed a substantial amount of 22% of total production, the contribution from the cell aggregates of < 355, and > 1500 μm was small.

3.15 Surface colour intensity in different size cell aggregates

In contrast to the profile of anthocyanin content, the surface colour intensity, as measured in terms of the red-green coordinate value of CIELAB Color Space, showed a steady increase with the increase in cell aggregate diameter (Fig. 3.18). The cell aggregates were intense red in colour with the peak surface colour intensity of 2.8 units on the red-green coordinate of the colour space. The high surface colour intensity and the low anthocyanin content in the larger cell aggregates indicate a steep radial gradient of anthocyanin content along the radius of the cell aggregates. Figure 3.19 shows the cross-sectional view of a cell aggregate of *D. carota* in suspension cultures.

3.16 PAL activity in different size cell aggregates

The phenylalanine ammonia lyase (PAL) activity in different size groups of cell aggregates showed considerable variations. The profile of PAL activity showed a similar pattern of anthocyanin content in different size cell aggregates. The PAL activity showed an initial increase with the increase in cell aggregate size with a peak specific activity of 4.3 unit mg"[1] protein in the cell aggregates of diameter of 500 - 850 μm, and decreased with a further increase in the cell aggregate size (Fig. 3.20). Though the overall pattern of PAL activity was similar to that of anthocyanin content in different size groups of cell aggregates, the differences in PAL activity were not that pronounced as that of anthocyanin content in different size cell aggregates. Further, there was no appreciable change in the PAL activity during the batch culture of *D. carota* cells.

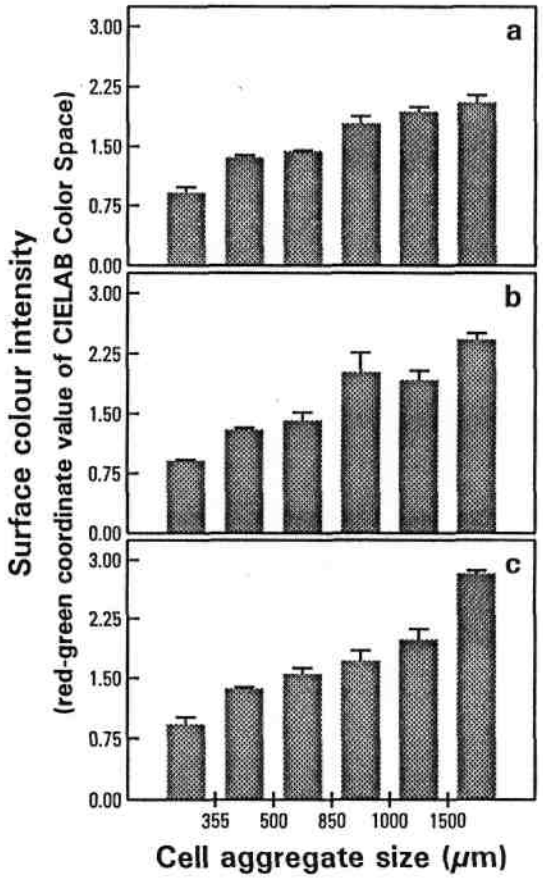


Figure 3.18. Surface colour intensity in different size groups of cell aggregates of *Daucus carota* in suspension cultures: 0 day (a); 6th day (b); 12th day (c). (mean ± s.d.; n = 3).

Figure 3.19. Gradient of anthocyanin in cell aggregate of *Daucus carota* in suspension cultures.

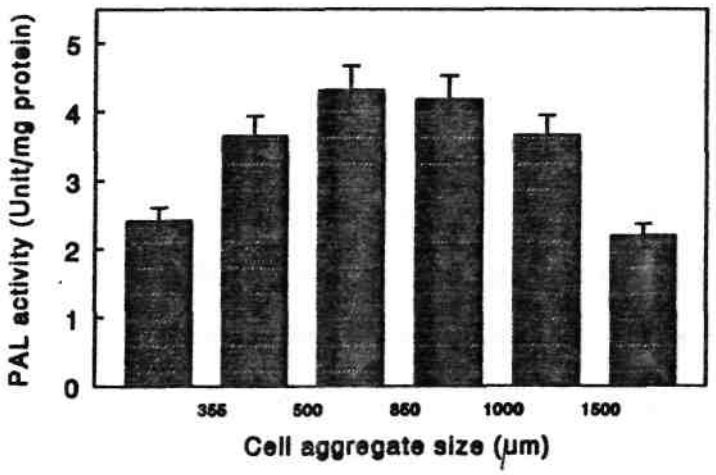


Figure 3.20. Phenylalanine ammonia lyase activity in different size groups of cell aggregates of *Daucus carota* in suspension cultures (mean ± s.d.; n = 3).

3.17 Consumption of sugars

Sucrose was the sole source of carbon in *D. carota* and *C. frutescens* cell cultures. The analysis of sucrose, glucose, and fructose levels in the culture medium indicated a rapid hydrolysis of sucrose during the lag phase in both the culture systems (Fig. 3.21). Nearly 46 and 48% of the initial sucrose was hydrolysed into glucose and fructose in two days after inoculation of *D. carota* and *C. frutescens* cells, respectively. The specific rate of sucrose hydrolysis was 4.79, and 5.19 g g^{-1} day^{-1} in *D. carota* and *C. frutescens,* respectively, during the lag phase. No sucrose was detected after 5 days after inoculation in both the culture systems.

Glucose and fructose consumption curves showed that glucose was consumed more compared to fructose. The overall consumption of glucose was 86.8, and 50.6% more compared to that of fructose in *D. carota* and *C. frutescens* cultures, respectively. The apparent dry biomass yield coefficient (Yχ/cHO) of carbohydrates was 0.86, and 0.83 g g^{-1} in *D. carota* and *C. frutescens,* respectively.

3.18 Consumption of ammonia and nitrate

An initial nitrogen concentration of 840.7 mg 1^{-1} in the MS medium was provided by NH_4N0_3, and $KN0_3$. The time-course study of residual ammonia and nitrate concentrations in the nutrient medium showed the rapid uptake of ammonia and a slower uptake of nitrate in both *D. carota* and *C. frutescens* suspension cultures (Fig. 3.22). There was no appreciable change in the ammonia or nitrate levels during the lag phase of growth. However, both the nitrogen sources were absorbed rapidly and continuously during the exponential growth of cells. 54, and 32% of the initial ammonia, and 42 and 29% of the initial nitrate was taken up by the cells of *D. carota* and *C. frutescens,* respectively, in the first ten days of culture. The consumption of ammonia, and nitrate continued to progress, and the ammonium was consumed till its almost complete exhaustion, while about 8% of the initial nitrate was left unutilized. The apparent yield coefficient of dry biomass on ammonium (Yχ/NH3) ^{was} *0.94,* and

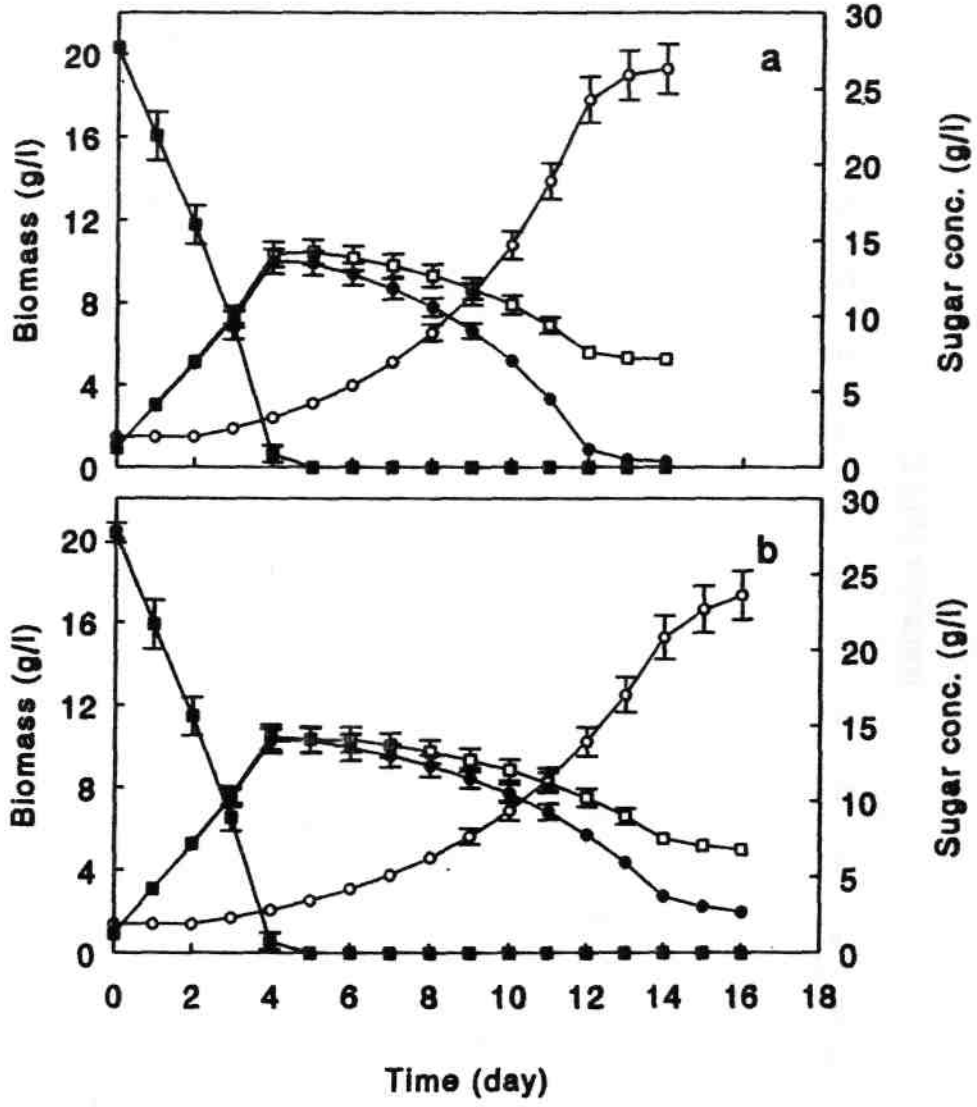


Figure 3.21. Time course of biomass accumulation, and sugar consumption by the cells of *Daucus carota* (a), and *Capsicum frutescens* (b) in suspension cultures: Biomass (o), sucrose (■), glucose (•), and fructose (□) (mean ± s.d.; n = 3).

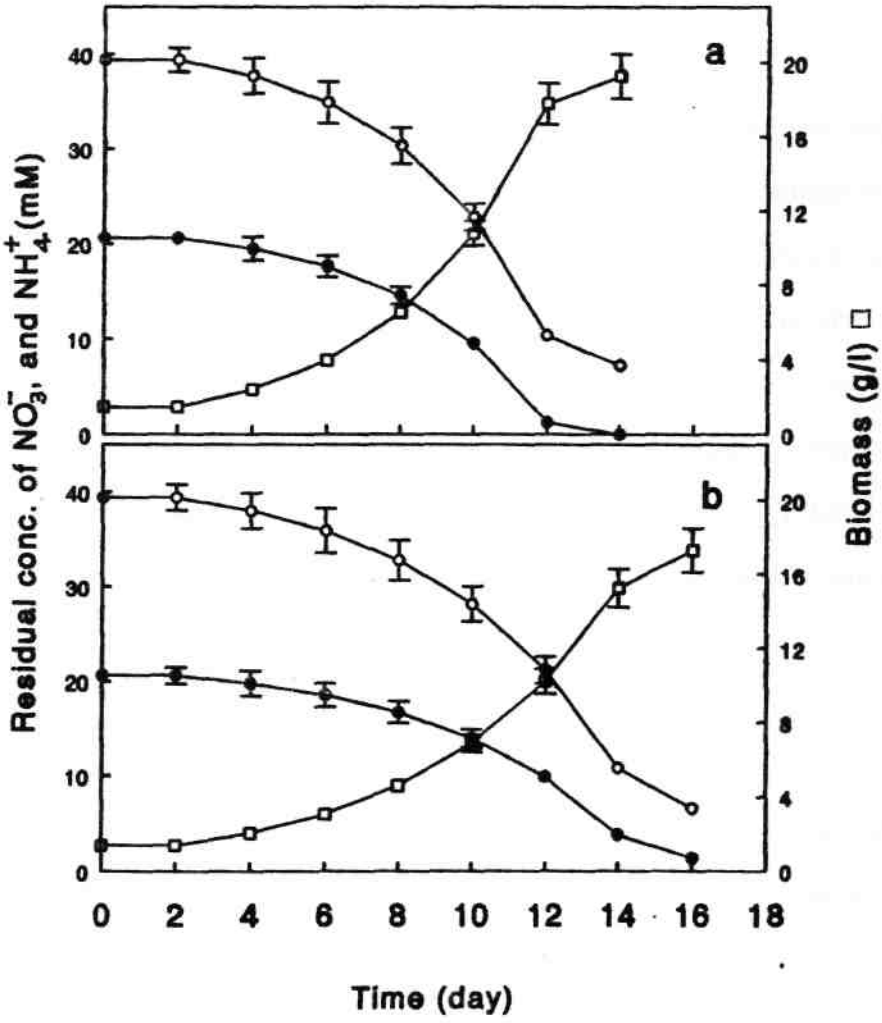


Figure 3.22. Consumption of ammonia (•), and nitrate (o) by *Daucus carota* (a), and *Capsicum frutescβns* (b) cells in suspension cultures (mean ± s.d.; n = 3).

0.89 g $mmol^{-1}$, while the corresponding yield coefficient of nitrate (Y_{X/NO_3}) was 0.58, and 0.52 g $mmol^{-1}$ in *D. carota* and *C. frutescens,* respectively.

3.19 Consumption of phosphate

An initial phosphate concentration of 118.7 mg 1^{-1} (1.25 mM) in the MS medium was provided by KH_2P0_4. The time course of phosphate depletion was similar to that of ammonia (Fig. 3.23). Although there was no appreciable change in the phosphate concentration during the lag phase of growth, the utilization of phosphate increased rapidly and continued till the end of log phase. Only 3, and 6.4% of the initial phosphate was left unutilized by the cell cultures of *D. carota* and *C. frutescens,* respectively. The yield coefficient of dry biomass on phosphate ($Y_{\chi/po4}$) was 15.95, and 14.79 g $mmol^{-1}$ in *D. carota* and *C. frutescens* cell suspension cultures, respectively.

3.20 Changes in medium conductivity

Changes in the electrical conductivity of the nutrient medium in *D. carota* and *C. frutescens* cell suspension cultures were monitored in order to estimate the relationship between the conductivity and the biomass density (Fig. 3.24). The conductivity of the culture medium decreased slowly and steadily with the progress in culture period. The analysis of medium conductivity and the biomass density showed a linear decrease in the conductivity with the increase in the biomass of *D. carota* and *C. frutescens* cells (Fig. 3.25). The relationship could be represented by the following equation:

$$\Delta X = k\,\Delta C \qquad \text{Eq. 3.1}$$

where ΔX is the increase in dry cell biomass, k is an empirical constant, and ΔC is the nutrient medium conductivity.

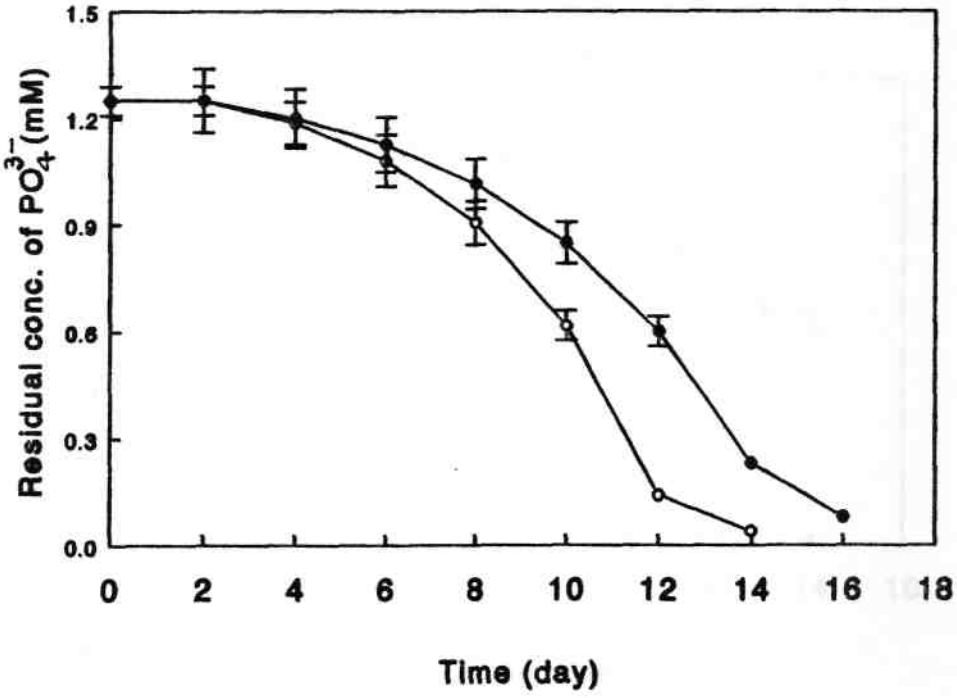


Figure 3.23. Consumption of phosphate by *Daucus carota* (o), and *Capsicum frutescens* (•) cells in suspension cultures (mean ± s.d.; n = 3).

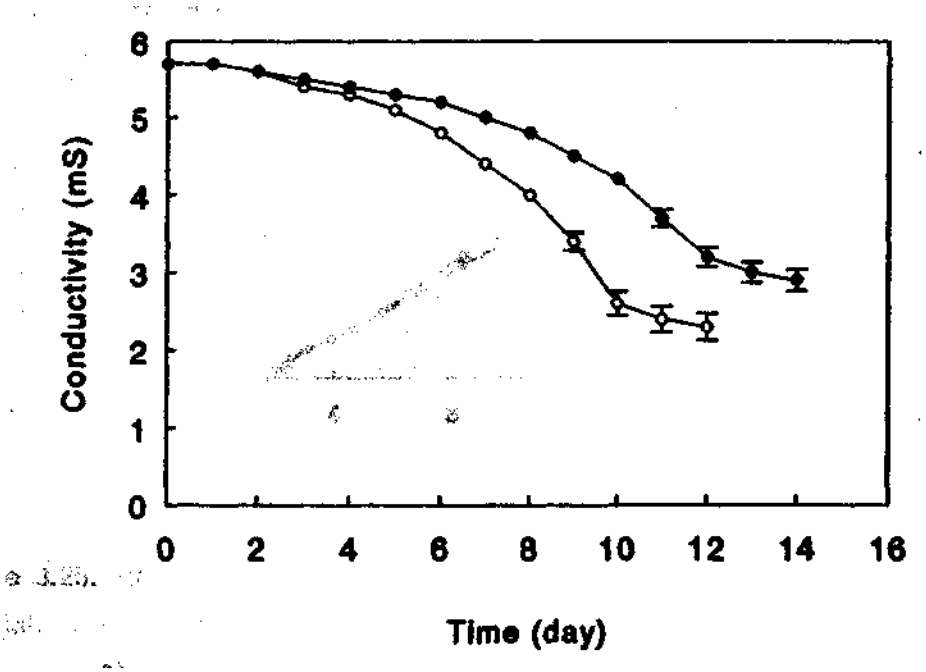


Figure 3.24, Progressive decrease in the electrical conductivity of culture media of *Daucus carota* (o), and *Capsicum frutescens* (•) (mean **+** s.d.; n = 3).

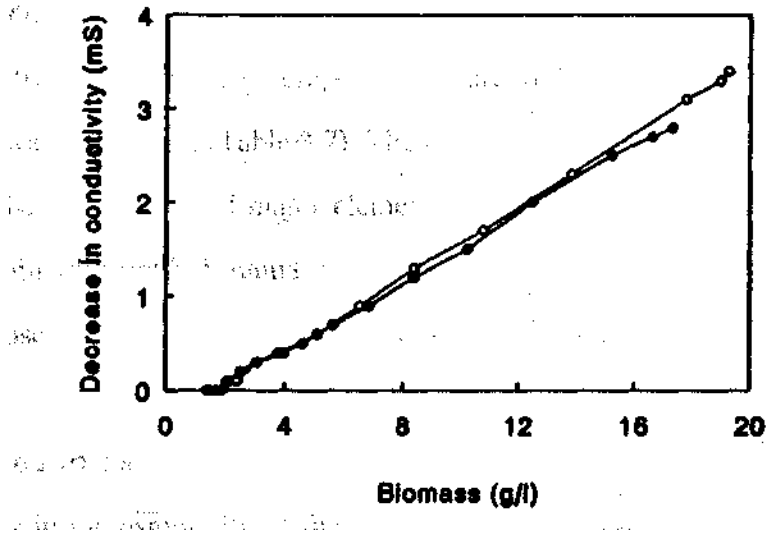


Figure 3.25. Relationship between the conductivity of culture medium and cell biomass of *Daucus carota* (o), and *Capsicum frutescens* (•) (mean; n = 3).

From the linear-regression analysis of the conductivity and biomass density, it was estimated that the proportionality constant *(k)* in the above equation was -5.29, and -5.56 g l^{-1} mS^{-1} for *D. carota* and *C. frutescens* cell cultures, respectively. The coefficient of linearity *(r^2*) between the conductivity and the biomass density was 0.997, and 0.998 in *D. carota* and *C. frutescens* cell cultures, respectively. An analysis of the conductivity of individual components in the Murashige and Skoog's medium with sucrose was carried out (Table 3.7). The medium conductivity was influenced by nitrates, $CaCl_2$, and minor and major elements. The addition of organic constituents such as vitamins did not substantially alter the conductivity. The addition of either sucrose, glucose or fructose resulted in decreased conductivity of the culture medium.

3.21 Changes in osmolarity of the medium

Changes in the osmolarity of the nutrient medium during the culture period of *D. carota* and *C. frutescens* were studied. In both the culture systems osmolarity of the medium increased sharply as a result of the rapid hydrolysis of sucrose into glucose and fructose (Fig. 3.26). The osmolarity of the culture medium reached the peak value of 257, and 260 mOsmol kg"[1] in *D. carota* and *C. frutescens* cultures, respectively, and decreased subsequently. An analysis of the contribution of individual components of Murashige and Skoog's medium was carried out (Table 3.7). In contrast to the conductivity of the medium, the osmolarity was largely influenced by the concentration of sucrose, glucose, or fructose. While NH_4N0_3 and $KN0_3$ showed positive influence on the osmolarity of the culture medium, many micronutrients did not contribute substantially.

3.22 Estimation of biomass based on osmolarity

The suitability of osmolarity as an index for estimation of biomass was investigated. Figure 3.27 shows the relationship between the osmolarity and the density of biomass in *D. carota* and *C. frutescens* cell cultures. With the exception of the

Table 3.7. Contribution of individual MS medium components toward conductivity and osmolarity.

Component	Concentration (mg l^{-1})	Conductivity (μs)	Osmolarity (mOsmol kg^{-1})
NH_4NO_3	1650	2600	41
KNO_3	1900	2000	37
H_3BO_3	6.2		
KH_2PO_4	170	130	3
KI	0.83	2.5	
$NaMoO_4 . 2H_2O$	0.25		
$CuSO_4 . 5H_2O$	0.025		
$CoCl_2 . 6H_2O$	0.025	22.5	
$ZnSO_4 . 7H_2O$	8.6		
$MnSO_4 . 7H_2O$	22.3		
$CaCl_2 . 2H_2O$	440	735	9
$MgSO_4 . 7H_2O$	370	350	3
Na_2-EDTA	37.25	55	
$FeSO_4 . 7H_2O$	27.85		
Thiamine HCl	1.0		
Pyridoxine HCl	1.0		
Nicotinic acid	1.0	3.3	
Glycine	4.0		
Myo-inositol	100		3
Sucrose	30000	-150	90
Glucose	15000	-450	90
Fructose	15000	-300	90

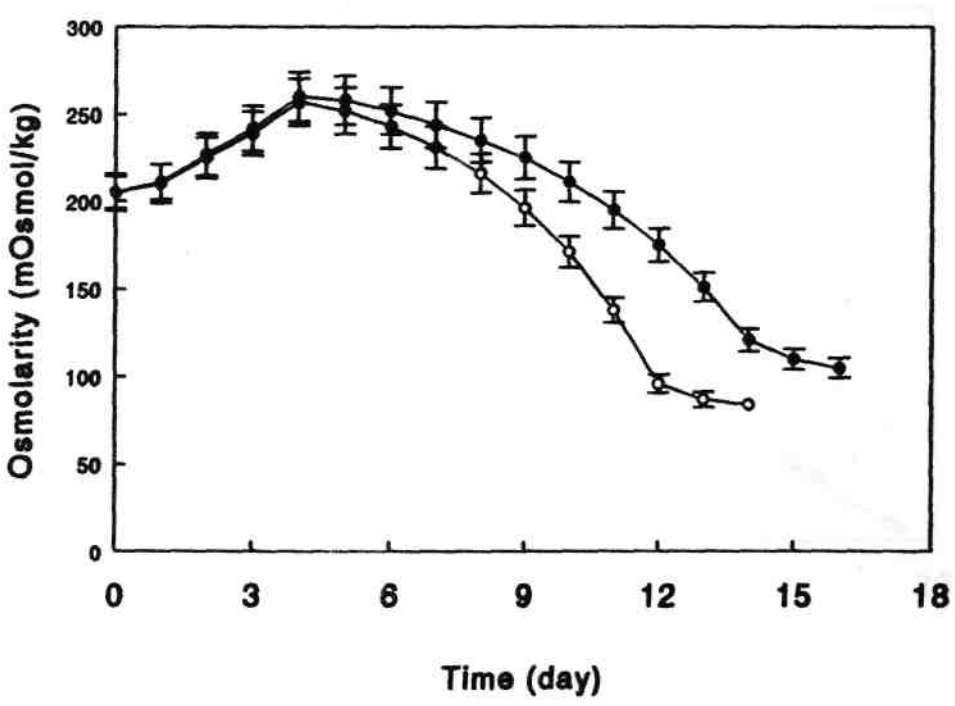


Figure 3.26. Time course of the osmolarity of culture media of *Daucus carota* (o), and *Capsicum frutescens* (•) (mean ± s.d.; n = 3).

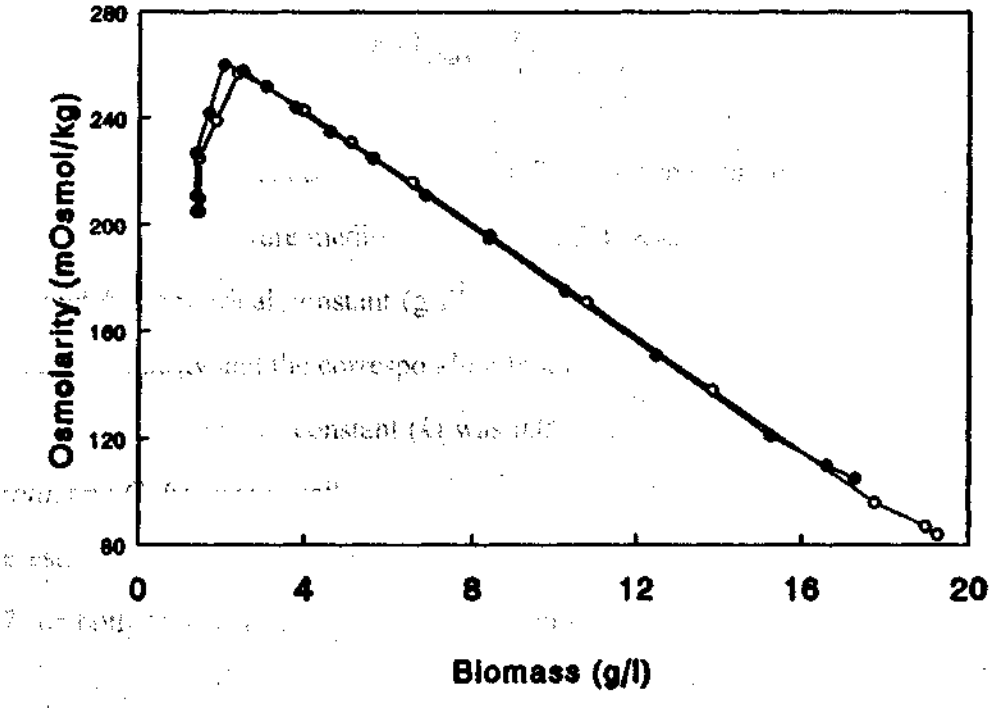


Figure 3.27. Relationship between the osmolarity of culture medium and cell biomass of *Daucus carota* (o), and *Capsicum frutescens* (•) (mean; n = 3).

initial increase, the osmolarity linearly decreased with the increase in the biomass density in both the cell culture systems. The following equation was formulated to describe the relationship between the osmolarity and the biomass density.

$$X = X_{Tmax} + k\,(T_{max} - T) \qquad \textbf{Eq. 3.2}$$

where X = dry cell concentration (g l^{-1}); T_{max} = maximum osmolarity reached; T = osmolarity of the culture medium (mOsmol kg); X_{Tmax} = dry cell concentration at T_{max}; and *k* = empirical constant (g l^{-1} kg $mOsmol^{-1}$). From the linear-regression analysis of osmolarity and the corresponding biomass density it was estimated that the value of the proportionality constant *(k)* was 0.096, and 0.094 g l^{-1} kg $mOsmol^{-1}$ for *D. carota,* and *C. frutescens* cell cultures, respectively. The correlation coefficient (r^{-2}) for the estimation of biomass based on the osmolarity of culture medium was over 0.9997 for both *D. carota* and *C. frutescens* cell culture systems.

3.23 Extracellular polysaccharides and proteins in culture medium

There was a continuous secretion of polysaccharides and proteins by both *D. carota* and *C. frutescens* cells into the liquid nutrient medium (Fig. 3.28). By the end of the log phase of growth, there was an accumulation of 1.2, and 1.05 mg ml^{-1} of polysaccharides, and 0.78, and 0.71 mg ml of proteins in the cell free media of *D. carota* and *C. frutescens,* respectively. Microscopic observation of the cell free medium showed the presence of cell debris.

3.24 Estimation of biomass based on the turbidity of cell free medium

As a consequence of the secretion of polysaccharides and proteins by the cells of *D. carota* and *C. frutescens,* the nutrient media gradually became turbid and viscous. Figure 3.29 shows the relationship between the increase in the turbidity of the cell free medium and the growth of cell biomass. The relationship was represented by

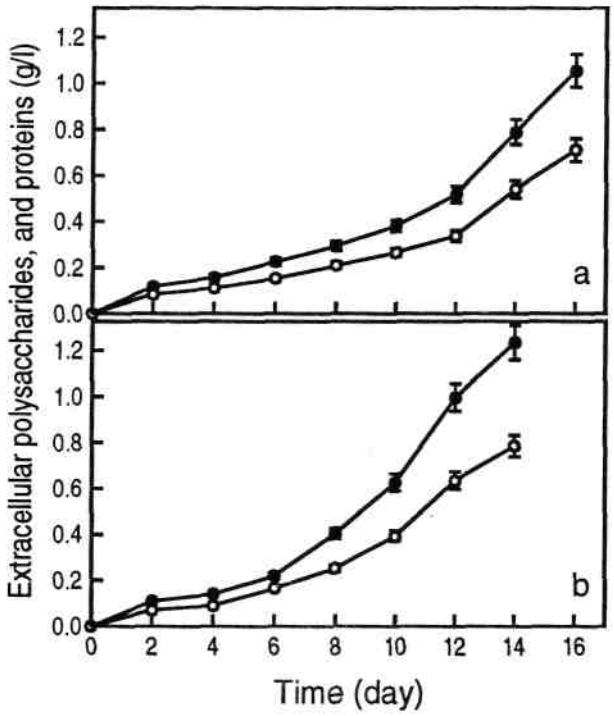


Figure 3.28. Time course of extracellular polysaccharides (•), and proteins (o) in *Capsicum frutescens* (a), and *Daucus carota* (b) cell suspension cultures (mean + s.d.; n = 3).

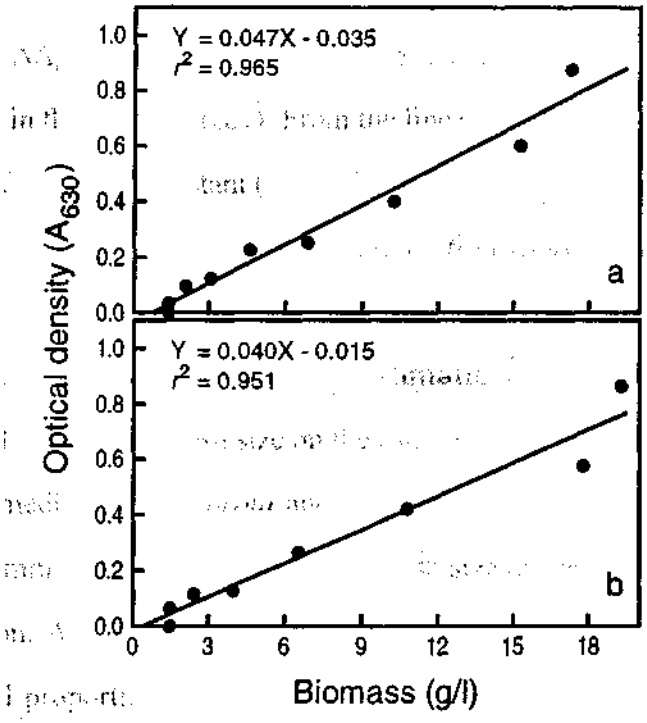


Figure 3.29. Relationship between the turbidity of cell-free culture medium and biomass density in *Capsicum frutescens* (a), and *Daucus carota* (b) cell suspension cultures (mean; n = 3).

the following equation:

$$\Delta X = k\, \Delta A_{630} \qquad \text{Eq. 3.3}$$

where, ΔA_{630} is the change in the optical density of the cell free medium per unit increase in the biomass (ΔX). From the linear-regression analysis it was estimated that the proportionality constant *(k)* was 24.93, and 21.26 with a correlation coefficient (r^{-2}) of 0.982, and 0.975 for *D. carota* and *C. frutescens,* respectively.

3.25 Effect of filter mesh size on estimation of biomass

The effect of filter mesh size on the estimation of biomass based on the turbidity of cell free medium in *D. carota* and *C. frutescens* suspension cultures was studied. Table 3.8 summarizes the effect of filter mesh size on the sensitivity and accuracy of the estimation. A fine mesh (37 μm) gave a better correlation but a lower value for the empirical proportionality constant *(k)*. On the other hand, a wider mesh (88 μm) gave a higher value for *k* with a poor correlation. Microscopic examination of the cell free medium obtained by the filter of mesh size 88 μm showed the presence of single cells at low density. However, in spite of the presence of cells, there was a moderate correlation between the turbidity of the cell free medium and the increase in the biomass in both the culture systems.

Table 3.9 compares the biomass estimation methods based on the conductivity, osmolarity, and turbidity of cell-free culture medium. It is apparent that the estimation based on osmolarity of the culture medium had the highest sensitivity, while turbidity of cell-free culture medium gave a quick rough estimate of biomass.

3.26 Production of anthocyanin in stirred tank bioreactor

The growth of biomass and production of anthocyanin from *D. carota* cells was studied at 2 L level using a stirred tank reactor. Figure 3.30 shows the time course of

Table 3.8. Effect of filter mesh size on the measurement of biomass based on the turbidity of cell-free culture medium.

	Daucus carota		*Capsicum frutescens*	
Filter mesh size (μm)	Empirical constant (k, g l^{-1})	Correlation coefficient (r^2)	Empirical constant (k, g l^{-1})	Correlation coefficient (r^2)
37	32.16	0.982	37.65	0.973
53	21.26	0.965	24.93	0.951
88	15.47	0.912	16.02	0.908

Table 3.9. Comparison of biomass estimation methods based on conductivity, osmolarity, and turbidity of cell-free medium.

Method	*Daucus carota*	*Capsicum frutescens*	
Conductivity[1]			
Empirical constant (*k*)	-5.29	-5.56	$g\ l^{-1}\ mS^{-1}$
Correlation coefficient (r^2)	0.997	0.998	
Osmolarity[2]			
Empirical constant (*k*)	0.096	0.094	$g\ l^{-1}\ kg\ mOsmol^{-1}$
Correlation coefficient (r^2)	>0.999	>0.999	
Turbidity of cell-free medium[3]			
Empirical constant (*k*)	21.26	24.93	$g\ l^{-1}$
Correlation coefficient (r^2)	0.965	0.951	

[1]See equation 3.1 on page **83**

[2]See equation 3.2 on page **91**

[3]See equation 3.3 on page **94**

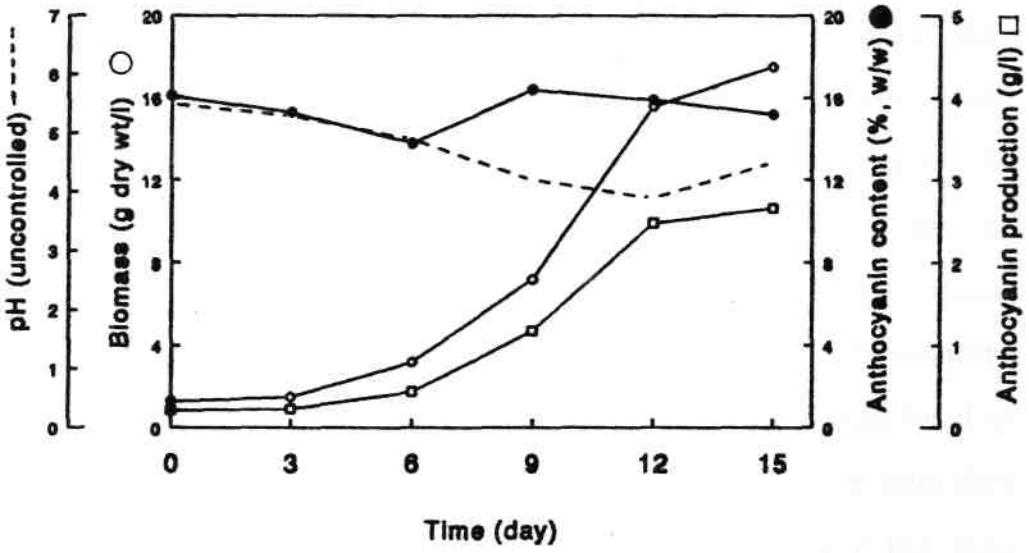


Figure 3.30. Growth and production of anthocyanin from *Daucus carota* cells in 2 L stirred tank bioreactor (mean; n = 3).

cell biomass, anthocyanin content and the production. There was about 18 g l^{-1} of dry cell biomass after 14 days of culture. The total anthocyanin production was 2.7 g l^{-1} . The growth and product formation profiles were essentially similar to those observed in shake flasks. The mean cell viability was over 90% throughout the culture period. The size distribution of cell aggregates was comparable to that in shake flasks indicating that the cells had a fair shear tolerance. In spite of the extracellular polysaccharides and proteins, there was no considerable frothing of the medium. The foam level controller, set at a height of 3 cm above the level of liquid medium, did not add any antifoam emulsion. The pH of the culture broth tended to drift towards acidic, and if not controlled, it dropped to 3.2 by 12th day of culture. The automatic pH controller added few ml of alkali intermittently. Estimation of biomass based on the conductivity and osmolarity using the empirical constants obtained with shake flask cultures, correlated well with the sample analyses, and demonstrated that these parameters can be used at large-scale cultivation of *D. carota* cells. Figure 3.31 shows the bioreactor used for the study.

3.27 Oxygen consumption rate

The oxygen requirement of *D. carota* and C. *frutescens* cells was estimated. The experimental set up used for the measurement of oxygen consumption rate is described in Section 2.23 of Material and Methods. Figure 3.32 shows the time course of oxygen depletion from the liquid nutrient medium in the presence of *D. carota,* and C. *frutescens* cells. The overall pattern of oxygen uptake was similar for both the culture systems studied. There was a steady and linear decrease in the dissolved oxygen up to a level of 30, and 25% of air saturation (2.4, and 2.0 mg l^{-1}) with *D. carota,* and *C. frutescens* cells, respectively. Below this concentration, the oxygen utilization rate suddenly dropped.

From the linear-regression analysis of the amount of oxygen consumed during different time intervals, it was estimated that the specific oxygen uptake rate (q_{02}) of

Figure 3.31. Stirred tank bioreactor (2 L) used for studies on growth and anthocyanin production from *Daucus carota* cell culture.

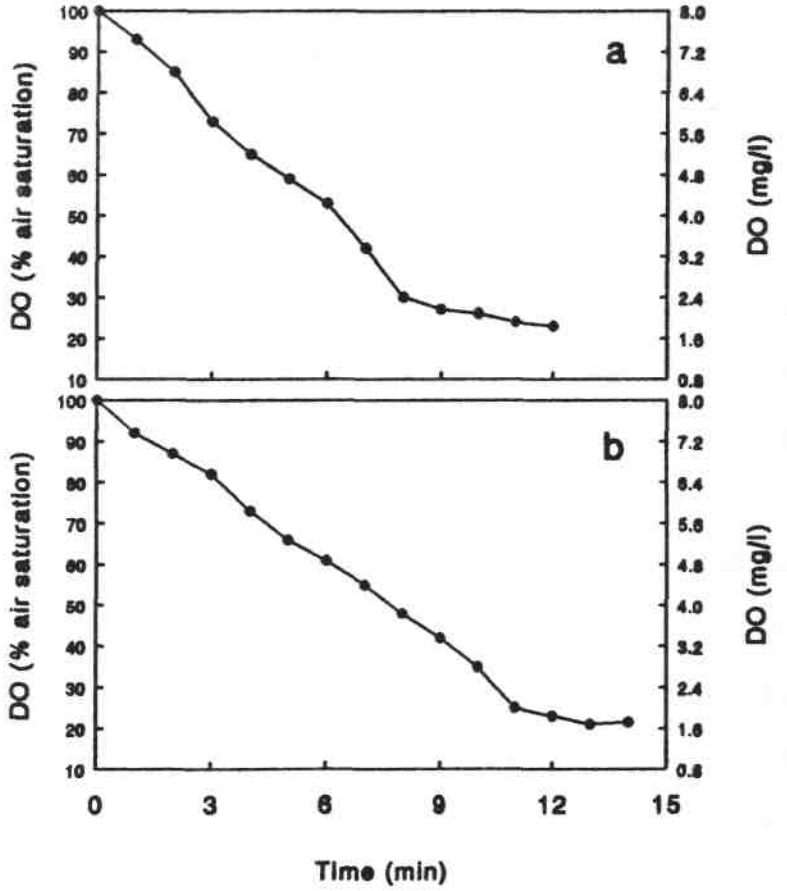


Figure 3.32. Oxygen uptake by *Daucus carota* (a), and *Capsicum frutescens* (b) cells (mean; n = 3).

D. carota, and *C. frutescens* cells was 0.75, and 0.57 mmol g h , or 0.58, and 0.44 g g^{-1} day^{-1} , respectively. From the Figure 3.32 it can be seen that the critical oxygen concentration was 30, and 25% air saturation for *D. carota,* and *C. frutescens* cells, below which the respiratory rate was affected. There was no considerable change in the specific oxygen consumption rate with culture age in both the culture systems.

3.28 Rheoiogical characteristics of the culture broth

Rheological characteristics of the culture broth of *D. carota* in the stirred tank bioreactor were studied. Samples were drawn from the reactor at 3 day intervals. In view of the practical limitation associated with the instrument used in for study, larger cell aggregates were removed from the samples by passing the culture broth through a sieve of mesh size 1500 μm. It is assumed that this would not affect the various estimations since the fraction of cell aggregates larger than 1500 μm was very small.

The viscosity (η) of the culture broth did not increase appreciably till the 6th day of culture. Figure 3.33 shows the flow curve (shear stress vs shear rate) of the 3-day old culture broth. The near linear curve passing through the origin indicates that the culture broth was Newtonian in rheology with no yield stress. This rheological nature is the property of most of the common fluids like water. The apparent viscosity of the culture broth, as estimated from the linear regression analysis of shear stress and shear rate data, was about 3 cP which is comparable to that of 1 cP for water.

However, viscosity of the culture broth rapidly increased from day 6 onwards diαe to the exponential growth of biomass. As evident from Figure 3.33, rheology of the culture broth at late exponential stage is strikingly different from that of the culture broth of initial stage. The flow curve became curvilinear indicating the non-Newtonianity. Among the various common flow models applied, the Power-Law model was the best fit to describe the flow behaviour of the culture broth. As estimated from non-linear regression analysis, the consistency index (k) was 132 (mPa $s)^{0.57}$, and the flow behaviour index (n) was 0.57 for the 12 day old culture broth.

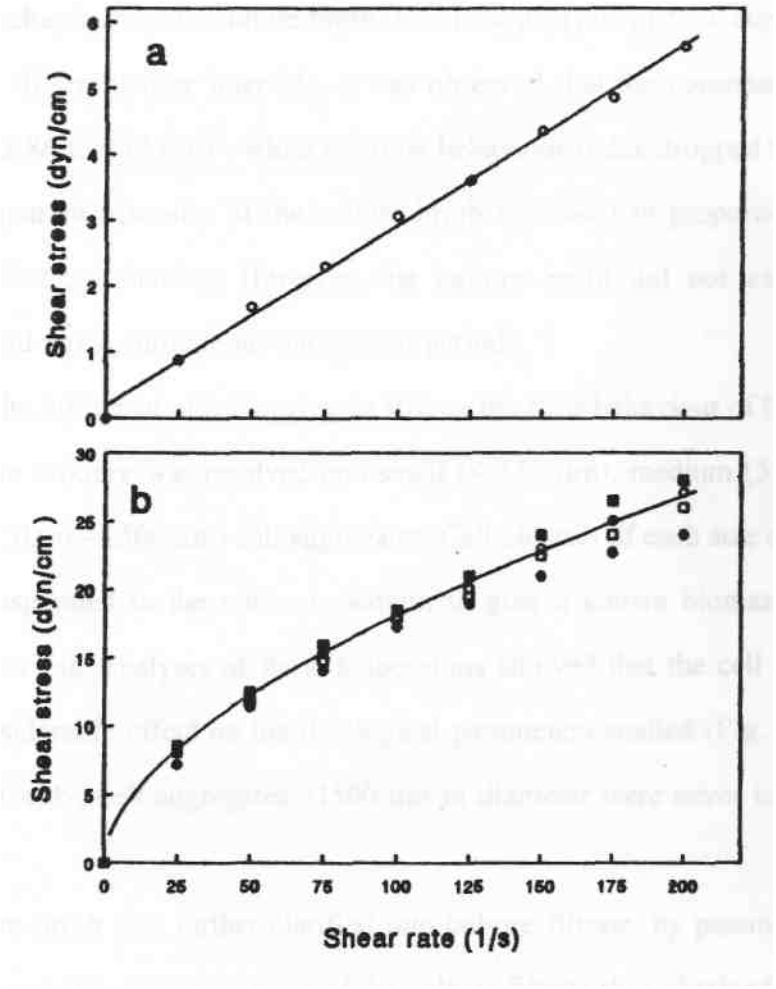


Figure 3.33. Flow curve of *Daucus carota* culture broth in stirred tank bioreactor. 3rd day (a); 12th day (b). culture broth (o); Small size (■); medium size (D); large size (•) cell aggregates (mean; n = 3).

The above results suggest that the culture broth slowly turned from Newtonian to non-Newtonian regime with increase in the cell density. Figure 3.34 shows the time course of flow behaviour of the culture broth. From the analysis of flow curves of the culture broth at different time intervals, it was observed that the consistency index increased from 3.86 to 132 $(cP)^n$, while the flow behaviour index dropped from 0.93 to 0.57. The apparent viscosity of the culture broth increased in proportion of 3rd power of the biomass density. However, the culture broth did not exhibit any considerable yield stress throughout the growth period.

To study the influence of cell aggregate size on the flow behaviour of the culture broth, the culture mixture was resolved into small (< 550 μm), medium (550 - 1000 μm), and large (1000 - 1500 μm) cell aggregates. Cell clusters of each size class were pooled and resuspended in the nutrient medium to give a known biomass density. Shear stress-shear rate analyses of these suspensions showed that the cell aggregate size has no considerable effect on the rheological parameters studied (Fig. 3.33). As mentioned previously, cell aggregates >1500 μm in diameter were never included in the study.

The culture broth was further clarified into culture filtrate, by passing through a sieve of mesh size 35 μm. Flow curve of the culture filtrate thus obtained indicated that the broth devoid of cells did not contribute much to the viscosity of the total culture fluid. The culture filtrate of the late log phase was purely Newtonian in flow behaviour with a viscosity of about 4 cP.

3.29 Characterization of anthocyanins

a) Separation and purification of anthocyanins

The methanolic extract of *D. carota* cells was separated into three distinct compounds using paper chromatography. Of the various solvent systems used, n-butanol-acetic acid-water (4:1:5) gave the best resolution. The three compounds had Rf values of 0.36, 0.38, and 0.48, which were designated as AN-I, II, and III,

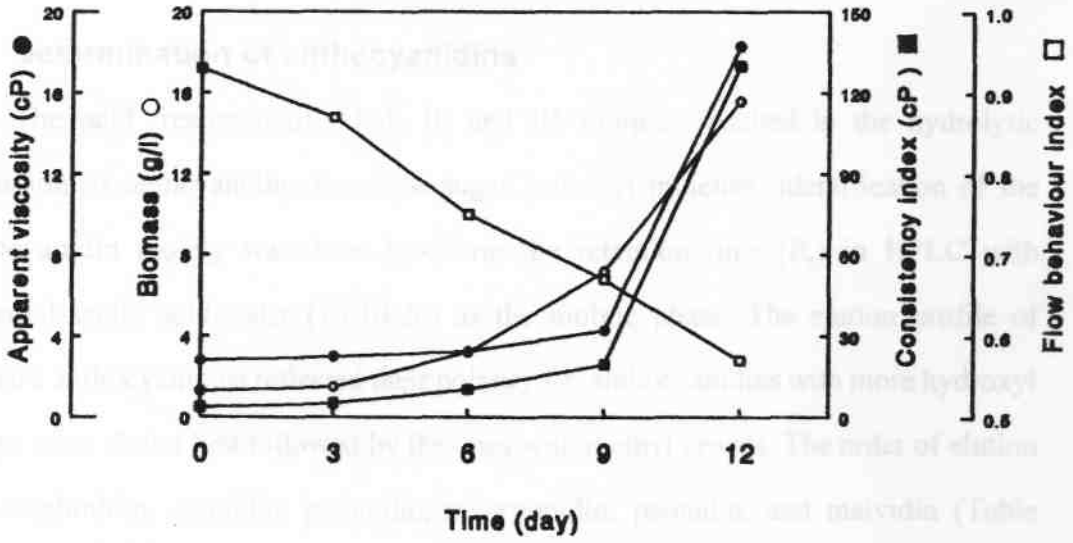


Figure 3.34. Time course of biomass and flow behaviour parameters of *Daucus carota* culture broth in 2 L stirred tank bioreactor (mean; n = 3).

respectively.

The spectral data of these separated bands showed the absorption maxima (λ_{max}) in the range of 525 - 535 nm in the visible region, and 250 - 290 nm in the UV region (Table 3.10), while the crude extract had λ_{max} of 238, and 535 nm (Fig. 3.35).

b) Determination of anthocyanidins

The acid treatment of AN-I, II, and III samples resulted in the hydrolytic separation of anthcyanidins from the sugar and acyl moieties. Identification of the anthocyanidin moiety was done based on the retention time (R_t in HPLC with methanol-acetic acid-water (70:10:20) as the mobile phase. The elution profile of standard anthocyanidins reflected their polarity *i.e.* anthocyanidins with more hydroxyl groups were eluted first followed by the ones with methyl groups. The order of eiution was delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin (Table 3.11). Based on the retention time, the anthocyanidins in the internal standards were identified.

All the three paper chromatographically separated compounds of the cell extract, AN-I, II, and III, gave a single peak at a retention time in the range of 9.3 - 9.5 min indicating that the cyanidin was the only anthocyanidin in the high yielding cell line of *D. carota.* Figure 3.36 shows the HPLC profile of *D. carota* cell extract.

c) Identification of sugars

Paper chromatography of the aqueous extracts of acid hydrolysed AN-I, II, and III indicated the presence of three different sugars, namely glucose, galactose, and xylose (Table 3.12). AN-I gave two distinct spots with R_f values comparable to those of galactose (0.29), and xylose (0.47), while AN-II, and AN-III showed a single spot with Rf value equal to that of glucose, and galactose, respectively. These results suggest that AN-I is a cyanidin diglucoside, while AN-II, and AN-III are cyanidin monoglucosides. The relative optical densities of AN-I, II, and III showed that the

Table 3.10. Spectral absorbtion of anthocyanins from *Daucus carota* cells in suspension cultures.

Anthocyanin	λ_{max}	
	UV	Visible
Cell extract	287	535
(crude)	328	
AN-I	255	530
	365	
AN-II	255	535
AN-III	272	525

*In acidified methanol (HCl, 1%).

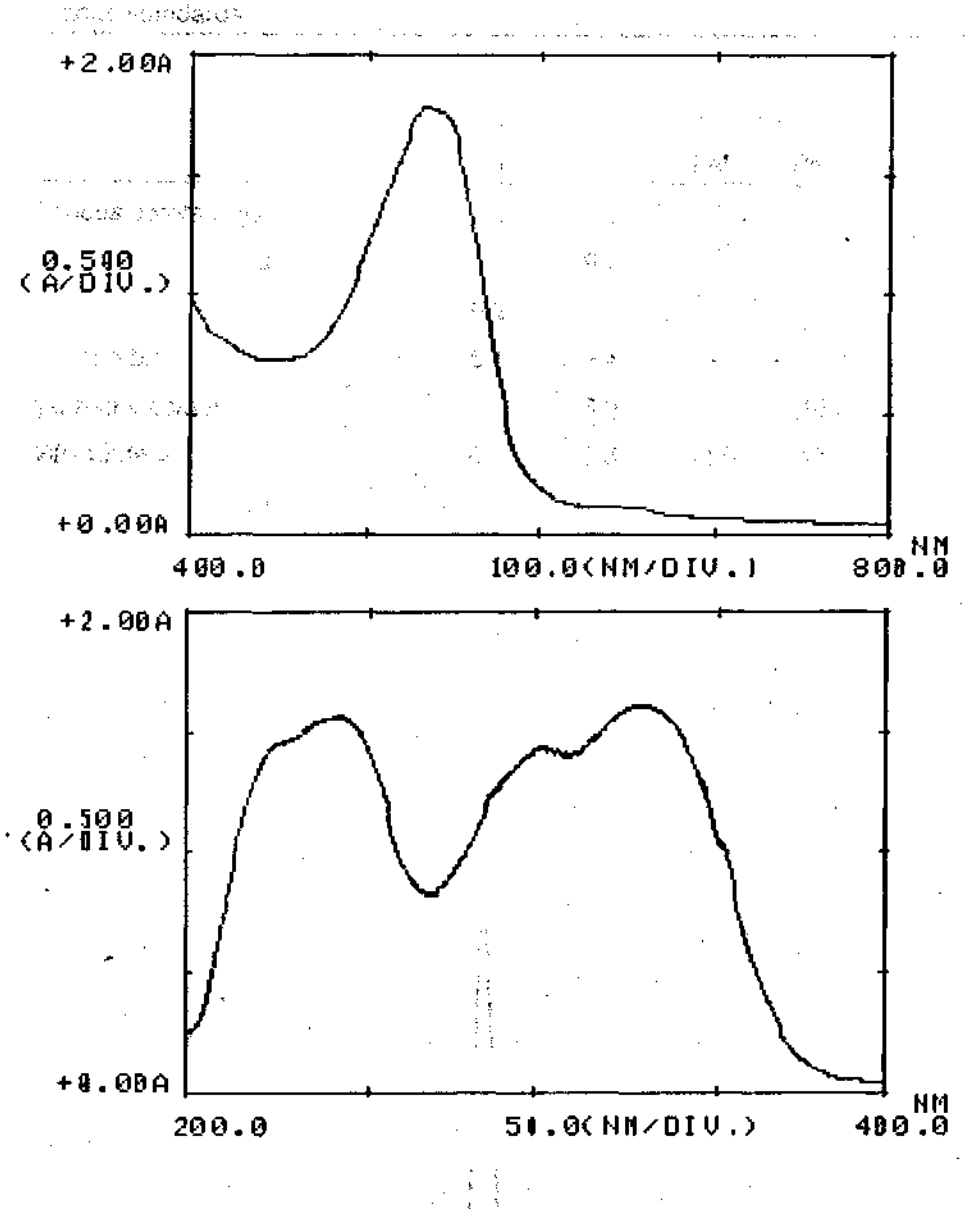


Figure 3.35. UV-Vlsible absorption spectra of *Daucus carota* cell extract.

Table 3.11. Retention times in HPLC of anthocyanidin in *Daucus carota* cell extract, and various standards.

Sample	Retention time (min)				
	Del.	Cyan.	Pet.	Peon.	Mal.
Daucus carota cells	-	9.4	-	-	-
Hibiscus rosasinesis	-	9.7	-	-	-
Solanum melongena	6.3	-	-	-	-
Banana bract	6.1	9.4	-	-	-
Ipomea tricolour	-	8.9	-	16.6	-
Vitis vinifera	6.3	9.3	11.6	17.0	20.7
Malvidin-3-glucoside	-	-	-	17.4	21.1

Del. = Delphinidin; Cyan. = Cyanidin; **Pet.** = Petunidin;
Peon. = Peonidin; **Mai.** = Malvidin.

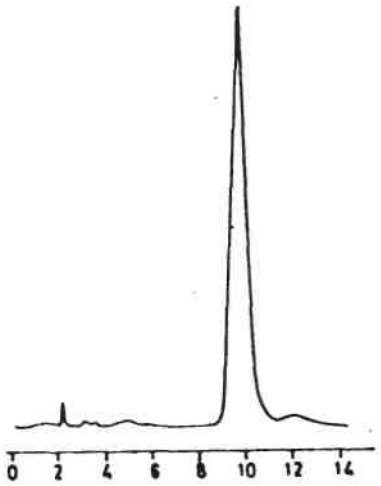


Figure 3.36. HPLC profile of *Daucus carota* cell extract.

HPLC conditions:

Column : C_{18} (μ-Bondapak 300 mm; 10μ)
Solvent : Methanol-acetic acid-water (70:10:20)
Flow rate : 1.5 ml min^{-1}
Detection : Absorbabnce at 530 nm.

Table 3.12. Paper chromatographic seperation of glycon moieties of anthocyanins from *Daucus carota* cells.

Sample	Rf value		
Cell extract (crude)	0.46	0.33	0.30
AN-I	0.47	-	0.31
AN-II	-	0.34	-
AN-III	-	-	0.30
Glucose	-	0.33	-
Galactose	-	-	0.30
Xylose	0.46	-	-

Solvent: n-butanol-benzene-pyridine-water (5:1:3:3).

cyanidin diglucoside was the major anthocyanin in *D. carota* cells.

No appreciable optical absorbance by AN-I, II, or III in the range of 300 -340 run suggested the absence of acyl groups.

3.30 Production of capsaicin using bubble column reactor

Continuous production of capsaicin from immobilized *C. frutescens* cells using alginate matrix was studied in an 1 litre bubble column reactor. The alginate beads encapsulating the cells were about 5 mm in diameter after hardening in $CaCl_2$ solution and harboured about 25 mg of cells per bead. About 4000 beads containing approximately 100 g of fresh cells were loaded into an 1 litre glass column. There was about 750 ml of void space and the beads were free floating.

Liquid nutrient medium containing the MS salts (Murashige and Skoog, 1962) and sucrose (3%, w/v) supplemented with 2,4-D (2 mg l^{-1}) and kinetin (0.5 mg 1) was passed through the column at a constant volumetric flow rate of 25 ml $hour^{-1}$, which corresponds to a dilution rate of 0.03 $hour^{-1}$. Assuming a perfect mixing, the mean residence time of the nutrient medium was about 30 hours. The reactor was aerated through a sparger located at the bottom of the column at a constant flow rate of 250 ml min^{-1} (0.25 VVM).

The spent medium collected through the outlet the column was analyzed for capsaicin. Figure 3.37 shows the time course of capsaicin productivity. The productivity rapidly increased to a maximum of 2.5 mg 1^{-1} day^{-1} in the initial four days and remained stable for the next 10 days. Thereafter the productivity slowly came down and after three weeks there was no synthesis of capsaicin. The total production of capsaicin was 35 mg l^{-1} over a period of three weeks of continuous culture. Figure 3.38 shows the bubble column reactor used for the continuous production of capsaicin.

Gradual softening, and collapse of the alginate beads was observed after three weeks of continuous operation.

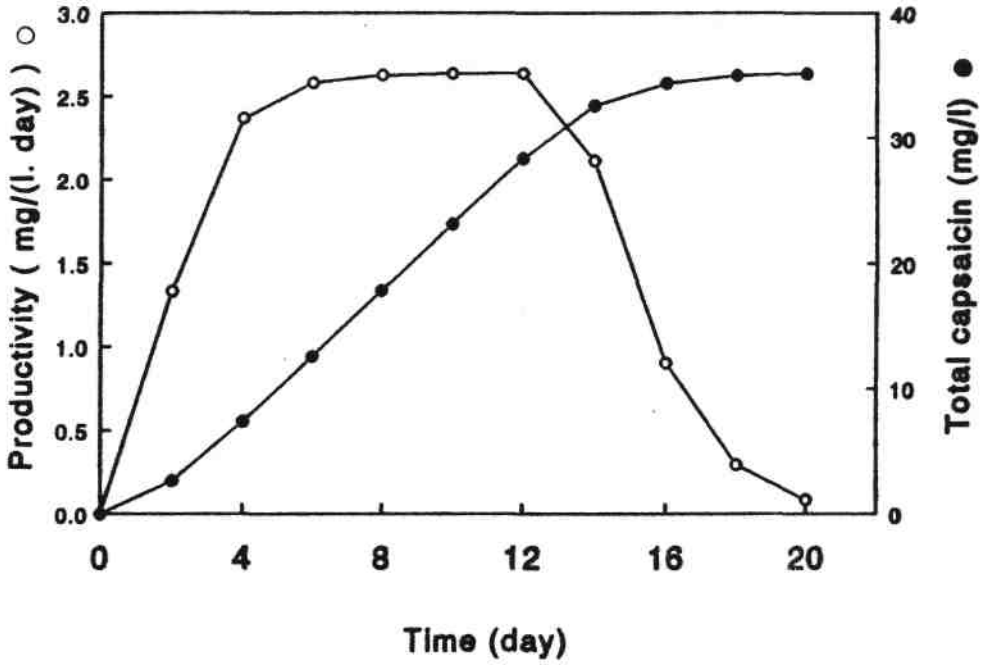


Figure 3.37. Continuous production of capsaicin from bubble column reactor with *Capsicum frutescens* cells immobilized in alginate matrix (mean; n = 3).

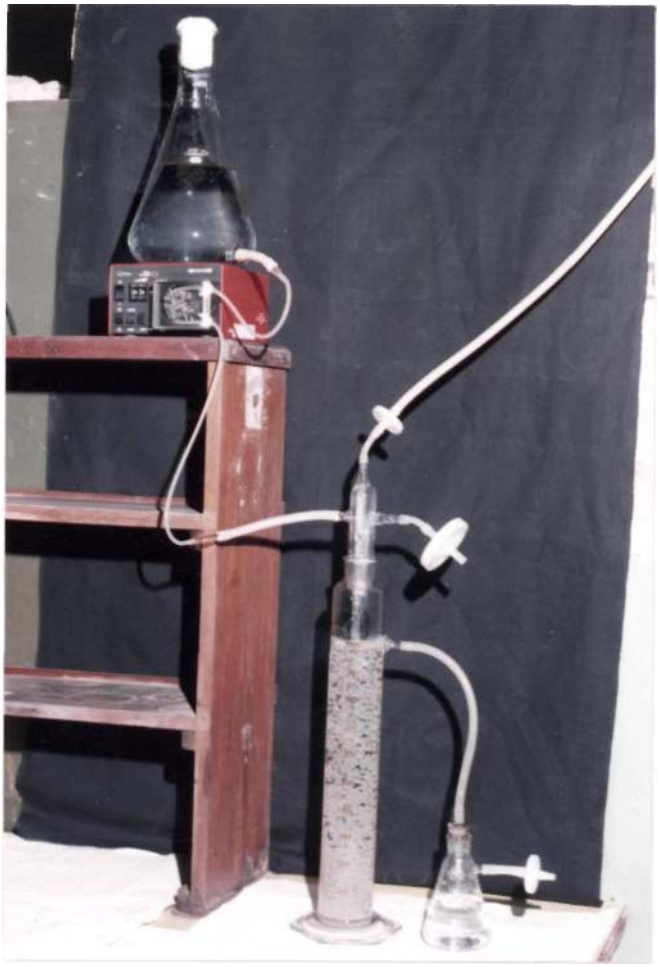


Figure 3.38. Bubble column reactor (1 L) used for studies on continuous production of capsaicin from *Capsicum frutescens* cells immobilized in alginate beads.

3.31 Entrapment of *C. frutescens* cells in polyurethane foam

The suitability of polyurethane foam (PUF) as an immobilization matrix for *C. frutescens* cells was studied. The PUF used in the present study was highly reticulate with over 95% of void space. PUF of 2.0 cm thickness was cut into circular discs of 5.5 cm in diameter and incubated in the cell suspension cultures of *C. frutescens.* Figure 3.39 shows the time course of cell loading into PUF. The cells were entrapped in the foam and with the subsequent growth of cells in side the foam, nearly 40% of the available space was filled with the cells in 14 days of incubation. This resulted in a cell loading of 0.37 g fresh cells cm of PUF. Cross sectional view of the PUF discs under the microscope showed a uniform cell density.

3.32 PRODUCTION OF CAPSAICIN USING PACKED-BED REACTOR

Continuous production of capsaicin from *C. frutescens* cells packed in polyurethane foam (PUF) bed was studied. PUF discs loaded with cells were aseptically packed into a glass column to a bed volume of 1 litre. The total cell biomass in the packed bed was over 380 g fresh weight. There was about 630 ml of void space and the nutrient medium could seep through the bed slowly.

Liquid nutrient medium containing the MS salts (Murashige and Skoog, 1962) and sucrose (3%, w/v) supplemented with 2,4-D (2 mg 1^{-1}) and kinetin (0.5 mg l^{-1}) was trickled through the packed-bed at a constant volumetric flow rate of 25 ml hour-[1], which corresponds to a dilution rate of 0.04 hour . Assuming no channelling, the mean residence time of the nutrient medium was about 25 hours. The feed nutrient medium saturated with oxygen supplied the required oxygen for the respiration of cells inside the foam.

The spent medium collected at the downstream of the column was analyzed for capsaicin production. Figure 3.40 shows the time course of capsaicin productivity. The profile of capsaicin production from the packed-bed column reactor was similar to that of bubble column reactor. The productivity rapidly increased to a maximum of about

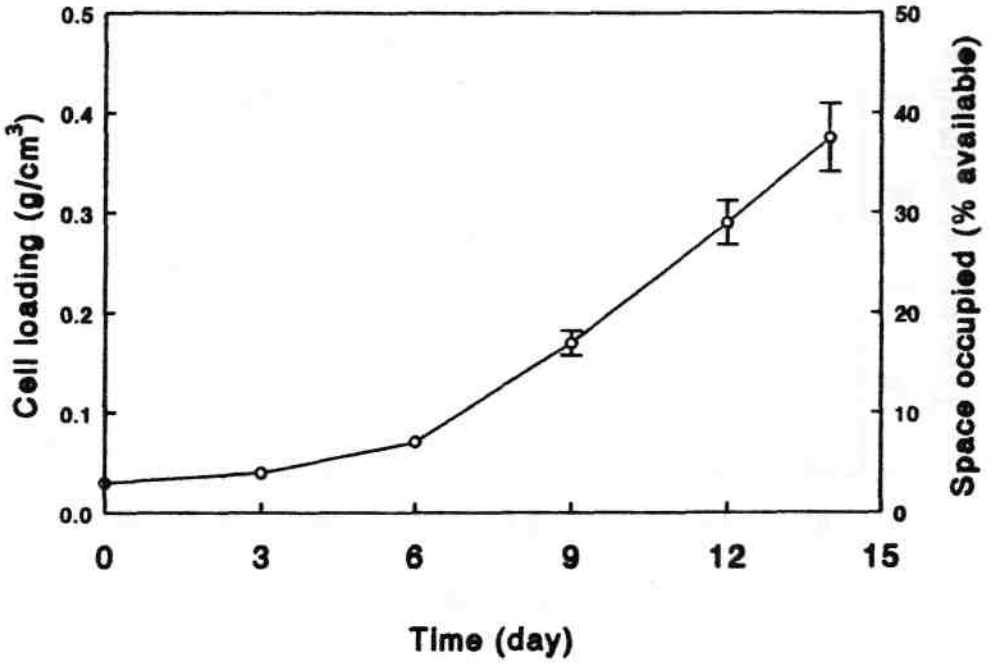


Figure 3.39. Time course of *Capsicum frutescens* cell loading into polyurethane foam (PUF) (mean ± s.d.; n = 3).

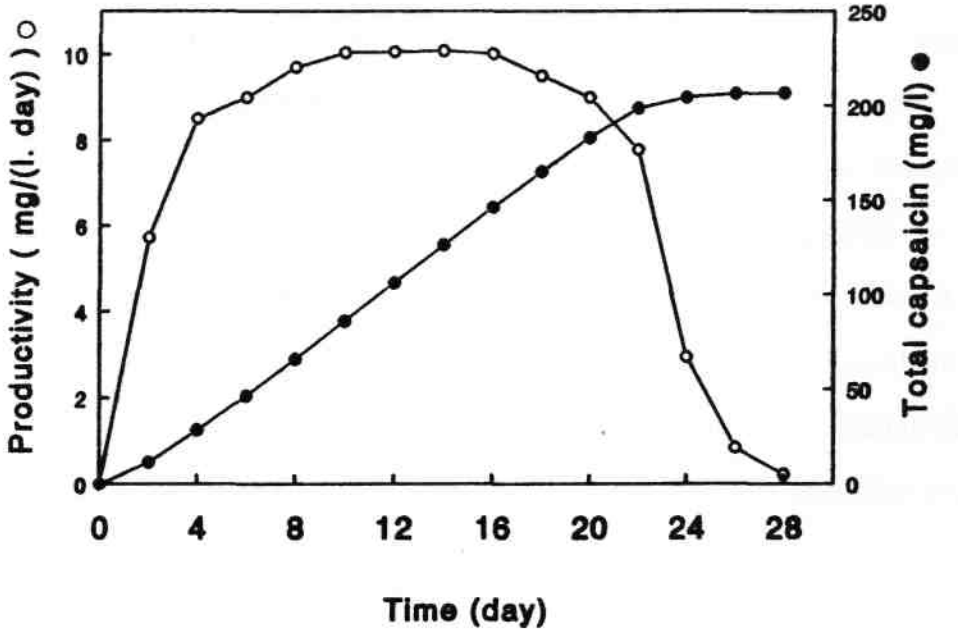


Figure 3.40. Continuous production of capsaicin from packed-bed column reactor of *Capsicum frutescens* cells immobilized in polyurethane foam bed (mean; n = 3).

10 mg 1^{-1} day^{-1} in the initial four days and remained stable for the next 20 days. Thereafter the productivity slowly came down and after four weeks there was no synthesis of capsaicin. The total production of capsaicin was 207 mg 1^{-1} over a period of three weeks of continuous culture. Figure 3.41 shows the packed-bed column reactor used for the continuous production of capsaicin.

Though the volumetric productivity of about 10 mg l^{-1} day is high as compared to that in the bubble column reactor, the specific productivity of capsaicin by C. *frutescens* cells in both the immobilizing matrices was approximately same which was about 25 µg g^{-1} fresh cells day^{-1} . Thus, due the high cell density, there was about 4 fold increase in the volumetric productivity of packed-bed reactor as compared to that of the bubble column reactor. Further, the synthesis of capsaicin from the cells immobilized in PUF sustained for longer time.

No physical deterioration of the foam was observed even after four weeks of continuous operation.

3.33 Enhancement of capsaicin production with fungal elicitor

In an effort to enhance the production of capsaicin, the application of a fungal elicitor was studied. *Aspergillus niger* mat extract was used in the feed nutrient medium for the packed-bed reactor at a concentration of 10 g equivalent of fresh mycelium per litre of medium. The elicitor and the concentration were selected based on the extensive work carried out earlier in our laboratory on elicitation of *in vitro* Gapsaicin synthesis (Sudhakar Johnson, 1991).

There was about 60% increase in the total production of capsaicin resulting in a maximum production of 330 mg 1^{-1} in a period of four weeks.

Table 3.13 compares the performance of the bubble column reactor, and the packed-bed reactor.

Figure 3.41. Packed-bed reactor (1 L) used for studies on continuous production of capsaicin from *Capsicum frutescens* cells immobilized in polyurethane foam.

Table 3.13. Comparison of continuous production of capsaicin from bubble column, and packed-bed reactors.

Bioreactor		
Bubble column reactor		
Biomass density	100	g fresh wt. l^{-1}
Average productivity	1.7	mg l^{-1} day^{-1}
Average specific productivity	17	μg g^{-1} fresh wt. day^{-1}
Max. duration of continuous operation	21	days
Total production	35	mg l^{-1} in 21 days
Packed-bed reactor		
Biomass density	380	g l^{-1}
Average productivity	7.4	mg l^{-1} day^{-1}
Average specific productivity	19.5	μg g^{-1} fresh wt. day^{-1}
Max. duration of continuous operation	28	days
Total production	207	mg l^{-1} in 28 days
Production under fungal elicitation	330	mg l^{-1} in 28 days

DISCUSSION

4.1 Development of a high yielding cell line of *D. carota*

4.2 Physico-chemical properties of plant cell culture media

4.3 Optimization of culture conditions for *D. carota* cells

4.4 Growth of *D. carota* and production of anthocyanin

4.5 Size distribution of cell aggregates of *D. carota*

4.6 Nutrient consumption in *D. carota* and *C. frutescens* cell cultures

4.7 Measurement of biomass in plant cell cultures

4.8 Growth and production of anthocyanin in bioreactor

4.9 Characterization of anthocyanin

4.10 Studies on continuous production of capsaicin

Higher plants produce a great variety of compounds, many of which are commercially important as pharmaceuticals, food additives, natural flavours, dyes, pesticides etc. *In vitro* culture of plant cells has long been recognised as an alternate source for high value phytochemicals. In the last two decades there have been intense efforts to develop cell culture processes for a number of compounds of commercial importance. However, there are only a few processes operative on commercial scale. These include shikonin production from *Lithospermum erythrorhizon* cell cultures by Mitsui Petrochemical Industries Ltd., Tokyo (Curtin, 1983), production of berberine from *Coptis japonica* cell cultures, by the same company, and few other compounds such as digoxin, geraniol, rosmarinic acid etc. (Mor, 1991).

The major limitations in commercial realization of this technology for a wider range of compounds are the slow growth of plant cells, low yields of the metabolites, and difficulties in scale-up of the processes. Hence, development of a stable high yielding cell line, optimization of various culture parameters, detailed studies on growth and product formation kinetics form the bases for the scale-up and evaluation of economic feasibility of the cell culture processes.

The present investigation dealt with the studies on production of anthocyanin from *Daucus carota* cell cultures, and capsaicin from immobilized cells of *Capsicum frutescens* at shake-flask and lab-scale reactor level.

4.1 Development of a high yielding cell line of *D. carota*

A stable and high yielding cell line is an important component of a fermentation process. As the secondary metabolites do not add any selective advantage or disadvantage to the proliferation of plant cells, a number of methods have been developed for improvement of the desired metabolite content of the cells. Zenk and co-workers (1977) presented a scheme to establish a high producing cell culture. The suggested scheme starts with a search for highly productive plants for culture

initiation, followed by the development of a special production medium for the highest formation of the desired compound. Finally, a screening of the heterogenous cell population for variant clones containing the highest levels of the desired products was to be employed. Following this procedure, several highly productive cell lines were established (Constabel *et al.,* 1981; Kurz *et al,* 1985; Tremouillaux-Guiller *et al.,* 1987). The screening of coloured compounds by the naked eye assisted by quantitative spectrophotometric analyses has given the most convincing results (Kinnersley and Dougall, 1980; Yamamoto *et ah,* 1982; Nozue *et al.,* 1987). However, automated cell sorting using flow-cytometer coupled with radio-immuno-assay (RIA), fluorescent-immuno-assay (FIA), or luminescent-immunosorbent-assay (LIA) were used for high throughput (Matsumoto *et al.,* 1980).

In the present study a high yielding cell line of *D. carota* was developed by repetitive selection for high anthocyanin containing cell clusters (Fig. 3.9). Interestingly, the growth rate of biomass also increased considerably. The very process of culture maintenance by periodic subculturing selectively enriches the fraction of fast growing cells. The cell line exhibited an impressive stability in terms of the anthocyanin content and growth rate.

4.2 Physico-chemical properties of plant cell culture media

Several hundreds of nutrient media have been formulated for plant cell, tissue, and organ culture *in vitro* (George *et al,* 1987). These media containing macro and micro nutrients, vitamins, and other organic supplements are used along with a variety of combinations of phytohormones. Various basal media, which differ in their source of nitrogen, phosphorous, potassium, and other minerals, were devised primarily for an optimum biological response *viz.,* growth, regeneration, accumulation of secondary metabolites, in the target species (Murashige and Skoog, 1962; Schenk and Hildebrandt, 1972; Litvay *et al,* 1985). However, the different sources of these

nutrients and the minerals have a bearing on the physico-chemical properties of the culture medium as well which, in turn, influence the biological responses. Osmotic pressure and ionic strength of culture medium influence the membrane transport (De Klerk-Keibert and van der Plas, 1985). pH and ionic strength affect the activities of extracellular enzymes and secretion of secondary metabolites (Zink and Veliky, 1979; Reid *et ah,* 1980; Ambid and Fallot, 1987; Husemann *et al.,* 1990). Several workers have observed that the plant cell culture media, in general, have poor buffer capacity (Butenko *et al.,* 1984; Skirvin *et al.,* 1986). Attempts were made to improve the buffer capacity of the medium by incorporating external buffers such as MES, PIPES, HEPES, and Tris with some success (Parfitt *et al.,* 1988; Benthorpe and Brown, 1990). However, there are no quantitative data on the buffer capacity of the plant cell culture media. Viscosity and density determine the buoyancy of cells and cell aggregates in suspension cultures and influence the solubility of oxygen and mass transfers (Blanch and Clark, 1996). To our knowledge there is no comprehensive report on the physico-chemical properties of plant cell culture media. In the present study osmolarity, conductivity, buffer capacity and the solubility of oxygen of few commonly used plant cell culture media were studied.

Considerable differences were recorded for both conductivity and osmolarity of various media studied (Table 3.1). Though a high conductivity, in general, indicates a high ionic strength, it was not possible to estimate the absolute value of ionic strength because of the differential equivalent conductances of the compounds. While the experimentally determined conductivity values are comparable to the calculated ones of George *et al.* (1987), the osmolarity strength of various plant cell culture media determined in the present study deviated from the calculated values. This may be due to the complete dissociation of all the electrolytes, and the incomplete dissociation of the non-electrolytes and the partial hydrolysis of sucrose during autoclaving. Osmotic pressure of the nutrient medium is known to exert a crucial

effect on growth (Wiznsama *et al,* 1986), regeneration (Kavi Kishore, 1987), and secondary metabolite production (Rajendran *et al.,* 1992). Normally, the osmolarity of the culture medium is less than that of the cytoplasm to enable water to flow into the callus (Kirkham and Holder, 1981). The cells of many plants which are native of sea shores or deserts (for example, many cacti) characteristically have a low water potential and in consequence may not be cultured in media with a relatively low osmotic strength. Such plants needed media with high sucrose concentration (4 - 6%) (Mauseth, 1979; Lassocinski, 1985).

All the nutrient media studied showed a poor buffer capacity (Fig. 3.6). The differences in the buffer capacities of the various nutrient media studied were due to the differences in the concentrations of $P0_4$ and $B0_3$ ions (Table 3.1). It is well documented that the pH of the culture medium plays an important role in growth, regeneration, and secondary metabolite production. The success of Lloyd and McCown (1981) as a woody plant medium may be explained in the light of its moderately high buffer capacity (576 μmole 1^{-1} at pH 5.5) which may prevent the woody tissues from being subjected to extreme acidity from leaching phenolic acids. While the addition of external buffers such as 2-(N-morpholino)ethanesulphonic acid (MES), tris (hydroxymethyl)aminomethane (Tris) etc., could enhance the buffer capacity of the plant tissue culture media (Tiburcio *et al.,* 1989, Koop *et al.,* 1983), these compounds are reported to have side biological effects which are unrelated to their buffering capacity (Klein and Manos, 1960). As suggested by Owen *et al.* (1991), only the development of plant tissue culture media with increased buffer capacity could alleviate the effect of pH changes on culturing.

The solubility of oxygen in the various cell culture media studied ranged from 7.01 - 7.79 mg $l-^{1}$ (Table 3.1), corresponding to 87 - 96% of the solubility in distilled water. Because of a phenomenon called 'salting out,' slightly less oxygen dissolves in plant cell culture media than in water due to the presence of salts and non-electrolytes

such as sucrose.

There are varied reports on the effect of partial pressure of dissolved oxygen. Growth rate of *Euphorbia pulcherima* cells in suspension cultures was maximum when the partial pressure of oxygen was 60%) of saturation and declined rapidly only when the $P0_2$ dropped below 10%> (Preil *et ai,* 1988). On the other hand, embryogenic alfalfa suspensions needed over 70% saturation for maximum growth (Stuart and Strickland, 1987). DO concentration has a critical effect on the growth and differentiation of carrot tissue (Kessell and Carr, 1972). However, from the literature, it appears that the differential biological responses of various plant cell culture media may not be due to the differences in the solubility of oxygen in view of the close range of oxygen solubility observed in the present study.

Selection of plant tissue culture media for *in vitro* studies has been largely empirical. However, there are fragmentary information on the type of tissues correlating with their specific requirements for a desired response. In the present study, it was tried to relate the physico-chemical properties of certain media to their biological responses based on the literature data. It was beyond the scope of this study to see the behaviour of various tissue cultures under varied physico-chemical properties. However, the information obtained from this study may interest some researchers to look into physico-chemical parameters also in addition to the well known requirements of phytohormones and other adjuvants for tissue culture.

4.3 Optimization of culture conditions

Influence of basal medium

Macro and micro nutrients have been reported to have considerable influence on growth and secondary metabolite formation in cultured plant cells (Dougall, 1980). Generally, increased levels of nitrate, potassium, ammonium, and phosphate tend to support rapid cell growth while depletion or deficiency of some of these nutrients is

associated with growth limitation and a concomitant enhanced secondary metabolism (Rajendran *et al.,* 1992). B5 medium (Gamborg, 1968) was found to be suitable for rosmarinic acid production from *Anchusa officinalis* cultures (DeEknamkul and Ellis, 1985). Fujita *et al.* (1981) found that the White's (1942) medium supports high shikonin content in *Lithospermum erythrorhizon* cell cultures while there was a reduction in the growth. This led to the development of a two stage culture system, wherein LS medium was used for biomass accumulation followed by the White's medium. A similar two-stage strategy was used by Yamamoto and Yamada (1987) for reserpine production from *Rauwolfia serpentina.* Zenk *et al.* (1977) compared the growth of cell cultures of *Catharanthus roseus* on seven commonly used culture media. While cells grew well on all the media tested, alkaloid content varied widely with the MS medium supporting maximum levels of the indole alkaloid accumulation.

The results obtained in the present study are in contrast to the above findings. Of the four different basal media studied, MS was found to be optimum in terms of both the biomass and anthocyanin content (Fig. 3.7). This is obviously due to the growth-associated nature of the product in the present cell line. The similar response has been noted in several other culture systems (Takeda, 1988; Hirasuna *et al.,* 1991).

The above findings imply a simple single stage culture format for large scale cultivation of the *D. carota* cell line developed in the present study.

Influence of hormones

Hormonal composition is often a critical factor in secondary metabolite production. The type and concentration of auxin and cytokinin, usually in combination, have been known to profoundly influence the product formation as well as growth of plant cells in culture (Gamborg *et al.,* 1971).

Experiments were conducted to find out the hormonal supplementation required to maximize the production of anthocyanin. 2,4-D showed a dose dependent increase

in the biomass, while its presence was inhibitory to anthocyanin biosynthesis. On the other hand, another auxin, IAA, promoted anthocyanin content as well as biomass (Table 3.2). While cytokinins, singly, did not influence considerably either the growth or anthocyanin formation (Table 3.3), a combination of IAA and Kn resulted in the maximum production of anthocyanin (Fig. 3.8).

Several studies showed that 2,4-D has an inhibitory effect on the synthesis of a wide range of secondary metabolites (Furuya *et al.,* 1971; Zenk *et al.,* 1975; Mantell and Smith, 1983). Zenk *et al.* (1977) observed that indole alkaloid accumulation in *Catharanthus roseus* was generally depressed by the addition of 2,4-D to media. The substitution of 2,4-D by another auxin, IAA, allowed high levels of the indole alkaloid accumulation in non-dividing cultures, while the inclusion of NAA allowed simultaneous alkaloid accumulation and cell division (Morris, 1986,). In general, the presence of 2,4-D in the medium causes dedifferentiation of the tissues, and consequently diminishes the levels of secondary products. However, there are a few examples where 2,4-D supported the biosynthesis of secondary metabolites. Production of L-DOPA by *Mucuna pruriens* (Brain, 1976), formation of ubiquinone in *Nicotiana tabacum* cells (Ikeda *et al.,* 1976), and diosgenin in *Dioscorea deltoidea* tissues (Staba and Kaul, 1971) was stimulated by high levels of 2,4-D. Incidentally, capsaicin production from *Capsicum frutescens,* the another cell line used in the present studies on continuous production in bioreactors, was optimized using 2,4-D in shake flasks (Suvarnalatha *et al,* 1993).

A survey of the plant hormone literature will quickly reveal contradictory results, and species/tissue differences in response preclude a clear general view of the primary effects of these substances, and make prediction of likely responses in culture experiments almost impossible.

The present study found that, from the balance of growth and anthocyanin content, the combination of IAA and Kn (2 mg l^{-1}, and 0.2 mg l^{-1}, respectively) was

optimum for the maximum production of anthocyanin from *D. carota* cells.

Influence of pH

Little work on the effect of pH on growth and product accumulation in cell cultures has been published. Koul *et al.* (1983) showed that the growth and alkaloid accumulation were affected to different extents by the initial medium pH in cultures of *Hyoscyamus.* Mukundan and Hjorsto (1991) reported that the growth and thiophene accumulation by hairy root cultures of *Tagetes patula* were maximum at a pH 5.7, followed by pH 6.0. The yield was lowest at pH 4.0. Optimal growth in plant cell cultures usually occurs in media with the initial pH values in the range of 5-6. In the present study, it is observed that both the growth and production of anthocyanin were maximum at pH 5.5.

Influence of temperature

Temperature is an another culture parameter which needs to be optimized. Experiments on the effect of temperature on growth and production of anthocyanin from *D. carota* cells revealed that 25 °C is the optimum (Fig. 3.14). Both the lower and higher temperatures than the optimum resulted in a substantial decrease in the anthocyanin content even though the biomass accumulation was unaffected.

Though there are not many studies, the optimum temperature for growth and production of secondary metabolites is generally in the range of 20 - 35 °C. Cell cultures of different species showed varied degree of sensitivity for temperature (Tulecke and Nickell, 1960; Matsumoto *et al,* 1972; Rose and Martin, 1975; Kato *et al,* 1976; Morris, 1986a). Lockwood (1984) showed that temperature extremes had little effect on the pattern of alkaloids accumulated by four *Papaver* species in culture. Courtois and Guern (1980) have previously studied the effect of temperature on growth and indole alkaloid accumulation in *C. roseus* cultures and found a ten fold

increase in the cellular alkaloids at 16 °C compared to the accumulation at a temperature of 27 °C, which, however, gave higher growth rate. However, in terms of the alkaloid productivity, no net gain was observed at the low temperature. On the other hand, Morris (1986b) reported that a cell line of the same species, *C. roseus,* had a maximum growth rate at 35 °C while the alkaloid content was less, and concluded that 25 °C is the optimum for the maximum yield of the alkaloid. Hilton and Rhodes (1990) found that the concentration of hyoscyamine in the cultured hairy roots of *Datura stramonium* was only 60% of that found at 30 °C. Hirasuna *et al.* (1996) reported that the taxol production from cultured *Taxus baccata* cells varied seasonally. They pointed out that the unavoidable fluctuation in temperature is one of the reasons. They also noted that the growth rate is virtually constant in spite of the temperature changes.

The optimum temperature of 25 °C observed in the present study is near to the ambient temperature and, hence, there may not be any difficulty in controlling the temperature in large scale culture of *D. carota* cells. Further, the metabolic heat generation is almost negligible in all plant cell cultures.

Optimum inoculum density

Plant cells are usually cultured at a certain cell density in order to initiate cell division. This is called the minimum critical inoculum density, below which the cells cannot start multiplying. Mori *et al.* (1993) observed that the strawberry cells took long time to grow when the inoculum was small (1 g fresh cells in 100 ml). It is believed that there is a conditioning factor which is required at a certain level to stimulate the cell division (Stuart and Street, 1969). At small initial inoculum density, concentration of this factor becomes low, and it takes long time for build-up of the same resulting in a prolonged lag phase. Yamakawa *et al.* (1985) reported that the conditioning factor is functional even after autoclaving at 120 °C for 10 min.

Inoculum size has been reported to influence secondary metabolite production (Rokem and Goldberg, 1985; Liang *et ah,* 1991). Su and Lei (1993) reported that rosmarinic acid productivity in perfused *Anchusa officinalis* cultures increased with the inoculum size, up to 4 g dry cells 1^{-1} , while the maximum cell concentration was not affected by inoculum size between 1 and 11 g dry cells 1^{-1} . Stimulatory effects of inoculum size on anthocyanin accumulation in a carrot suspension culture (Ozeki and Komamine, 1985), and shikonin production by *Lithospermum erythrorhizon* cell cultures (Fujita and Hara, 1985) were also reported. In suspension cultures of *Holarrhena antidiysenterica,* total alkaloid concentration increased with increasing inoculum level up to 3 g 1^{-1} , giving a final cell concentration of 15.5 g 1^{-1} and alkaloid concentration of 23.5 mg 1^{-1} at this level (Panda *et ah,* 1992). In addition, Matsubara *et al* (1989) demonstrated that for high density fed-batch cultures of *Coptis japonica,* the cell concentration and berberine production increased with the increasing inoculum size up to 8 g dry cells 1^{-1}. At the highest inoculum concentration, the biomass obtained was 55 g dry wt. 1^{-1} with a production of 3.5 g berberine 1^{-1} medium. Apart from the inoculum size, the age of the inoculum is also influential on growth and secondary metabolite formation. Stafford *et al.* (1985) showed that the young, rapidly multiplying cells of *Catharanthus roseus* possessed the greatest ability to form biomass and also to accumulate alkaloids.

The results of the present study on the level of inoculum density indicated (Table 3.5) that the minimum critical inoculum density for the cell line used is rather low, and suggests an inoculum density of about 20 g fresh cells 1^{-1} would be optimum for a batch culture period of 14 days.

4.4 Growth and production of anthocyanin

The growth cycle of a typical plant cell suspension culture is essentially similar to that of microorganisms, although the time scale is much longer. The lag,

exponential, stationary, and death phases can be interpreted as adaption, rapid growth, nutrient limitation, and cell lysis, respectively (Warren, 1992). The growth profile of the *D. carota* cell line employed in the present study showed this typical pattern (Fig. 3.10). The estimated specific growth rate (μ) of 0.22 day^{-1} , which corresponds to a doubling time of 3.2 day, is within the range reported for several plant cell cultures. However, the most rapidly growing plant cell culture that has been reported is *Nicotiana tabacum* (Noguchi *et al,* 1982) which has a doubling time of 18 h.

However, several complex growth profiles have reported for plant cell cultures (de Gunst *et al.,* 1990). These varied patterns of growth appear to have an underlying constant somaclonal variations and adaptation of cells to the dynamic environment.

Numerous investigations on the time-course of product accumulation in relation to the growth of cells have revealed two basically different patterns (Sakuta and Komamine, 1987). A non-growth associated product formation, in which the product is formed during the late exponential, or stationary phase. This indicates an inverse relationship between the growth and product. Anthocyanin formation in *Daucus carota* cultures has often been cited as an example for this type of product pattern (Barz *et al.,* 1990). However, the *D. carota* cell line used in the present study exhibited the other type of product formation, namely the growth-associated product formation (Fig. 3.11). Interestingly, the unselected primary cultures of *D. carota* did show the former product formation pattern (Fig. 3.5). This reveals that there was a shift in the product formation pattern with the selection of cells. However, Payne *et al.* (1987) made a strong remark that many reports in the plant cell literature have erroneously interpreted a constant level of product in the cells of a growing culture to mean that product synthesis was not occurring when in fact the production rate was directly proportional to the growth rate.

It appears that the so-called growth-, and non-growth associated product patterns are only idealized situations representing the two extremes. The accumulation of

secondary metabolites in cultures is the result of a dynamic balance between biosynthetic, biotransformational, and biodegradative processes. This situation is complex and is undoubtedly the reason why mainly empirical approaches have been adopted so far in studies on cultural factors which influence the accumulation of secondary metabolites.

4.5 Size distribution of cell aggregates

Plant cells in suspension cultures tend to aggregate and form clumps, resulting in a heterogeneous population of cell clusters, ranging from a few cells to several thousands of cells, some times measuring a few centimetres in diameter. The size of cell aggregates has implications in the design and operation of bioreactors, and in the downstream processing (Panda *et al.,* 1989; Doran, 1993). Various biochemical parameters such as cytochrome oxidase, peroxidase, and catalase activities, rate of protein synthesis, pool size of free amino acids, and secondary metabolite accumulation and regeneration have been found to depend on cell cluster size (Street, 1977; Gana *et al.,* 1995). The cluster size is presumably governed by the degree of cohesiveness of the cells which is a function of the composition of cell wall. High auxin concentrations have been reported to result in fine suspensions. Cell aggregation can be controlled by culture methods, such as repetitive selection for small cell aggregates and cell plating (Dixon, 1985). Lowering of Ca^{2+} (Takayama *et al.,* 1977), and the addition of sorbitol and cell-wall degrading enzymes, such as pectinase and cellulase (King *et al.,* 1974), lessen the aggregation of cells in suspension cultures. In bioreactors, aeration and agitation operations exert hydrodynamic stresses, affecting the cell aggregation (Tanaka *et al.,* 1988). While a smaller size of cell aggregates is preferred from the stand-point of process engineering, a certain degree of cell-cell contact (Lindsey and Yeoman, 1983) and cell differentiation (Steward *et ah,* 1958) is often required for the synthesis of secondary metabolites. A detailed knowledge of the

biological and engineering aspects of the size of cell aggregates is a prerequisite for the optimization of process parameters.

In the present investigation, therefore, size distribution of cell aggregates, the effect of cell aggregate size on anthocyanin content, and the biochemical differences in various size groups of cell aggregates were studied in detail.

The profile of biomass distribution, in terms of percentage of total dry weight, remained unchanged during the culture with over 92% of biomass present in the aggregates of 500 - 1500 μm in diameter (Fig. 3.15). This observation is in agreement with that of Tanaka *et al.* (1992), who reported that there was only a slight change in the size distribution of *Catharanthus roseus* cell aggregates during the batch culture in a liquid medium. The cell aggregates of *Oryza sativa* were, however, smaller in the stationary phase than in the logarithmic growth phase. Mavituna and Park (1987) reported that the size distribution of cell aggregates of *Capsicum frutescens* in suspension cultures was Gaussian with a mean and standard deviation of 2.29 and 1.21 mm, respectively.

The anthocyanin content showed an increase with the increase in cell aggregate size up to a diameter of 500 - 850 μm (Fig. 3.16). This can be expected, because a certain degree of cell aggregation is required for secondary metabolite synthesis. Buitelaar and Tramper (1992) have reported that the dispersed cells of *Tagetes patula* could not synthesize pyrethrins. Gana *et al.* (1995) have reported that, for regeneration of *Avena* spp. cell suspension cultures, the minimum cluster size is 3 mm. However, the anthocyanin content decreased sharply, when the cell aggregate size exceeded 500 - 850 μm (Fig. 3.16). A similar observation, that a cell aggregate diameter of 12 mm was critical for the maximum production of thiophenes in *Tagetes patula* cell cultures has been reported by Hulst *et al.* (1989). These findings suggest that while the aggregation of cells into clumps can impart some sort of rudimentary organization, other factors, such as mass transfer resistances, come into effect. These opposing

factors perhaps get balanced at a particular size of cell aggregate for the maximum rate of secondary metabolism.

In contrast to the profile of anthocyanin content, the surface colour intensity, as measured in terms of the red-green coordinate value of CIELAB Color Space, showed a steady increase with the increase in cell aggregate diameter (Fig. 3.18). The high surface colour intensity and the low anthocyanin content in the larger cell aggregates indicate a steep radial gradient of anthocyanin content along the radius of the cell aggregates. Based on the measured respiration, and on an effective diffusion coefficient of 1.9 x 10^{-9} m^{-2} s^{-2} of the cell aggregates of *Tagetes patula,* Hulst *et al.* (1989) calculated that the oxygen supply would not be limiting to cell aggregates of up to 3 mm diameter. The maximum cell aggregate size in the present study was much less than 3 mm. Spier (1995) has opined that, in view of the slow growth and metabolic rates, the plant cells do not seem to suffer from any mass transfer resistances along the radius of the small cell aggregates. One plausible explanation for the observed gradient of anthocyanin in the cell aggregates is the availability of light, which is an important factor in the biosynthesis of anthocyanins and flavonoids (Zhong *et al.,* 1991; 1993a). The intensity of light decreases along the radius of the cell aggregate, with the result that the cells at the core receive less light compared to those in the outer layers. A photoprotective role of the anthocyanins, in the sub-epidermal layer of cotyledons and hypocotyls of tomato seedlings, has been considered recently as part of a mutual, negative regulation of anthocyanin accumulation at early stages of chloroplast development (Bowler and Chua, 1994). It has been speculated that anthocyanins function as green light filters in vegetative tissues (Stafford, 1994).

The present study has shown that there is a positive relationship between the anthocyanin content and the cell aggregate size, and that there exists a steep radial gradient of anthocyanin content along the radius of the cell aggregates. Light probably playing a role, a critical size of cell aggregate is necessary for maximum production

of anthocyanin.

4.6 Nutrient uptake

Quantitative information on growth kinetics, sugar utilization, respiratory requirements, biomass and product yields, yield coefficients of key nutrients is essential and forms the basis for process optimization, design, monitoring and control, and scale-up strategies. Further, these are the key parameters which form the basis of the analysis of process economics. Much can be gained in our understanding of physiology of growth process by studying the nutrient uptake kinetics.

Yield coefficients are biological variables, which are used to relate the ratio between various consumption and production rates of mass and energy. They are typically assumed to be time-independent and are calculated on an overall basis. The yield coefficients are very useful, particularly in the cases, like the *D. carota* cell line used in the present study, where the biomass composition is quite uniform, product selectively does not change much during the exponential growth (Fig. 3.11), and growth associated production (Dunn *et al.,* 1992).

Consumption of sugars

Sucrose is the most commonly used carbon source in plant cell culture media. The first metabolic step in the conversion of this disaccharide into biomass and other secondary metabolites is its hydrolysis. Majority of the sucrose is extracellularly hydrolysed into glucose and fructose by the membrane bound invertases. Invertases, especially, the acid invertases, are ionically bound to the cell wall (Leigh *et al.,* 1979; Lauriere *et al.,* 1988; Obenland *et al.,* 1993). Some amount of sucrose is also hydrolysed in the cytoplasm of cells (Ricardo and ap Rees, 1970). In both *D. carota* and *C. frutescens* cultures, the sucrose was rapidly hydrolysed at a rate of about 4.8, and 5.2 g sucrose g^{-1} dry wt. day^{-1}, respectively (Fig. 3.21). *Vitis vinifera* cells

hydrolysed the sucrose present in the liquid medium at a higher specific rate of 7.2 g sucrose g^{-1} dry wt. day (Pepin *et al.,* 1995). Though the rapid hydrolysis of sucrose is a commonly reported feature in plant cell cultures (Thorn *et al.,* 1981; Zamski and Wyse, 1985; Kanabus *et al.,* 1986), there are several cell cultures in which sucrose was hydrolysed slowly, and a fair amount of unhydrolysed sucrose was detected even at the end of the culture period (Zhong *et al.,* 1993.).

The overall consumption of glucose was 86.8 and 50.6% more compared to that of fructose in *D. carota* and *C. frutescens* cultures, respectively (Fig. 3.21). The preferential uptake of glucose is almost always reported in plant cell cultures (Fett-Neto *et al.,* 1994; Pepin *et al,* 1995; Pestchanker *et al,* 1996). However, Wickremesinhe and Arteca (1994) have reported that the growth of *Taxus* cell suspensions was faster during a period of increased fructose availability, and they suggest that this may be due to an increased entry of fructose into the cells coupled with increased fructokinase activity.

As the culture period progressed, glucose and fructose gradually and proportionately decreased. While the glucose exhausted almost completely, a fair amount of fructose, about 6 g l^{-1} , was left unutilized at the end of culture period in both the cultures of *D. carota* and *C. frutescens* cells (Fig. 3.21). While this is the common pattern of carbohydrate uptake (Panda *et al.,* 1992; van Gulik *et al,* 1993), there are cases on both the extremes of this utilization pattern. The sugar uptake of *Taxus baccata* cells in suspension cultures was initially slow and limited to only about 2 g l^{-1} during the first 10 days (Srinivasan *et al.,* 1995). These cells utilized the intracellularly stored carbohydrate carried over from the previous culture. On the other hand, substantial amount of carbohydrate was sequestered inside the cells of *Taxus cuspidata* in a Wilson-type bioreactor and redistributed through cell division (Pestchanker *et al.,* 1996).

The yield coefficients of dry biomass on carbohydrate of over 0.8 g g^{-1} were

observed in the present study, which are slightly higher than those generally reported for batch culture of plant cells for an initial carbohydrate concentration of 30 g l^{-1} (King, 1977; Dougall *et al,* 1982; van Gulik *et al.,* 1989). Ducos and Pareilleux (1986), for instance, have reported cell yields ranging from 0.39 to 0.55 g g^{-1}, depending on the aeration rate, for *Catharanthus roseus* cell cultures. On the other hand, cell yields of 0.77 g g^{-1} have been reported for *Medicago sativa* cells grown on lactose (30 g 1^{-1}) as the sole carbon source (Pareilleux and Chaubet, 1981). The slightly higher values of the apparent yield coefficient mean a low maintenance coefficient for the culture systems used in the present study. However, measurement of the actual maintenance coefficient requires mass balance analysis which necessitates the inlet and outlet gas analysis, and elemental analysis of the biomass. The apparent yield coefficient estimated over the entire culture period is often used for comparisons. However, the batch culture period of plant cell and hairy root cultures varies widely from 7-60 days. In cultures of long period, considerable amount of the carbohydrate gets converted into energy required to maintain the metabolism, and, hence, comparisons based on the apparent yield coefficients lead to considerable discrepancies.

Uptake of ions

The typical uptake pattern of NH_4^+, $N0_3^-$, and $P0_4^{-3}$ correlating well with the accumulation of biomass was observed with dry biomass yield coefficients of 0.94, 0.58, and 15.95 g $mmol^{-1}$ of NH_4^+, $N0_3^-$, and $P0_4^{-3}$, respectively, for *D. carota,* and 0.89, 0.52, and 14.79 g $mmol^{-1}$, respectively, for *C. frutescens* cells in suspension cultures (Fig. 3.22; Fig. 3.23). The uptake of ammonia was rapid compared to that of nitrate. Kwok and Doran (1995) observed a linear relationship between biomass production and consumption of nitrate, and ammonium ions, in *Atropa belladonna* hairy root cultures in MS medium. A similar linear relationship between the uptake

of $N0_3^{-1}$, $S0_4^{-2}$–, K^+ , and Ca^{+2} , and the accumulation of biomass, with the only exception of $P0_4^{-3}$, which was entirely taken up in the first 24 hours, was reported in tobacco cell cultures (Kato *et al,* 1977). This linear relationship appears to be the general pattern of ion uptake by plant cell cultures. In fact, it is this relationship which forms the basis of estimation of biomass based on conductivity of the culture media, which is one of the commonly used method of biomass estimation, especially in bioreactor cultivation of plant cells.

The present study on carbohydrate, and ion uptake revealed the patterns of their utilization and generated quantitative kinetic data.

4.7 Measurement of biomass in plant cell cultures

Medium conductivity and estimation of biomass

A linear relationship between the uptake of $N0_3^{-1}$, $S0_4^{-2}$–, K^+ , and Ca^{+2}, and the accumulation of biomass is the typical pattern of ion uptake in plant cell cultures (Kato *et al.,* 1977; Kwok and Doran, 1995). Electrical conductivity of a nutrient medium gives a measure of the ionic concentration in the medium and forms a convenient means of biomass estimation without the recourse to direct cell sampling.

Changes in the conductivity of the nutrient medium in *D. carota* and *C. frutescens* cell suspension cultures were monitored in order to estimate the relationship between the conductivity and the biomass accumulation (fig. 3.24). The analysis of medium conductivity and the biomass density showed a linear decrease in conductivity with the increase in the biomass of the both *D. carota,* and *C. frutesences* cells (Fig. 3.25).

In an early study of plant cell suspension cultures, Hahlbrock and Kuhlen (1972) reported a concomitant decrease in medium conductivity and increase in fresh weight during the growth of cell of *Petroselinum hortense* and *Glycine max.* Though the pattern of decrease in the conductivity appeared graphically to mirror that of increase

in cell concentration, the precise relationship between the two variables was not quantified. From a comprehensive study, Taya *et al.* (1989a) reported linear correlations between increase in cell dry weight and decrease in conductivity for shake-flask cultures of *Coffea arabica, Nicotina tabacum, Withania somnifera,* and *Catharanthus roseus.* The direct proportionality between changes in dry weight and conductivity was expressed in the form of the Eq. 3.1 on page 83. The values of the proportionality constant *(k)* for the above four species were -3.6, -2.8, -3.2, and -4.1 g dry wt. 1^{-1} mS^{-1} , respectively. *D. carota* and *C. frutescens* cell cultures, investigated in the present study, showed the similar linear relationship with the proportionality constant of-5.29, -5.56 g 1 mS^{-1} , respectively. There is a considerable difference in the values of the proportionality constant from species to species, and the values observed for the culture systems used in the present study are slightly higher compared to those reported by Taya *et al.* (1989a). This is obviously due the differences in the yield coefficients of biomass on different ionic compounds, and a higher value for the proportionality constant implies higher yield of biomass on ionic nutrients. While Taya *et al.* (1989b), in another study, reported a value of 2.13 g 1^{-1} mS^{-1} for *Armoracia rusticana* roots, Ryu *et al.* (1990) reported a proportionality constant of 4.6 g 1^{-1} mS^{-1} for suspension cultures of *Pyrus communis L.*

However, there are several limitations in the use of conductivity for estimation of biomass. Firstly, it is less sensitive to measure small increases in biomass as it depends only on the electrolyte concentration of the nutrient medium, and ignores the sugars which are present in more concentration than all the other nutrients put together. An investigation on the individual contribution of different media constituents showed that the sugars did not contribute positively towards the medium conductivity (Table 3.7). Instead, the presence of sugars slightly reduced the conductivity value due their characteristic lowering of ionic mobility, which was also observed earlier by Taya *et al.* (1989a). This means that the decreasing concentrations of sugar, due to the

uptake of cells, would push up the conductivity value. Thus the negative contribution of sugars towards the conductivity results in its less sensitivity to measure biomass. Further, the conductivity failed to correlate to growth of *Catharanthus roseus* hairy roots (Toivonen *et al.,* 1990), and *Medicago sativa* cell suspensions (McDonald and Jackman, 1989). This is possibly due to the non-linear relationship of ionic uptake and the growth of biomass. Kwok *et al.* (1992) studied the limitations associated with conductivity measurement for monitoring the growth in *Atropa belladonna* cell cultures and found that it also depends on the inoculum density.

Medium osmolarity as a measure of growth

Having studied the limitations on the sensitivity of conductivity for measurement of biomass, another similar property of nutrient medium, namely the osmolarity, was explored for its suitability as an index for estimation of biomass. It was found that there is a fair linear relationship between the increase in biomass and the decrease in osmolarity of the culture media of both *D. carota* and *C. frutescens* cell culture systems (Fig. 3.27).

However, there was a sharp increase in osmolarity of the culture media as a result of the hydrolysis of sucrose into glucose and fructose. The equation 3.2 presented on page 91 could relate all the variables. As all the constituents contributed towards the osmolarity of the culture medium, its measurement correlated well with the increase in biomass in both the cultures studied. In culture systems where sucrose is hydrolysed rapidly before the onset of log phase, the parameter X_{Tmax} in the equation becomes equal to the inoculum density. On the other hand, if sucrose is hydrolysed slowly, the extent of initial enhancement of osmolarity will be reduced because of the reduction in formation of glucose and fructose. The concomitant growth of cells that tend to use glucose and fructose would push down the osmolarity. This leads to less sensitivity and a more difficult calibration.

However, the rapid hydrolysis of sucrose has been reported in several cell culture systems (Thorn *et al.,* 1981; Zamski *et al.,* 1985; Kanabus *et al.,* 1986), and, hence, the method of biomass estimation based on the osmolarity of culture medium, developed from the present study, may be applicable to many plant cell cultures. Further, osmolarity is an important culture parameter in itself.

Estimation of biomass based on the turbidity of cell-free medium

Turbidimetric measurement of biomass is the most commonly used method in bacterial cultures. Sung (1976) showed that this method can be used for very fine suspension cultures of plant cells. However, turbidimetric measurements are satisfactory only in cases where the suspensions are fine, or have small cell aggregates with a narrow size distribution (Lescure, 1966; Wilson *et al.,* 1971). In most of the cases, the plant cells in suspension culture tend to aggregate and form clumps, ranging from a few cells to aggregates measuring a few centimetres in diameter. Consequently, the turbidimetric measurement of biomass of plant cells in liquid suspension cultures has been difficult. Further, since the density of the plant cell aggregates is usually higher than that of most of the liquid nutrient media, the cell aggregates sediment immediately after the mixing is stopped. Tanaka *et al.* (1992) developed a specialized spectrophotometric system - with a rotating magnetic bar inside the sample cuvette - to keep the culture fluid homogeneous during the measurement. Aoyagi *et al.* (1992) suggested the use of solutions of different densities to prevent the plant cell aggregates from sedimenting during the measurement of optical density. Kubek and Shuler (1978) developed a sonication-optical density method for estimation of plant cell biomass. However, it is very difficult to obtain a representative sample in many plant cell culture systems. Zhong *et al.* (1993b) used a laser turbidimeter for on-line measurement of biomass.

Plant cells of many species in suspension culture are known to secrete a variety

of polysaccharides, proteins, peptides, fatty acids, polymers of pectic acids, etc. (Morvan, 1982; Hale *et al,* 1987; Otsuji et al., 1994; Wangsamuth and Doran, 1994), resulting in the culture medium to becoming turbid and viscous. In the present study, the relationship between the turbidity of cell-free culture medium and the cell biomass was investigated for its possible use as an index for the estimation of biomass using *Capsicum frutescens* and *Daucus carota* cell cultures.

There was a continuous secretion of polysaccharides and proteins by both the cultures into the nutrient medium (Fig. 3.28). By the end of the log phase of growth, there was an accumulation of 1.05 and 1.2 g l^{-1} of polysaccharides, and 0.71 and 0.78 g l^{-1} of proteins in the cell-free media of *D. carota* and *C. frutescens* cultures, respectively. It is well known that the plant cells in liquid medium secrete a variety of polysaccharides (Becker *et al,* 1964; Mante and Boll, 1975; Akiyama and Kato, 1982). Otsuji *et al.* (1994) reported 4.6 g l^{-1} of extra cellular polysaccharides in *Polianthus tuberosa* cell cultures. Hale *et al.* (1987) observed that the liquid nutrient medium became highly viscous with the secretion of polysaccharides from *Phleum pratense* cells in suspension cultures. Microscopic observation of the cell-free medium showed the presence of cell debris. Bruke *et al.* (1974) found that the composition of extra cellular polysaccharides in Sycamore cell suspension cultures was very similar to that of the cell-wall polysaccharides.

As a consequence of the secretion of polysaccharides and proteins by the cells of *C. frutescens* and *D. carota,* the nutrient media gradually became turbid and viscous. Figure 3.29 shows the relationship between the increase in the turbidity of the cell-free medium and the growth of cell biomass. Recently, Glicklis et al. (1998) studied kinetics of extracellular polysaccharides in *Symphylium offlcinalis* cell cultures. Though the relationship between the extracellular polysaccharides and biomass is not linear in this culture system, the predicted values by a non-linear model correlated well with the experimental values. This definite relationship, linear or non-linear,

demonstrates the possibility of using the turbidity of cell-free medium for estimation . of biomass in plant cell suspension cultures.

The sensitivity of this method depends on the magnitude of the empirical constant *(k)* (Eq. 3.3 on page 94). A lower value for *k* means a large change in the turbidity of the cell-free medium for a relatively small increase in the biomass. Table 3.8 summarizes the effect of filter mesh size on the estimation of biomass in *D. carota* and *C. frutescens* cell suspension cultures. As it can be seen, a fine mesh (37 μm) gave a better correlation, but a higher value for the empirical proportionality constant *(k)*. On the other hand, a wider mesh (88 μm) gave a lower value for *k* but a poor degree of correlation. Microscopic examination of the cell-free medium obtained by the filter of mesh size 88 μm showed the presence of single cells at low density. The reason for a moderate correlation between the turbidity of the cell-free medium and the increase in the biomass in spite of the presence of cells may be due to the near constant size distribution of cell aggregates in both the culture systems (Mavituna and Park, 1987).

However, in view of the fact that a number of factors such as medium composition, phytohormones, aeration, and agitation influence the degree of the secretion of extra cellular compounds (Hale *et al,* 1987; Otsuji *et al,* 1994), for a given culture system an optimum size of the filter needs to be selected for the estimation of biomass based on the turbidity of cell-free culture medium.

The present study demonstrates a linear relationship between the turbidity of cell-free medium, and the increase in biomass of *D. carota* and *C. frutescens* cells with a good correlation, and suggests its possible use as an index for estimation of biomass.

4.8 Growth and production of anthocyanin in bioreactor

The mass cultivation of plant cells in bioreactors has been demonstrated long back (Tulecke and Nickell, 1960). Since then a large number of plant cell cultures

were studied using bioreactors for understanding and optimization of process parameters, and for feasibility studies (Byrne and Koch, 1962; Hahlbrock *et al.,* 1974; Smart and Fowler, 1984; Srinivasan *et al.,* 1995; Bhqme *et al,* 1997).

In the present investigation, *D. carota* cells were cultured in a stirred tank reactor of 2 L volume. As it is observed in the present study (Fig. 3.30), the growth, product formation, and nutrient uptake kinetics are usually comparable in shake-flask cultures and low volume bioreactors (Fowler, 1986). There was a total production of 2.7 g l^{-1} of anthocyanin in 14 days of culture. Kobayashi *et al.* (1993) reported a 1.09 g l^{-1} of anthocyanin from *Aralia cor data* cell cultures in a 500 L fermenter in 16 days of culture. The special feature of this cell line was that it does not require light irradiation for growth and anthocyanin formation.

The absence of the breakage of cell aggregates, and the high viability of cells observed in the present experiments in the bioreactor, demonstrate the shear tolerance of the *D. carota* cell line employed in the studies. Though it was thought earlier that plant cells are sensitive to shear stress (Mandels, 1972), the shear tolerance of plant cells was subsequently demonstrated for several species (Kato *et al.,* 1975; Noguchi *et al.,* 1977; Scragg *et al.,* 1988). *Catharanthus roseus* cell suspensions exposed to 1000 rpm in a 3 L stirred tank bioreactor, showed no loss of viability.

The observations on the yields of biomass and product, and the shear tolerance of cells in the present study are encouraging and suggest that a stirred tank reactor system may be employed for large scale cultivation of the *D. carota* cell line used in the studies. Stirred tank reactor has several advantages over many other types of bioreactors. Further, the growth-associated formation of anthocyanin, observed in the present study, eliminates the need for a two-stage culture.

Oxygen requirement of *D. carota* and *C. frutescens* cells

Oxygen is an important nutrient required for the respiration of plant cells, and

it is the least soluble among all the other compounds of the nutrient media. Plant cells require less oxygen than microorganisms because of their slow metabolism. However, little quantitative work has been published on the effects of gas regime to which the cells are exposed. Various studies show that plant cells are sensitive to dissolved oxygen concentration. High oxygen concentrations depress cell growth (Smart and Fowler, 1981), secondary metabolite accumulation (Yamakawa *et al.,* 1983), and other metabolic activities (Pareilleux and Chaubet, 1981). Growth and product formation kinetics were altered by high DO (Zenk *et al.,* 1977). High aeration rates may strip-off nutrients such as $C0_2$ from the culture broth (Maurel and Paeilleux, 1985; Ducos and Pareilleux, 1986). $C0_2$ is often considered as an essential nutrient in the culture of plant cells and has a positive effect on growth (Gathercole *et al.,* 1976; Maurel and Pareilleux, 1985). Some workers have used the low agitation and high aeration in stirred tank reactors to supply oxygen in a reasonable mixing range (Kato *et al.,* 1977; Drapeu *et al.,* 1986). The rate of oxygen transfer from gas phase to the cell site is generally expressed in terms of K_{La} (volumetric mass transfer coefficient of oxygen, in $h"^{1}$) (Aiba *et al.,* 1973). The K_{La} value depends on several factors such as the rate of agitation, aeration, the nature of the particular plant cell as well as the culture fluid, dimensions and geometry of the reactor vessel. High K_{La} means better oxygen transfer, but reported to cause poor cell growth (Tananka, 1981), and even cell death (Kato *et al.,* 1975; Pareilleux and Vinas, 1983). On the other hand, low K_{La} do not support good growth. Reports suggest that the suspension cultures of *Nicotiana tabacum* (Kato *et al.,* 1975), *Catharanthus roseus* (Smart and Fowler, 1981), and *Vitis* spp. (Yamakawa *et al.,* 1983) cells grow better at a restricted range of K_{La}.

Quantitative data on the exact requirement of oxygen, and profiles of oxygen consumption rate is essential for designing the reactor vessel, balancing agitation and aeration rates, and scale-up.

In the present investigation the oxygen requirement of *D. carota* and

C. frutescens cells was estimated (Fig. 3.32). The specific oxygen uptake rate (qo2) of *D. carota* was 0.75 mmol g^{-1} h^{-1} , while it was 0.57 mmol g h for *C. frutescens* cells in suspension cultures. This low oxygen consumption rate is within the range of 0.2 - 1.0 mmol g^{-1} h^{-1} reported for various plant cell culture systems (Drapeau *et al,* 1986; Spear, 1986; van Gulik *et al,* 1989; Snape *et al,* 1989; Taticek *et al,* 1990; Schlatmann *et al,* 1994). However, there are varied reports on the changes in oxygen demand with culture age. Pepin *et al* (1995) reported that the specific oxygen consumption rate of about 0.6 mmol g^{-1} h^{-1} remained constant, as observed in the present study, during the exponential growth of *Vitis vinifera* cells. However, q_{02} of *Taxus baccata* cells in suspension cultures increased from about 0.1 mmol g^{-1} h^{-1} after inoculation to 0.28 mmol g^{-1} h^{-1} around day 11 and decreased gradually thereafter (Srinivasan *et al,* 1995). This fluctuation in the q_{02} was attributed to the change in the specific growth rate. However, both the cell culture systems used in the present study had a fairly constant specific growth rate.

Another finding from the study was that both the cell culture systems showed the minimum critical DO concentration of about 30% of air saturation below which the respiratory rate was affected (Fig. 3.32). This is expected because the low concentration of oxygen in the bulk fluid limits the supply of oxygen to the site of its utilization. The critical DO concentration for oxygen uptake by suspended plant cells has been reported to be 16 - 20% of air saturation under average culture conditions (Kessel and Carr, 1972; Payne *et al,* 1987). The minimum critical DO concentration increases with higher aggregation of cells. Yu and Doran (1994) reported that hairy root cultures of *Atropa belladonna* needed 100% of air saturation below which the growth rate was severely affected due to the high root density. Bligny *et al* (1985) studied the effect of low oxygen concentration on Sycamore *{Acer pseudoplantanus)* in suspension cultures. They observed that at 10 μM 0_2 (approx. 4% of air saturation), the molar proportion of fatty acids dramatically changed in all lipid classes of the cell

membrane.

The present study quantifies the oxygen requirement of *D. carota* and *C. frutescens* cells in suspension cultures and shows that about 40% of air saturation would be optimum for these cells for cultivation in bioreactors. Bohme *et al.* (1997) used a DO level of 30% of air saturation for culture of *Aesculus hippocastanum* cells in a newly designed membrane stirrer reactor.

Rheological studies on *D. carota* culture broth

The measurement and control of the rheological properties of culture broth are of importance as they significantly affect heat and mass transfer, mixing behaviour of the broth, recovery and purification of products and the response of various sensors used to monitor and control of culture parameters. Hence, rheological considerations are critical in the interpretation, planning and control of basic experiments, the development of rational scale-up procedures, design and operation of monitoring and control systems, control of bioprocesses, operation and design of recovery equipment and in increasing the yield and productivity of fermentation processes (Charles, 1978).

To generate the data needed for scale-up of anthocyanin production from *D. carota* cell cultures, some rheological properties of the culture broth was studied in 2 L bioreactor.

As indicated by the near linear curve passing through the origin of the flow diagram (Fig. 3.33), the culture broth of *D. carota* was initially Newtonian in rheology with an apparent viscosity of 3 cP without any considerable yield stress. The apparent viscosity of the culture broth remained low during the first 6 days of culture, and increased rapidly after thereafter to a maximum of 18 cP on 12 day. A similar pattern of increase in viscosity was observed by Kato *et al.* (1978) for tobacco cell suspensions. At the beginning, their culture had an apparent viscosity of 1.2 cP and it rose to 33 cP (at a shear rate of 60 s^{-1}) when the biomass reached to the maximum

of 13.1 g dry wt. 1^{-1} . The effect of biomass concentration on viscosity of the culture broth was investigated by Zhong *et al.* (1992) using *Perilla frutescens* cell suspension. They found that the apparent viscosity of the culture was approximately constant when the cell concentration was below 5 g 1^{-1} , but the effect of the cell density on viscosity of the culture broth was very strong when the biomass concentration exceeded 13 g 1^{-1} . The viscosity was roughly proportional to the fourth power of the cell concentration.

The cell aggregate size in the range of <300 - 1500 µm did not show any considerable change in the flow behaviour of the culture broth (Fig. 3.33), conforming the similar observation by Zhong *et al.* (1992).

The culture broth in the late exponential stage, showed shear-thinning pseudoplastic flow behaviour with a consistency index (k) of 132 (mPa s) 0.57 , and the flow behaviour index (n) of 0.57 (Fig. 3.33). This non-Newtonian behaviour was reported for several other cell cultures, such as *Catharanthus tricuspidata* Bureu, *Nicotiana tabacum* L (Kato *et al.,* 1978; Tanaka, 1982), and *Perilla frutescens* (Zhong *et al,* 1992). The flow behaviour of *D. carota* cell culture broth observed in the present study including the absence of yield stress is qualitatively similar to that of *Nicotiana tabacum* cell suspensions (Kato *et al.,* 1978). However, the culture broth of *Perilla frutescens* exhibited the Bingham plastic flow behaviour with an yield stress of over 0.3 dyn cm^{-2} (Zhong *et al.,* 1992). Wagner and Vogelmann (1977) found that the rheological behaviour of the cell suspension of *Morinda citrifolia* (after 16 days cultivation) was rather complicated, and they observed shear thinning and thixotrophic behaviour in addition to yield stress.

The varied behaviour of culture broths of different species even at a comparable cell densities may be due to an interaction between cell aggregates at high densities (Oka, 1974), morphology, and the ratio of cell dry weight to fresh weight (Zhong *et al.,* 1992). There are only a very few reports on the detailed study of these aspects

(Tanaka, 1982), while they are extensively studied for microbial cultures (Sato, 1961; Ghildyal *et al.,* 1987).

However, the culture broths separated from cells in all the above discussed species, including *D. carota* used in the present study, were always purely Newtonian and the viscosity was not considerably high.

The present study on the rheology of *D. carota* cell culture broth in 2 L stirred tank bioreactor reveals that the culture suspension changed from Newtonian to non-Newtonian regime at high cell density.

4.9 Characterization of anthocyanins produced by *D. carota* cells

The classical paper chromatography method for separation of anthocyanins, originally developed by Harborne (1958a), was highly satisfactory and used by several workers for long time (Feluki and Francis, 1967; Feluki, 1969, Francis, 1982). He reported Rf values for most of the anthocyanins. These Rf values in combination with the spectral analysis of the extracts and the separated bands such as UV and visible absorption, the ratio of absorbance at 440 nm to that of maximum absorption (500 - 550 nm) were used to identify anthocyanins (Harborne, 1958b; 1967).

Of the different solvents used to separate anthocyanins in *D. carota* cell extract, n-Butanol-acetic acid-water (4:1:5) gave the best separation. The Rf values and the spectral data of the extract revealed the presence of at least three types of anthocyanins in the cultured cells. (Table 3.10).

Further analysis of the acid hydrolysed cell extract, by HPLC, showed a single peak in all the three paper chromatographically separated bands at the same retention time comparable to that of cyanidin. This clearly showed that the cultured cells synthesized only the cyanidin. (Fig. 3.36; Table 3.11). The method could clearly separate the six major anthocyanidins according to their polarity (in the order of: delphinidin, cyanidin, petunidin, pelargonidin, peonidin and malvidin). The *D. carota*

cells in culture used by Dougall (1980) also synthesized only the cyanidin glycosides.

Paper chromatographic analysis of the sugar moieties showed the presence of xylose, glucose and galactose (Table 3.12). Occurrence of these three sugars has been reported by Harborne (1976) in leaves, flowers, stems of *Daucus* species, and also in black carrot. Recent studies on wild carrot clones revealed the occurrence of all these sugars (Halaweish and Dougall, 1990).

The pigments separated by paper chromatography did not show any considerable absorption in the UV range of 300 - 330 nm suggesting the absence of acyl groups. The presence of non-acylated anthocyanins in the culture used in the present study is in contrast to results obtained with wild carrot, wherein, only acylated anthocyanins are found to accumulate (Hopp and Seitz, 1987; Dougall, 1989). A rare anthocyanin was isolated from mutant cells of *Daucus carota* (Vaush *et al.,* 1980) with an yield of 300 mg of purified anthocyanin per litre of cell suspension.

The above analyses suggest that the cell extract of *D. carota* contained three anthocyanins namely, cyanidin xylosyl galactose, cyanidin mono glucose and cyanidin galactose. The spectrophotometric estimations showed that above three were in the molar ratio of 5:3:2, respectively.

4.10 Studies on continuous production of capsaicin

In vitro production of capsaicinoids by immobilized cells of *Capsicum* spp. has been a subject of intense research (Lindsey *et al.,* 1983; Lindsey and Yeoman, 1984; Hau and Yeoman, 1991; Sudhakar Johnson and Ravishankar, 1996). Immobilization of biocatalysts offers several advantages (Table 1.6). Reports from our laboratory have shown that immobilized cells of *Capsicum annum* and C. *frutescens* produce capsaicinoids that leach out into the medium (Sudhakar Johnson *et al.,* 1990, 1991). Alginate immobilization is viewed as a mild process involving non-toxic components and a pH, osmolarity, and temperature suitable for preserving the viability of plant

cells. Douglas and Phillips (1997) have developed a method to produce small, monodispersed alginate beads for cell immobilization. Production of capsaicin from cells immobilized in alginate beads is governed by several factors, such as the bead strength, hormones, and elicitors. These factors have been extensively studied and optimized using a response surface methodology (Suvarnalatha *et al.,* 1993).

In the present study, the volumetric productivity of capsaicin has been studied using a bubble column reactor of 1 L volume. Under the conditions employed, the maximum productivity of capsaicin was about 2.5 mg 1 day^{-1} (Fig. 3.37). The total capsaicin produced was 35 mg 1^{-1} over a period of three weeks. The gradual softening and collapse of alginate beads after three weeks of continuous operation was inevitable.

In another experiment, the immobilization of cells in polyurethane foam (PUF) was studied. A high cell loading of 0.37 g fresh cells cm of PUF was achieved with 14 days of incubation (Fig. 3.39). The profile of cell loading into the PUF was qualitatively similar to the growth of cells in liquid medium (Fig. 3.21). In a similar study, Mavituna and Park (1985) observed that while the overall cell loading pattern was similar to that of growth pattern, the specific growth rate of cells inside the foam was slightly less compared to that in liquid medium. In a detailed study, Rhodes *et al.* (1987) investigated the factors affecting the immobilization of plant cells in polyurethane foam using three plant species. They found that the inoculum size and the length of the incubation period have a bearing on the loading of cells into the reticulate foam.

Continuous production of capsaicin from the cells immobilized in PUF was studied using a packed-bed reactor. Interestingly, the specific productivity of capsaicin with both the alginate and PUF matrix was same which was about 25 mg g^{-1} fresh cells day^{-1} (Fig. 3.40). However, by virtue of the high cell density, the volumetric productivity in the packed-bed reactor was about 4 fold high compared to that in the

bubble column reactor. Lindsey *et al.* (1983) found that the capsaicin production was increased by two fold after the immobilization of *C. frutescens* cells in the pores of PUF. In a circulating bed reactor, Mavituna *et al.* (1987) studied the capsaicin production from the cells immobilized in PUF. They found that the DO concentration between 0-20% of saturation level resulted in the appearance of capsaicin in the medium.

The influence of various fungal and bacterial elicitors on capsaicin production was extensively studied (Sudhakar Johnson *et al.,* 1991). In the present study, productivity of capsaicin from the packed-bed reactor under the influence of *Aspergillus niger* derived elicitor was studied. There was about 60% increase in the total production of capsaicin resulting in a maximum production of 330 mg 1^{-1} in a period of four weeks (Table 3.13).

SUMMARY & CONCLUSION

Plant cell culture is now undoubtedly a mature technology for the production of high value phytochemicals *in vitro.* The successful large-scale production of shikonin with cultured plant cells of *Lithospermum erythrorhizon* has demonstrated the technological feasibility of industrial scale culture of dispersed plant cells. This has led to the exploration of several plant cell culture based production systems. Though over a dozen of cell culture based processes are on the verge of commercialization, production of a large number of phytochemicals from plant cell cultures is still uneconomical. Primarily, low volumetric productivity, and difficulties in scale-up are the two major bottle necks in industrial application of this technology. However, the better understanding of *in vitro* physiology of plant secondary metabolism, and the recent advancements in bioreactor designs and improved bioprocessing, there have been augmented efforts to develop plant cell culture based processes.

The present investigation dealt with the studies on production of anthocyanin from *Daucus carota* cell cultures, and capsaicin production from immobilized cells of *Capsicum frutescens* at shake flask, and lab-scale reactor level. The highlights of the results are as follows.

Cell cultures of *Daucus carota,* and *Capsicum frutescens* were established and maintained by periodic subculture.

A high yielding cell line of *D. carota* was developed by repetitive selection for high anthocyanin containing cell clusters. The cell line had an average anthocyanin content of 15.88% (w/w, on dry basis) and a specific growth rate is 0.217 day^{-1} .

In order to quantify and understand the biological effects of physico-chemical properties, osmolarity, conductivity, buffer capacity, and the solubility of oxygen of twelve commonly used plant cell culture media have been investigated. While the solubility of oxygen in the various cell culture media studied varied only narrowly from 7.01 - 7.79 mg l^{-1}, osmolarity, conductivity, and buffer capacity showed considerable differences. The data generated would be useful in rational selection of basal media for plant cell and tissue culture, and in bioprocessing.

Nutritional requirements of the *D. carota* cell line were studied. Murashige and Skoog's (1962) basal medium with 3% (w/v) sucrose supplemented with indole-3-acetic acid (2 mg 1^{-1}) and kinetin (0.2 mg 1^{-1}) was found to be the optimum for the maximum production of anthocyanin.

Other culture conditions such as pH, temperature, inoculum density, and the dissolved oxygen concentration were studied. The optimum values of these parameters for maximum production of anthocyanin were worked out.

The growth and product formation kinetics of the *D. carota* cell line were studied in detail. The growth of cell biomass followed a typical sigmoidal curve, with a maximum cell density of 18.9 g dry weight 1^{-1}. The anthocyanin formation was associated with the growth of biomass with an average content of 15.9% (w/w, on dry basis). There was a total production of 3.1 g 1^{-1} in a period of 15 days of batch culture under the optimum conditions.

The size distribution of cell aggregates of *D. carota* in suspension cultures was studied in detail. The size of the cell aggregates spanned from <355 to >1500 μm in diameter with biomass distribution centred at between 500-850 μm. The size distribution of cell aggregates remained unchanged during the culture period.

An interesting profile of anthocyanin content, and the surface colour intensity in different size groups of cell aggregates revealed that there is a steep radial gradient of anthocyanin along the radius of the large cell aggregates. The light shielding effect by the outer layers of cells was implicated to be the plausible reason for the observed gradient of anthocyanin.

Kinetics of sugar utilization, and ion uptake were studied in detail in both the *D. carota* and *C. frutescens* cell culture systems. The specific rates of sucrose hydrolysis, consumption rates and biomass yield coefficients of sugars, ammonia, nitrate, and phosphate were estimated. The quantitative data obtained from the study

can form the basis for further studies on reactor design, monitoring and control, and scale-up strategies.

Estimation of biomass based on the conductivity of nutrient media in *D. carota* and *C. frutescens* was studied in detail. A linear relationship between the decrease in conductivity and the increase in biomass accumulation was observed. The values of the empirical constants were estimated.

The suitability of osmolarity as an index for estimation of biomass was shown. A linear relationship between the decrease in osmolarity and the increase in biomass of *D. carota* and *C. frutescens* has been reported. This established a new sensitive index for estimation of biomass in plant cell cultures. The detailed analysis of sensitivity of the method was carried out and a comparison between the conductivity and osmolarity was drawn.

Both *D. carota* and *C. frutescens* cell cultures secreted polysaccharides and proteins into the culture medium. The time course of extracellular polysaccharides and proteins was studied. The resulting turbidity was found to be in linear relationship with biomass. The study suggested that the turbidity of cell-free culture medium could be an yet another parameter for estimation of cell biomass in plant cell culture systems in which a considerable amount of polysaccharides and proteins are secreted.

The production of anthocyanin from *D. carota* cell cultures was studied at 2 L level using a stirred tank bioreactor. Growth and production were nearly comparable to those observed with shake-flask cultures. The total anthocyanin production was 2.7 g l^{-1} in 14 days of batch culture. The results obtained in the lab-scale bioreactor were indicative of the suitability of the conventional stirred tank bioreactor for large-scale cultivation of this high yielding cell line.

The specific oxygen consumption rates of *D. carota* and *C. frutescens* cells were

estimated to be 0.75, and 0.57 mmol g^{-1} h^{-1} , respectively. The minimum critical oxygen level for these cell cultures was about 30% of air saturation.

The rheological characteristics of the *D. carota* culture broth in the stirred tank bioreactor were studied. It was found that the culture broth changes from Newtonian to non-Newtonian regime at about 12th day in culture. The culture broth was found to obey the Power-law flow model. All the parameters of the model were estimated.

Preliminary studies on the characterization of anthocyanins produced by the cell culture of *D. carota* revealed that cyanidin is the major aglycon moiety which is glycosilated to glucose, and galactose. The results were indicative of the absence of acyl groups.

Continuous production of capsaicin from *Capsicum frutescens* cells immobilized in alginate matrix was studied using 1 litre bubble column reactor. With a biomass density of 100 g fresh cells l^{-1} , there was an average volumetric productivity of 1.7 mg 1^{-1} day^{-1} . The total capsaicin production was 35 mg 1^{-1} over a period of 21 days.

The suitability of polyurethane foam (PUF) as an immobilization matrix for C. *frutescens* cells was studied. A high cell loading of 0.37 g fresh cells cm^{-3} of PUF was achieved.

Continuous production of capsaicin from *C. frutescens* cells immobilized in PUF was studied using a 2 litre packed-bed reactor. Though the specific productivity of capsaicin (25 µg g^{-1} fresh cells day^{-1}) in the packed-bed reactor was comparable to that in the bubble column reactor, by virtue of the high cell density, there was about 4 fold increase in the volumetric productivity. Under the influence of a fungal elicitor, the capsaicin production was increased to a maximum of 330 mg 1^{-1} in a period of four weeks.

Further work is needed for commercial realization of the above processes. The future studies may include the following aspects.

- Scale-up of *D. carota* cell cultures in bioreactors. The conventional stirred tank bioreactor might be used. A constant DO level may be used as the criterion for the scale-up, while ensuring the adequate bulk mixing.

- Scale-up of capsaicin production from *C. frutescens* cells immobilized in polyurethane foam.

- Downstream processing for food-grade quality of the above products to be developed. *In situ* extraction, particularly in the case of capsaicin production, may be considered using polymeric adsorbents.

As the above aspects are multidisciplinary in nature, interdisciplinary efforts are needed to develop the commercial-scale processes for the production of anthocyanin and capsaicin.

LITERATURE CITED

Aiba, S., Humphrey, A. E. and Millis,N. F. 1973. *Biochemical Engineering* Academic Press: San Diego.

Akiyama, Y. and Kato, K. 1982. Methylation analysis of extracellular polysaccharides from suspension cultured cells of *Nicotiana tabacum. Phytochemistry* 21: 1325-1329.

Alfermann, A. W., Bergmann, W., Figur, C., Helmobold, U., Schwantag, D., Schuller, Land Reinhard, E. 1983. Biotransformation of B-methyldigitoxin to B-methyldigoxin by cell cultures of *Digitalis lanata.* In: *Plant Biotechnology* (S. H. Mantell and Smith, H. Eds.) Cambridge University Press: Cambridge, pp 67-74.

Ambid, C. and Fallot, J. 1987. Influence of external pH on alkaloid production and excretion by *Catharanthus roseus* resting cell suspensions. *NATO ASI Ser. A.* **134:** 529-533.

Amrhein, N., Dues, B., Gelirke, P. and Steinrucken, H. C. 1980. The site of the inhibition of the shikimate pathway by glyphosate II. Interference of glyphosate with chansmate formation *in vivo* and *in vitro. Plant Physiol.* 61: 830-834.

Anderson, W. C. 1978. Tissue culture propagation of rhododendrons. *In vitro* **14:** 334-335.

Aoyagi, H., Yokoi, H. and Tanaka, H. 1992. Measurement of fresh and dry densities of suspended plant cells and estimation of their water content. *J. Ferment. Bioeng.* 73: 490-496.

Balandrin, M. F. and Klocke, J. A. 1988. Medicinal, aromatic and industrial materials from plants. In: *Biotechnology in Agriculture and Forestry* (Y. P. S. Bajaj Ed.) Springer-Verlag: Berlin. Vol. 4. pp 3-36.

Banerjee, S., Zehra, M., Kukreja, A. K. and Sushil Kumar 1995. Hairy roots in medicinal plants. *Curr. Res. Med. Aromatic Plants* 17: (in press).

Barz, W., Beimen, A.,Drager, B., Jaques, U. Otto, Ch. Super, E. and Upmeier, B. 1990. Turnover and storage of secondary products in cell cultures. In: *Secondary Products from Plant Tissue Culture* (B. V. Charlwood and M. J. C. Rhodes Eds.) Oxford University Press: New York, pp 79-102.

Bauch, H. J. and Leistner, 1978. Aromatic metabolites in cell suspension cultures of *Galium mullugo* L. *Planta Med.* **33:** 105-123.

Becker, G. E., Hui, P. A. and Albersheim, P. 1964. Synthesis of extracellular polysaccharide by suspensions *of Acer pseudoplatanus* cells. *Plant Physiol.* 39: 913-920.

Benthorpe, D. and Brown, G. D. 1990. Growth and secondary metabolism in cell cultures of Tanacetum, Mentha and Anethum species in buffered media. *Plant Sci.* 67: 107-113.

Berlin, J., Forche, E., Wray, V., Hammer, J. and Hosel, W. 1983. Formation of benzophenanthridine alkaloids by suspension cultures of *Eschscholtzia californica. Z. Naturforsch.* 38: 346-352.

Berlin, J. 1984. Plant cell cultures - a future source of natural products. *Endeavour* 8: 5-8.

Bhattacharya, S. and Bhat, K. K. 1997. Steady shear rheology of rice-blackgram suspensions and suitability of rheological models. *J. Food Eng.* 32: 241-250.

Birch, J. R., Lambert, K., Thompson, P. W. Kenny, A. C. and Wood, L. A. 1987. Antibody Production with AirliftFermentors. In: *Large Scale Cell Culture Technology* (B. K. Lydersen Ed.) Hanser Publishers: Munich, pp 1-20.

Blanch, H. W. and Clark, D. S. 1996. *Biochemical Engineering.* Marcel Dekker Inc.: New York.

Bligny, R., Rebeille, F. and Douce, R. 1985. 0_2-triggered changes of membrane fatty acid composition have no effect on Arrhenius discontinuities of respiration in Sycamore *(Acer pseudoplantanus* L.) cells. /. *Biol. Chem.* **260:** 9166-9170.

Bohme, C, Schroder, M. B. Jung-Heiliger, H. and Lehmann, J. 1997. Plant cell suspension culture in a bench-scale fermenter with a newly designed membrane stirrer for bubble-free aeration. *Appl. Microbiol. Biotechnol.* 48: 149-154.

Bowler, C. and Chua, N.M. 1994. Emerging themes of plant signal transduction. *Plant Cell* 6, 1529-1541.

Brain, K. R. 1976. Accumulation of L-DOPA in cultures from *Mucuna pruriens. Plant Sci. Lett.* 7: 157-161.

Branen, A. L. 1990. Introduction to food additives. In: *Food additives* (A. L. Branen, P. M.Davidson and S. Salminen Eds.) Marcel Dekker Inc.: New York, pp 1-8.

Breuling, M., Alfermann, A. W. and Reinhard, E. 1985. Cultivation of cell cultures of *Berbiris wilsonae* in 20 L airlift bioreactors. *Plant Cell Rep.* 4: 220-223.

Brodelius, P. 1988. Immobilized plant cells as source of biochemicals *la: Bioreactor Immobilized Enzymes and Cells* (M. Moo-Young Ed.) Elsevier Essex: pp 167-196.

Bruke, D., Kaufman, P., McNeil, M. and Albersheim, P. 1974. The structure of plant cell walls. IV. A survey of the walls of suspension-cultured monocots. *Plant Physiol.* 54: 109-115.

Buitelaar, R. M. and Tramper, J. 1992. Strategies to improve the production of secondary metabolites with plant cell cultures. A literature review. *J. Biotechnol.* 23, 111-141.

Butenko, R. G., Lipsky, A. K., Chernyak, N. D. and Arya H. C. 1984. Changes in culture medium pH by cell suspension cultures of *Dioscorea deltoidea. Plant Sci. Lett.* **35:** 207-212.

Byrne, A. F. and Koch, M. B. 1962. Food production by submerged cultures plant tissue cells. *Science* **135:** 215-226.

Charles, M. 1978. Technical aspects of the rheological properties of microbial cultures. *Adv. Biochem. Eng.* 8: 1-59.

Charlwood, B. V. and Rhodes, M.J. C. 1990. *Secondary Products from Plant Tissue Culture* Clarendon: Oxford.

Chen, P. S.,Toribara, T.Y.and Warner, H. 1956. Microdetermination of phosphorous. *Anal. Chem.* 28: 1756-1758.

Constabel, F., Rambold, S. Chatson, K. B., Kurz, W. G. W. and Kutney, J. P. 1981. Alkaloid production in *Catharanthus roseus* (L.) G. Don. VI. Variation in alkaloid spectra in cell lines derived from one single leaf. *Plant Cell Rep.* 1: 3-5.

Courtois, D.and Guren, J. 1980. Temperature response of *Catharanthus roseus* cells cultivated in liquid medium. *Plant Sci. Lett.* 17: 473-482.

Curtin, E. 1983. Harvesting profitable products from plant tissue culture. *Bio/Technology* 1: 649-657.

Dalton, C. C. 1978. The culture of plant cells in fermenters. *Heliosynthase of Acquaculture seminar de Martiques.* CNRS. pp 1-11.

De-Eknamkul, W. and Ellis, B. E. 1985. Effect of auxins and cytokinins on growth and rosmarinic acid formation in cell suspension cultures of *Anchusa officinalis. Plant Cell Rep.* 4: 50-53.

de Gunst, M. C. M.,Harkes, P. A. A.,Val, J., van Zwet, W. R. and Libbenga, K. R. 1990. Modelling the growth of batch culture of plant cells: a corpuscular approach. *Enzyme Microbial Technol.* 12: 61-71.

De Klerk-Kiebert, Y.H. and Van der Plas, L.H.W. 1985. Relationship of respiratory pathways in soyabean cell suspensions to growth of the cells at various glucose concentrations. *Plant Cell Tiss. Org. Cult.* 4: 225-233.

Deus, S. N. B. and Zenk, M. H. 1982. Exploitation of plant cells for the production of alkaloids in *Catharanthus roseus* cell suspension cultures. *Planta Med.* 50:427-431.

Deus-Neumann, B. and Zenk, M. H. 1984. *Planta Med.* 54: 427-431.

Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical Laboratory Procedures 1972. Difco Laboratories, Inc.: Michigan.

Dixon, R. A. 1985. Isolation and maintenance of callus and cell suspension cultures. In: *Plant Cell Culture* (R. A. Dixon Ed.) IRL Press: Oxford, pp 1-20.

Dodds, J. H.and Roberts, L.W. 1985. *Plant Cell Culture* Cambridge University Press: Cambridge.

Doran, P. M. 1993. Design of bioreactors for plant cells and organs. *Adv. Biochem. Eng. Biotechnol.* 48: 115-168.

Dornenburg, H. and Knorr, D. 1995. Strategies for the improvement of secondary metabolite production in plant cell cultures. *Enzyme Microbial Technol.* 17:674-684.

Dornenburg, H. and Knorr, D. 1997. Challenges and opportunities for metabolite production from plant cell and tissue cultures. *Food Technol.* 51(11): 47-54.

Dougall, D.K. 1980. Nutrition and metabolism. In: *Plant tissue culture as a source of biochemicals* (E. J. Staba Ed.) CRC Press: Boca Raton, pp 21-58.

Dougall, D. K.,LaBrake, S. and Whitten, G. H. 1982. Plant cells grown inchemostats. In: *Plant Tissue Culture Proceedings of the Fifth International Congress of Plant Tissue and Cell Culture* (A. Fujiwara Ed.) Japanese Association for Plant Tissue Culture. Tokyo, pp 51-54.

Dougall, D. K. 1989. Sinapic acid stimulation of anthocyanin accumulation in carrot cell cultures. *Plant Sci.* 60: 259-262.

Douglas, B. S. and Phillips, J. A. 1997. Production of small, monodispersed alginate beads for cell immobilization. *Biotechnol. Prog.* 13: 562-568.

Drapeau, D., Blanch, H. W. and Wilke, C. R. 1986. Growth kinetics of *Dioscorea deltoidea* and *Catharanthus roseus* in batch culture. *Biotechnol. Bioeng.* 28: 1555-1563.

Dubois, M., Gillies, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28: 350-356.

Ducos, J. P. and Pareilleux, A. 1986. Effect of aeration rate and influence $pC0_2$ in lage-scale cultures of *Catharanthus roseus* cells. *Appl. Microbiol. Biotechnol.* 25:101-105.

Dunn, I. J., Heinzle, E., Ingham, J. and Prenosil, J. E. 1992. *Biological Reaction Engineering* VCH Publishers, Inc.: New York, pp 9-50.

Ebel, J., Schaller-Hekeler, B., Knoblock, K. H., Wellmann, E., Grisebach, H. and Hahlbrock, K. 1974. Coordinated changes in enzyme activities of phenylpropanoid metabolism during the growth of soybean cell suspension cultures. *Biochem. Biophys. Ada* 362: 417-424.

Ellis, B. E. 1980. Hoppe-Seyler's Z. *Physiol. Chem.* 361: 242.

Endress, R. 1994. *Plant Cell Biotechnology.* Springer-Verlag: Berlin, pp 121-255.

Eriksson, T. 1965. Studies on the growth requirements and growth measurements of *Happlopapus gracilis. Physiol. Plant.* 18: 976-993.

Feluki, T. and Francis, F. J. 1967. A new developing solvent for paper chromatography of various phenolic compounds, sugars and amino acids. *J. Chromatog.* 26: 404-411.

Feluki, T. 1969. Anthocyanins of Strawberry, Rhubarb, Radish and Onion. *J. Food Sci.* 34: 365-369.

Fett-Neto, A.G., Zhang, W.Y.DiCosmo, F. 1994. Kinetics of taxol production, growth, and nutrient uptake in cell suspensions of *Taxus cuspídata. Biotechnol. Bioeng.* 44: 205-210.

Fowler, M.W. 1986. In: *Plant Cell Culture Technology* (M. M. Yeoman Ed.) Blackwell Scientific: Cambridge, pp 202-207.

Fowler, M. W. 1987. Process systems and approaches for large scale plant cell culture. In: *Plant Tissue and Cell Culture* (C. E. Green, D. A. Somers, W. P. Hackett and D. D. Biesboer Eds.) Liss: New York, pp 459-471.

Fowler, M. W. and Scragg, A. H. 1988. Natural products from higher plants and plant cell culture. In: *Plant Cell Biotechnology* (M.S. S. Pais, F. Mavituna and J. M. Novais Eds.) NATO ASI Series. Springer-Verlag: Berlin, vol. 18 pp 165- 177.

Fowler, M. W. and Stafford, A. M. 1992. Plant cell culture, process systems and product synthesis. In: *Plant Biotechnology* (M. W. Fowler, G. S. Warren) Pergamon Press: Oxford, pp 79-98.

Francis, F. J. 1982. Analysis of anthocyanins. In: *Anthocyanins as food colours* (P. M. Markakis Ed.) Academic Press: New York, pp 181-207.

Francis, F. J. 1989. Food colorants: Anthocyanins. *Crit. Rev. Food Sci. Nutr.* 28: 273-314.

Fujino, K. Koga, N. Matsushita, T. and Funatsu, K. 1992. B-carotene production by DO-stat semi-continuous culture of carrot cells in an air-lift column with draft tube. In: *Biochemical Engineering for 2001* (S. Furusaki, I. Endo, R. Matsumo Eds.) Springer-Verlag. Hong Kong, pp 274-276.

Fujita, Y.,Hara, Y., Ogino, T., Suga, C. and Morimoto, T. M. 1981. Production of shikonin derivatives by cell suspension cultures of *Lithospermum erythrorhizon.* II. A new medium for the production of shikonin derivatives. *Plant Cell Rep.* 1: 61-63.

Fujita, Y. and Hara, Y. 1985. The effective production of shikonin by cultures with an increased cell population. *Agric. Biol. Chem.* 49: 2071-2075.

Fukui, H.,Nakagawa, K. Tsuda, S. and Tabata, M. 1983. In: *Plant Tissue Culture* (A. Fujiwara Ed.) Japanese Association for Plant Tissue Culture Association: Tokyo, pp 313-314.

Furuya, T.,Kojinma, H. and Syono, K. 1971. Regulation of nicotine biosynthesis by auxins in tobacco callus tissues. *Phytochemistry* 10: 1529-1532.

Furuya, T. and Ishii, T. 1972. The manufacturer of Panax plant tissue culture containing crude saponins and crude sapogenins which are identical with those of natural Panax roots. *Japanese patent.* 48-31917.

Furuya, T. Yoshikawa, T., Orihara, Y. and Oda, H. 1984. Studies on the culture conditions for *Panax ginseng* in jar fermenters. /. *Nat. Prod.* 47: 70-75.

Gabelman, A. 1994. Genetic engineering and other advanced technologies. In: *Bioprocess Production of Flavour, Fragrance and Color Ingredients* (A. Gabelman Ed.) John Wiley & Sons Inc.: New York, pp 227-230.

Gal, I. E. 1965. Neuere angaben uber capsicdin. *Experientia* 21: 383.

Gamborg, O. L.,Miller,R. A. and Ojima, K. 1968. Nutrient requirements of suspension cultures of soyabean root cells. *Exp. Cell Res.* 50: 151- 158.

Gamborg, O. L., Constabel, F., La Rue, T. A. G., Miller, R. A. and Steck, W. 1971. The influence of hormones on secondary metabolite formation in plant cell cultures. In: *Les cultures de Tissue de Plantes* Centre National de La Recherche Scientifique: Paris, pp 335-344.

Gana, J. A.,Sharma, G. C., Zipf, A., Saha, S., Roberts, J. and Wesenburg, D. M. 1995. Genotypic effects on plant regeneration in callus and suspension cultures of *Avena. Plant Cell Tiss. Org. Cult.* 40: 217-224.

Gathercole, R. W. E., Mansfield, K. J. and Street, H. E. 1976. Carbon dioxide as an essential requirement for cultured Sycamore cells. *Physiol. Plant.* 37: 213-217.

Gautheret, R. J. 1939. Sur la possibilite de realiser la culture indefinee de tissus de tubercules de carotte. *C. R. Acad. Sci.* **208:** 118-130.

George, E. F., Puttock, D.J. M. and George, H.J. 1987. *Plant Tissue Culture Media.* Exegetics Ltd.: Westbury.

George, P. S. and Ravishankar, G. A. 1995. Induction ofcrocin and crocetins in callus cultures of *Gardenia jasminoides* Ellis. *Food Biotechnol.* 9:29-38.

Ghildyal, N. P., Thakur, M. S., Srikanta, S., Jaleel, S. A.,Prapulla, S. G.,Prasad, M. S. Devi, P. N. and Lonsane, B. K. 1987. Rheological studies on *Streptomyces fradiae* ScF-5 in submerged fermentation. /. *Chem. Technol. Biotechnol.* 38: 221-234.

Glicklis, R., Mills, D., Sitton, D., Stortelder, W. and Merchuk, J. C. 1998. Polysaccharide production by plant cells in suspension: Experiments and mathematical modelling. *Biotechnol. Bioeng.* 57: 732-740.

Govindarajan, V. S. and Ananthakrishna, S. M. 1974. Paper chromatographic determination of capsaicin. *The Flavour Ind.* 5: 176-178.

Greenberg, A. E., Clesceri, L. S., Eaton, A. D. (Eds.) 1992. In: *Standard Methods for the examination of water and wastewater.* American Public Health Association: Washington, DC.

Gresshoff, P. M. and Doy, C. H. 1974. Transgenosis of bacterial genes from *E. coli* to cultures of haploid *Lycopersion esculentum* and haploid *Arabidopsis thaliana* plant cells. Z. *Pflanzenphysiol.* 73: 132-138.

Gutsu, E. V.,Baleshova, N. N., Lazur'evskii, G. V. and Timina, O. O. 1982. Izv. Akad. Nauk, Mold. SSR. Ser. Biol. Khim. Nauk. 24.

Haberlandt, G. 1902. Culturversuche mit isolierten Pflanzenzellen. *Sitz Akad. Wiss. Wien.* **Ill:** 69-92.

Hahlbrock, K., Sutter, A., Wellmann, E., Ortmann, R. and Grisebach, H. 1971. Relationship between organ development and activity of enzymes involved in flavone glycoside biosynthesis in young parsley plants. *Phytochemistry* **10:** 109-116.

Hahlbrock, K.,Kuhlen, E. 1972. Relationship between growth of parsley and soybean cells in suspension cultures and changes in the conductivity of the culture medium. *Planta* **108:** 271-178.

Hahlbrock, K.,Ebel, J. and Oaks, A. 1974. Determination of specific growth stages of plant cell cultures by monitoring conductivity changes in the medium. *Planta* **118:** 75-84.

Halaweish, F. T. and Dougall, D. K. 1990. Sinapoyl glucose synthesis by extracts of wild carrot cell cultures. *Plant Sci.* 71: 179-184.

Hale, A. D., Pollock, C. J. and Dalton, S. J. 1987. Polysaccharide production in liquid cell suspension cultures of *Phleum pratense* L. *Plant Cell Rep.* 6: 435-438.

Hamill, J. D.,Parr, A.J. Rhodes, M.J. C, Robins, R. J. and Walton, N.J. 1987. New routes to plant secondary products. *Biotechnol.* 5: 800-804.

Harborne, J. B. 1958a. The chromatographic identification of the anthocyanin pigments. *J. chromatog.* 1: 473-488.

Harborne, J. B. 1958b. Plant polyphenols. 1. Anthocyanin production in the cultivated potato. *Biochem. J.* 74: 262-269.

Harborne, J. B. 1967. Comparative biochemistry of the flavanoids. In: *Chemistry and Biochemistry of Plant Pigments* Academic Press: New York.

Harborne, J. B. 1976. In: Chemistry and Biochemistry of Plant Pigments (T. H. Goodwin Ed.) Academic Press: New York, Vol. I. pp 736-778.

Harborne, J. B. 1982. *Introduction to ecological biochemistry* Academic Press: New York.

Hau, R. D. and Yeoman, M. M. 1991. The influence of intracellular pools of phenylalanine derivatives upon the synthesis of capsaicin by immobilized cell cultures of the chilli pepper *Capsicum frutescens. Planta* **185:** 72-80.

Havkin-Frenkel, D., Podstolski, A. and Knorr, D. 1996. Effect of light on vanillin formation by *in vitro* cultures of *Vanilla planifolia. Plant Cell Tiss. Org. Cult.* 45: 133-136.

Herlich, K. 1990. In: *Official Methods of Analysis of the Association of Analytical Chemists* (Association of Official Analytical Chemists) William Byrd Press, Inc.: Virginia, pp 1014-1015.

Hildebrandt, A.C. 1977. Single cell cultures, protoplasts and plant viruses. In: *Plant Cell Tissue and Organ Culture* (J. Reinert and Y. P. S. Bajaj Eds.) Springer Verlag: Berlin, pp 581-597.

Hilton, M. G. and Rhodes, M.J. C. 1990. Growth and hyoscyamine production of hairy root cultures of *Datura stramonium* in a modified stirred tank reactor. *Appl. Microbiol. Biotechnol.* 33: 132-138.

Hirasuna, T.J.,Shuler, M. L.,Lackney, V.K. and Spanswick, R. M. 1991. Enhanced anthocyanin production in grape cell cultures. *Plant Sci.* 78: 107-120.

Hirasuna, T. J., Pestchanker, L. J., Srinivasan, V. and Shuler, M. L. 1996. Taxol production in supension cultures *Taxus baccata. Plant Cell Tíss. Org. Cult.* 44: 95-102.

Hoagland, D. R. and Arnon, D. I. 1950. Circular 347, California Agr. Exp. Stat. Berkely.

Hoekstra, S. S., Harkes, P. A. A. and Libbenga, K. R. 1988. Screening of calli and compact globular structures of *Cinchona ledgeriana* for production of quinone alkaloids. In: *Manipulating Secondary Metabolism in Culture* (R. J. Robins and M. J. C. Rhodes Eds.) University Press: Cambridge, pp 96.

Hooker, B. S., Lee, J. M. and An, G. 1990. Cultivation of plant cells in a stirred vessel: Effect of impeller design. *Biotechnol. Bioeng.* 35: 296-304.

Hopp, W. and Seitz, H. U. 1987. The uptake of acylated anthocyanin into isolated vacuoles from a cell suspension culture of *Daucus carota. Planta,* 170: 74-78.

Hulst, A. C. and Tramper, J. 1989. Immobilized plant cells: a literature survey. *Enzyme Microbial Technol.* 11: 546-558.

Hulst, A. C, Meyer, M.M. T.,Breteler, H. and Tramper, J. 1989. Effect of aggregate size in cell cultures of *Tagetes patula* on thiophene production and cell growth. *Appl. Microbiol. Biotechnol.* 30: 18-25.

Husemann, W., Amino, S., Fischer, K., Herzbeck, H. and Callis, R. 1990. Light dependent growth and differentiation processes in photoautotropic cell culture. In: *Progress in plant cellular and molecular biology* (H. J. J. Nijkamp, L. H. W. Van der Plas and Van AartrijkEds.) Kluwer Academic Publishers: Dortrecht Netherlands, pp 373-378.

Ikeda, T.,Matsumoto, T. and Noguchi, M. 1976. Formation ofubiquinone by tobacco plant cells in suspension culture. *Phy to chemistry* 15: 568-569.

Ilker, R. 1987. *In vitro* pigment production: An alternative to color synthesis. *Food Technol.* 41: 70-72.

Jala, M. A. F. and Collin, H. A. 1979. Secondary metabolism in tissue cultures of *Theobroma cocoa. New Phytol.* 83: 343-349.

Jones, A. and Veliky, I. 1981. Effect of medium constituents on the viability of immobilized plant cells. *Can. J. Bot.* 59: 2095-2101.

Kanabus, J., Bressan, M. R. and Hodges, T. K. 1986. Carbon assimilation in carrot cells in liquid culture. *Plant Physiol.* 82: 363-368.

Kargi, F. and Rosenberg, M.Z.1987. Plant cell bioreactors: present status and future trends. *Biotechnol. Prog.* 3: 1-8.

Kato, A.,Shimizu, Y.and Nagai, S. 1975. Effect of initial K_{La} on the growth of tobacco cells in batch culture. *J. Ferment. Technol.* 53: 744-751.

Kato, A., Hashimoto, Y.,Soh, Y. 1976. Effect of temperature on the growth of tobacco cells. *J. Ferment. Technol.* 54: 754-757.

Kato, A.,Fukasawa, A.,Shimizu, Y.,Soy, Y.and Nagai, S. 1977. Requirements of $P0_4^{3}$", $N0_3$", $S0_4$ ", K^+,and Ca^+ for the growth of tobacco cells in suspension culture. /. *Ferment. Technol.* 55: 207-212.

Kato, A.,Kawagoe, S. and Soy, Y. 1978. Viscosity of the broth of tobacco cells in suspension cultures. /. *Ferment. Technol.* 56: 224-228.

Kavi Kishore, P. B. 1987. Energy and osmotic requirements for high frequency regeneration of rice plants from long term cultures. *Plant Sci.* 48: 189-194.

Kessel, R. H. J. and Carr, A. H. 1972. The effect of dissolved oxygen concentration on growth and differentiation of carrot *(Daucus carota)* tissue. *J. Exp. Bot.* 23: 996-1007.

King, P. J., Cox, B.J., Fowler, M. W. and Street, H. E. 1974. Metabolic events in synchronized cell cultures of *Acer pseudoplantanus.* L. *Planta 111:* 109-122.

King P. J. 1977. Studies on the growth in culture of plant cells XXII. Growth limitation by nitrate and glucose in chemostat cultures of *Acer pseudoplantanus* L./. *Exp. Bot.* 28: 142-155.

Kinnersley, A. M. and Dougall, D. K. 1980. Increase in anthocyanin yield from wild carrot cell cultures by a selection based on cell-aggregate size. *Planta* 149: 200-204.

Kirkham, M.B. and Holder, P. L. 1981. Water osmotic and turgor potentials ofkinetin treated callus. *Hortic. Sci.* 16: 306-307.

Klein, R. M. and Manos, G. E. 1960. Use of metal chelates for plant tissue cultures. *Ann. New York Acad. Sci.* 88: 416-425.

Knorr, D., Caster, C, Dornenburg, H., Dorn, R., Graf, S., Havkin-Frankel, D., Podstolski, A. and Werrman, U. 1993. Biosynthesis and yield improvement of food ingredients from plant cell and tissue cultures. *Food Technol.* 47(2): 57-63.

Knuth, M. E. and Sahai, O. P. Flavour Composition and method. US patent no. 5,057,424, Oct. 15, 1991.

Ko, K. S., Noguchi, H.,Ebizyka, Y. and Sanakawa, U. 1989. Oligoside production by hairy root cultures transformed by Ri plasmid. *Chem. Pharm. Bull.* 37: 245-248.

Kobayashi, Y., Akita, M., Sakamoto, K, Hongfeng-Liu, Shigeoka, T., Koyano, T., Kawamura, M.and Furuya, T. 1993. Large-scale production of anthocyanin by *Aralía cor data* cell suspension cultures. *Appl. Microbiol. Biotechnol.* 40: 215-218.

Koichi, N. and Hisashi, K. 1990. Pharmacology of anthocyanosides from wild blue berries. Part 3. Shokuhin. *Kogyo-Japan* 33: 57-62.

Komamine, A.,Tabata, M.and DiCosmo, F. 1991. *Plant Cell Culture in Japan* CMC Co. Ltd.: Tokyo.

Koop, H. U., Weber, G. and Schiweiger, H.G. 1983. Individual culture of selected single cells and protoplasts of higher plants in micro droplets of defined media. Z. *Pflanzenphysiol.* **112:** 21-34.

Koul, S., Ahuja, A. and Grewal, S. 1983. *Planta Med.* **47:** 11-16.

Kreis, W. and Reinhard, E. 1989. The production of secondary metabolites by plant cells cultivated in bioreactors. *Planta Med.* 55: 409-416.

Kubek, D.J. and Shuler, M.L. 1978. A rapid quantitation method to measure growth of plant cell suspension cultures. *Can. J. Bot.* 56: 2340-2343.

Kukreja, A. K.,Zehra, M., Sultana, A. and Banerjee, S. 1996. Hairy roots of medicinal plants: Potential and prospects. Oral Presentation: All India Symposium on Recent Advances in Biotechnological Applications of Plant Tissue and Cell Culture & XVIII Meeting of Plant Tissue Culture Association of India. June 22-24, 1995.

Kurz, W. G. W., Chatson, R. B. and Constabel, F. 1985. Biosynthesis and accumulation of indole alkaloids in *Catharanthus roseus* cultivars. In: *Primary and Secondary Metabolism of Plant Cell Cultures* (K. H. Neumann, W. Barz and E. Reinhard Eds.) Springer-Verlag: Berlin, pp 143-153.

Kwok, K. H., Tsoulpha, P. and Doran, P. M. 1992. Limitations associated with conductivity measurement for monitoring growth in plant tissue culture. *Plant Cell Tiss. Org. Cult.* 29: 93-99.

Kwok, K. H. and Doran, P. M. 1995. Kinetic and stoichiometric analysis of hairy roots in a segmented bubble column reactor. *Biotechol. Prog.* **11:** 429-435.

Lassocinski, W. 1985. Chlorophyll deficient cacti in tissue cultures. *Ada Hortic.* **167:** 287-293.

Lauriere, M.,Lauriere, C.Sturm, A.Faye, L.,Chrispeels, M.J. 1988. Characterization of B-fructosidase, an extracellular glycoprotein of carrot cells. *Biochemie* 70: 1483-1491.

Leckie, F., Scragg, A. H. and Cliffe, K. C. 1990. The effect of continuous high shear stress on plant cell suspension cultures. In: *Progress in Plant Cellular and Molecular Biology* (H. J. J. Nijkamp, L. H. W. van der Plas and J. Van Aartrijk Eds.) Kluwer Academic: Dordrecht, pp 689-693.

Leigh, R. A. ap Rees, T. Fuller, W. A. and Banfield, J. 1979. The location of acid invertase activity and sucrose in the vacuoles of storage roots of beetroot *{Beta vulgaris) Biochem. J.* **178:** 539-547.

Lescure, A.H. 1966. Relationship between the density of plant cells and their growth kinetics in submerged medium. *Exp. Cell. Res.* 44: 620-623.

Liang, S. Z., Zhong, J. J. and Yoshida, T. 1991. Review of plant cell culture technology for production of useful products (part 2) *Ch. J. Ind. Microbiol.* 21: 28-32.

Lindsey, K. and Yeoman, M. M. 1983. The relationship between growth rate, differentiation and alkaloid accumulation in cell cultures. *J. Exp. Bot.* 34: 1055-1065.

Lindsey, K., Yeoman, M. M., Black, G. M. and Mavituna, F. 1983. A novel method for the immobilization and culture of plant cells. *FEBS Lett.* **155:** 143-149.

Lindsey, K. and Yeoman, M. M. 1984. The synthetic potential of immobilized cells of *Capsicum frutescens* Mill.cv. annuum. *Planta* **162:** 495-501.

Litvay, J. D., Devi Verma, C. and Johnson, M. A. 1985. Influence of loblolly pine *(Pinus taeda* L.) culture medium and its components on growth and somatic embryogenesis of wild carrot *(Daucus carota* L.) *Plant Cell Rep.* 4: 325-328.

Lloyd, G. and McCown, B. 1981. Commercially feasible micropropagation of Mountain laurel, *Kalmia lalifolia,* by use of shoot tip culture. *Proc. Int. Plant Proc. Soc.* 30: 421-427.

Lockwood, G. B. 1984. Alkaloids of suspensions derived from four *Papaver* spp. and the effect of temperature stress. Z. pflanzenphysiol. **114:** 361-363.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. /. *Biol. Chem.* **193:** 265-275.

Luckner, M., Nover, L. and Bohm, H. 1977. Secondary metabolism and cell differentiation. *Mol. Biol. Biochem. Biophys.* **23.**

Madhavi, D.L.,Prabha, T. N., Singh, N. S. and Patwardhan, M.V. 1991. Biochemical studies with garlic *(Allium sativum)* cell cultures showing different flavour levels. /. *Sci. Food Agric.* 56: 15-24.

Mandels, M. 1972. The culture of plant cells. *Adv. Biochem. Eng.* 2: 201-215.

Mante, S. and Boll, W.G. 1975. Comparison of growth and extracellular polysaccharides of cotyledon cell suspension cultures of bush bean *(Phaseolus vulgaris* cv. Contender) grown in coconut-milk medium and synthetic medium. *Can. J. Bot.* 53: 1542-1548.

Mantell, S. H. and Smith, H. 1983. In: *Plant Biotechnology* (S. H. Mantell and H. Smith Eds.) Cambridge University Press: Cambridge pp 75-108.

Matsubara, K.,Yamada, Y.,Kitani, S., Yoshioka, T.,Morimoto, T. and Fujita, Y. 1989. High density culture of *Coptis japonica* cells increases berberine production. /. *Chem. Tech. Biotechnol.* **46:** 61-69.

Matsumoto, T., Ohunishi, K.,Nistrida, K. and Noguchi, M. 1972. *Agric. Biol. Chem.* **36:** 2177.

Matsumoto, T., Ikeda, T., Okimura, C, Obi, Y.,Kisaki, T. and Noguchi, M. 1982. Production of ubiquinone-10 by highly producing strains selected by a cell cloning technique. In: *Plant Tissue Culture* (A. Fujiwara Ed.) Maruzen: Tokyo, pp 275-276.

Maurel, B. and Pareilleux. A. 1985. Effect of carbon dioxide on the growth of cell suspensions of *Catharanthus roseus. Biotechnol. Lett.* 7: 313-318.

Mauseth, J. D. 1979. Cytokinin elicited formation of pith rib meristem and other effects of growth regulators on the morphogenesis of *Echino cereus* (Cactaceae) seedling shoot apical meristem. *Am. J. Bot.* 66: 446-451.

Mavituna, F. and Park, J. 1985. Growth of immobilized plant cells in reticulate polyurethane foam matrices. *Biotechnol. Lett.* 7: 637-640.

Mavituna, F. and Park, J.M. 1987. Size distribution of plant cell aggregates in batch culture. *Chem. Eng. J.* 35: 9-14.

Mavituna, F. Park, J., Williams, P. and Wilkinson, A. 1987. Characteristics of immobilized plant cell reactors. In: *Plant and Animal Cells: Process Possibilities* (C. Webb and F. Mavituna Eds.) Horwood: Chichester. pp 92-115.

McDonald, K. A. and Jackman, A. P. 1989. Bioreactor studies of growth and nutrient utilization in alfalfa suspension cultures. *Plant Cell Rep.* 8: 455-458.

Miller, G. L. 1959. Use of Dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31: 426-428.

Mor, J. R. 1991. Biotechnology in flavour synthesis. In: *Proc. 8th World Congress of Food Science and Technology* Canada.

Mori, T., Skurai, M., Shigeta, J., Yoshida, K. and Kondo, T. 1993. Formation of anthocyanins from cells cultured from different parts of strawberry plants. *J. Food Sci.* 58: 788-792.

Morris, P. 1986a. Regulation of product synthesis in cell cultures of *Catharanthus roseus* II. Comparison of production media. *Planta Med.* 52: 121-126.

Morris, P. 1986b. Regulation of product synthesis in cell cultures of *Catharanthus roseus.* Effect of culture temperature. *Plant Cell Rep.* 5: 427-429.

Morvan, H. 1982. Release of pectic acid polymers during growth of *Silene alba* cell suspension cultures. *Physiol. Veg.* 20: 671-678.

Mukundan, U.,Hjortso, M. A. 1991. Growth and thiophene accumulation by hairy root cultures of *Tagetes patula* in media of varying initial pH. *Plant Cell Rep.* 9: 627-630.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497.

Nassau, K. 1993. Color. In: *Encyclopaedia of Chemical Technology* (M.Howe-Grant Ed.) John Wiley & Sons, Inc.: New York. vol. 6. pp 841-876.

Nitsch, J. P. and Nitsch, C. 1969. Haploid plants from pollen grains. *Science* **163:** 85-87.

Noguchi, M., Matsumoto, T., Hirata, Y., Yamamoto, K., Katsuyama, A., Kato, A., Azechi, S. and Katoh, K. 1982. Improvements of growth rates of plant cell cultures. In: *Plant Tissue Culture and its Biotechnological Applications* (W. Barz, E. Reinhard and M. H. Zenk Eds.) Springer-Verlag: Berlin, pp 85-94.

Nozue, M., Kawai, J. and Yoshitama, K. 1987. Selection of a high anthocyanin producing cell line of sweet potato cell cultures and identification of pigments. *J. Plant Physiol.* **129:** 81-88.

Obenland, D. M., Simmen, U., Boiler, T. and Wiemken, A. 1993. Purification and characterization of three soluble invertases from barley *(Hordeum vulgare* L.) leaves. *Plant Physiol.* **101:** 1331-1339.

Ohta, S. and Yamazawa, M. 1982. In: Plant Tissue Culture (A. Fujiwara Ed.) Japanese Association for Plant Tissue Culture: Tokyo, pp 321-322.

Oka, S. 1974. Rheological - Biotechnology. Syokabo: Tokyo.

Otsuji, K.,Honda, Y.,Sugimura, Y.and Takei, A. 1994. Production ofpolysaccharides in liquid cultures of *Polianthus tuberosa* cells. *Biotechnol. Lett.* **16:** 943-948.

Owen, H. R., Wengerd, D. and Miller, A. R. 1991. Culture medium pH influenced basal medium, carbohydrate source, gelling agent, activated charcoal and medium storage method. *Plant Cell Rep.* **10:** 583-586.

Ozeki, Y. and Komamine, A. 1985. Effects of inoculum density, zeatin and sucrose on anthocyanin accumulation in a carrot suspension culture. *Plant Cell Tiss. Org. Cult.* 5: 45-53.

Panda, A. K.,Mishra, S., Bisaria, V. S. and Bhojwani, S. S. 1989. Plant cell reactors - a perspective. *Enzyme Microbial Technol.* 11: 386-397.

Panda, A. K., Mishra, S. and Bisaria, V. S. 1992. Alkaloid production by plant cell suspension cultures of *Holarrhena antidysenterica:* Effect of major nutrients. *Biotechnol. Bioeng.* 39: 1043-1051.

Pareilleux, A. and Chaubet, N. 1981. Mass cultivation of *Medicago sativa* growing on lactose: Kinetic aspects. *Eur. J. Appl. Microbiol. Biotechnol.* 11: 222-225.

Pareilleux, A. and Vinas, R. 1983. Influence of the aeration rate on the growth yield in suspension cultures of *Catharanthus roseus* (L.) G. Don. /. *Ferment. Technol.* 61: 429-433.

Parfitt, D. E., Almehdi, A. A. and Bloksberg, L.N. 1988. Use of organic buffers in plant tissue culture systems. *Scientia Hortic.* 36: 157- 163.

Park, J. M, Giles, K. L. and Songstad, D. D. 1990. Industrial production of sanguinarine from cell suspension cultures of *Papaver somniferum. Proc. Asia-Pacific Biochemical Engineering Conference.:* Korea April22-25.

Parr, A. J. 1988. Secondary products from plant cell culture. In: *Biotechnology in Agriculture: Advances in Biotechnological Processes* (A. Mazrahi and R. Alan Eds.) Liss Inc.: New York, pp 2-34.

Payne, G. F.,Shuler, M. L. and Brodelius, P. 1987. Large scale plant cell culture. In: *Large Scale Cell Culture Technology* (B. K. Lyderson Ed.) Hanser, Munich, pp 193-229.

Payne, G., Bringi,V.,Prince, C. and Shuler, M. L. 1992. *Plant Cell and Tissue Culture in Liquid Systems* Hanser Publishers: Munich.

Pepin, M. F., Archambault, J., Chavarie, C. and Cormier, F. 1995. Growth kinetics of *Vitis vinifera* cell suspension cultures: I. Shake flask cultures. *Biotechnol. Bioeng.* 47: 131-138.

Pestchanker, L.J.,Rodes, S. C. and Shuler, M. L. 1996. Kinetics of taxol production and nutrient use in suspension cultures of *Taxus cuspidata* in shake flasks and a Wilson-type bioreactor. *Enzyme Microbial Technol.* 19: 256-260.

Peterson, G.L. 1983. Determination of total protein. *Methods Enzymol.* 91: 95-121.

Peterson, M. and Alfermann, A. W. 1993. Plant Cell Cultures. In: *Biotechnology: Biological Fundamentals* (H. J. Rehm and G. Reed Eds.) VCH Publishers: Weinheim. Vol. I.,pp 577-614.

Preil, W., Florek, P., Wix, U. and Beck, A. 1988. Towards mass propagation by use of bioreactors. *Ada Horήc.* **226:** 99-106.

Radwan, S. S. and Kokate, C. K. 1980. Production of higher levels of trigonelline by cell cultures of *Trigonella foenum-graceum* than by the differentiated plant. *Planta* **147:** 340-344.

Rajasekhar, E. W., Edwards, M., Wilson, S. B. and Street, H. E. 1971. *J. Exp. Bot.* **22:** 107.

Rajendran, L.,Prathibha, K. R., Ravishankar, G. A. and Venkataraman, L. V. 1992. Anthocyanin production in callus cultures of *Daucus carota* as influenced by nutrient stress and osmoticum. *Biotechnol. Lett.* **14:** 707-712.

Rajendran, L.,Suvarnalatha, G., Ravishankar, G. A. and Venkataraman, L. V. 1994. Enhancement of anthocyanin production in callus cultures of *Daucus carota* L. under the influence of fungal elicitors. *Appl. Microbiol. Biotechnol.* **42,** 227-231.

Ravishankar, G. A. and Venkataraman, L. V. 1990. Food applications of plant cell cultures. *Curr. Sci.* 59: 914-920.

Reid, M. S., Paul, J. L. and Young, R. E. 1980. The effect of pH and ethephon on betacyanin leakage from beet root discs. *Plant. Physiol.* **66:** 1015-1016.

Rhodes, M. J. C. 1985. Immobilized plant cells. In: Topics in Enzyme and Microbiol Biotechnol (A.Wiseman Ed.) Ellis Horwood: Chichester. pp 51-88.

Rhodes, M. J. C, Smith, J. I. and Robins, R. J. 1987. Factors affecting the immobilization of plant cells on reticulated polyurethane foam particles. *Appl. Microbiol. Biotechnol.* **26:** 28-35.

Ricardo, C. P. P. and ap Rees, T. 1970. Invertase activity during the development of carrot roots. *Phytochemistry* 9: 239-247.

Rokem, J. S. and Goldberg, I. 1985. Secondary metabolites from plant cell suspension cultures: Methods for yield improvement, *la: Advances in Biotechnological Processes* (A. Mizrahi and A. L. van Wezel Eds.) Alan R Liss. New York, pp 241-274.

Rose, D.and Martin, S. M. 1975. Growth of suspension cultres of plant cells *(Ipomea* spp.) at various temperatures. *Can. J. Bot.* 53: 315-320.

Routier, J. B. and Nickell, L. G. 1956. Cultivation of plant tissue. US Patent 2, 747., 334.

Ruyter, C. M. and Stockigt, J. 1989. Novel natural products from plant cell and tissue culture - an update. *GIT Fachz. Lab.* 4: 283-293.

Ryu, D. D. Y.,Lee, S. O. and Romani, R. J. 1990. Determination of growth rate for plant cell cultures: comparative studies. Biotechnol. Bioeng. 35: 305-311.

Sakuta, M. and Komamine, A. 1987. Cell culture in phytochemistry. In: Cell Culture and Somatic Genetics of Plants (F. Constabel and I. K. Vasil Eds.) Academic Press: Sand Diego. Vol 4; pp 97.

Sato. K. 1961. Rheological studies on some fermentation broths I. Rheological analysis of kanamycine and streptomycine fermentation broths. /. *Ferment. Technol.* 39: 347-353.

Schenk, R. U. and Hildebrandt, A.C. 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Can. J. Bot.* 50: 199-204.

Schlatmann, J. E., Moreno, P. R. H.,Vinke, J. L.,ten Hoopen, H. J. G. Verpoorte, R. and Heijnen, J. J. 1994. Effect of oxygen and nutrient limitation on ajmalicine production and related enzyme activities in high density cultures of *Catharanthus roseus. Biotechnol. Bioeng.* 44: 461-468.

Schmauder, H. P. and Doebel, P. 1990. Plant cultivation as a biotechnological method. *Acta Biotechnol.* 10: 501-516.

Scragg, A. H., Morris, P., Allan, E. J., Bond, P. and Fowler, M. W. 1987. Effect of scale-up on serpentine formation by *Catharanthus roseus* suspension cultures. *Enzyme Microbial Technol.* 9: 619-624.

Scragg, A. H., Allan, E. J. and Leckie, F. 1988. Effect of shear on the viability of plant cell suspensions. *Enzyme Microbial Technol.* 10: 361-367.

Scragg, A. H. and Aston, S., Steward, R. D. and Allan, E. J. 1990. Growth and quassin accumulation by cultures of *Quassia amara. Plant Cell Tiss. Org. Cult.* 23: 165-169.

Scragg, A. H. 1992. Bioreactors for the mass cultivation of plant cells. In: *Plant Biotechnology* (M. W. Fowler, G. S. Warren, M. Moo-Young Eds.) Pergamon Press: Oxford, pp 45-62.

Segel, I. H. 1976. *Biochemical calculations.* John Wiley and Sons, Inc.: London.

Seitz, H. U. and Hiderer, W. 1988. Anthocyanins. In: *Cell Culture and Somatic Cell Genetics of Plants* (F. Constabel and I. K. Vasil Eds.) Acad. Press Inc.: San Diego. Vol 5. pp 49-76.

Shuler, M. L. 1981. Production of secondary metabolites from plant tissue culture -problems and prospects. *Ann. N.Y. Acad. Sci.* 369: 65-79.

Sim, K. S. 1964. Yakhak Hoechi 8: 69.

Singh, G. and Curtis, W. R. 1994. Reactor design for plant cell suspension culture. In: *Biotechnological Applications of Plant Cultures* (P. D. Shargool and T. T. Ngo Eds.) CRC Press: Boca Raton, pp 151-183.

Singh, G. 1997. Reactor design for plant cell culture of food ingredients and additives. *Food Technol.* 51(11): 62-66.

Skirvin, R. M., Chu, M. C, Mann, M. L., Young, H., Sullivan, J. and Fermanian, T. 1986. Stability of tissue culture medium pH as a function of autoclaving time and cultured plant type. *Plant Cell Rep.* 5: 292- 294.

Smart, N. J. and Fowler, M. W. 1981. Effect of aeration on large-scale cultures of plant cells. *Biotechnol. Lett.* 1: 171-176.

Smart, N. J. 1984. Plant cell technology as route to natural products. *Lab. Pract. Jan.* 11-35.

Smart, N. J. and Fowler, M. W. 1984. An airlift column bioreactor suitable for large-scale cultivation of plant cell suspensions. *J. Exp. Bot.* 35: 531-537.

Snape, J. B., Thomas, N. H. and Callow, J. A. 1989. How suspension cultures of *Catharanthus roseus* respond to oxygen limitation: Small-scale test with applications to large-scale cultures. *Biotechnol. Bioeng.* 34: 1058-1062.

Songstad, D.D., Giles, K. L.,Park, J., Novakovski, D. Epp. D. and Friesen, L. 1990. Use of nurse cultures to select for *Papaver somniferum* cell lines capable of enhanced sanguinarine production. *J. Plant Physiol.* **136:** 236-239.

Spear, K. 1986. Developments in food colouring: The natural alternatives. *Trends Biotechnol.* 6: 283-288.

Spier, R. E. and Fowler, M. W. 1983. Animal and plant cell culture. In: *Comprehensive Biotechnology* (A. T. Bull and H. Dalton Eds.) Pergamon Press: Oxford, pp 301-330.

Spier, R. E. 1995. Gradients in animal and plant cell technology systems. *Enzyme Microbial Technol.* 17: 91-92.

Srinivasan, V.,Pestchanker, L.,Moser, S., Hirasuna, T. J., Taticek, R. A. and Shuler, M. L. 1995. Taxol production in bioreactors: Kinetics of Biomass accumulation, nutrient uptake, and taxol production by cell suspensions of *Taxus baccata. Biotechnol. Bioeng.* 47: 666-676.

Staba, E. J. and Kaul, B. 1971. US Patent 3 628 287.

Staba, E. J. 1982. Production of useful compounds from plant tissue culture. In: *Plant Tissue Culture* (A. Fujiwara Ed.) Japanese Association for Plant Tissue Culture: Tokyo, pp 25-30.

Stafford, A., Smith, L. and Fowler, M. W. 1985. *Plant Cell Tiss. Org. Cult.* 4: 83-.

Stafford, A., Morris, P. and Fowler, M. W. 1986. Plant cell biotechnology - a perspective. *Enzyme Microbial Technol.* 8: 578-587.

Stafford, A. 1991. The manufacture of food ingredients using plant cell and tissue cultures. *Trends Food Sci. Technol.* 2: 116-122.

Stafford, A. 1994. Anthocyanins and betalaines: Evolution of the mutually exclusive pathways. *Plant Sci.* **101,** 91-98.

Steward, F. C., Mapes, M. O. and Mears, K. 1958. Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. *Amer. J. Bot.* 45, 705-708.

Stickland, G. G. and Sunderland, N. 1972. Production of anthocyanins, flavonols and chlorogenic acid by cultured callus tissues of *Haplopappus gracilis. Ann. Bot.* 36: 443-457.

Street, H. E. 1977. *Plant Tissue and Cell Culture* Blackwell: Oxford.

Stuart, D. A. and Strickland, G. A. 1987. Bioreactor production of alfalfa somatic embryos. *Hortic. Sci.* 22: 800-803.

Stuart, R. and Street, H. E. 1969. Studies on the growth in culture of plant cells. /. *Exp. Bot.* 20: 556-571.

Su, W. W. and Lei, F. 1993. Rosmarinic acid production in perfused *Anchusa officinalis* culture: effect of inoculum size. *Biotechnol. Lett.* 15: 1035-1038.

Sudhakar Johnson, T., Ravishankar, G. A. and Venkataraman, L. V. 1990. *In vitro* capsaicin production by immobilized cells and placental tissues of *Capsicum annuum* L. grown in liquid medium. *Plant Sci.* 70: 223-229.

Sudhakar Johnson, T. Ravishankar, G. A. and Venkataraman, L.V. 1991. Elicitation of capsaicin production in freely suspended cells and immobilized cell cultures of *Capsicum frutescens* Mill.*Food Biotechnol.* 5: 197-205.

Sudhakar Johnson, T. 1993. Studies on production of capsaicin in immobilized cells and placental tissues of *Capsicum annuum* and *C frutescens.* Ph.D. Thesis. University of Mysore: Mysore.

Sudhakar Johnson, T., Priyasethu, K. M. and Ravishankar, G. A. 1996. A rapid method of determining the viability of plant cells using a mixture of fluorescein diacetate and calcofluor white. *J. Med. Aromatic Plant Sci.* 18: pp 505-506.

Sudhakar Johnson, T. and Ravishankar, G. A. 1996. Precursor biotransformation in immobilized placental tissues of *Capsicum frutescens* Mill. I. Influence of feeding intermediate metabolites of the capsaicinoid pathway on capsaicin and dihydrocapsacin accumulation. /. *Plant Physíol* 147: 481-485.

Sujatha, V., Ravishankar, G. A. and Venkataraman, L. V. 1990. Induction of crocin, crocetin, picrocrocin and saffranal synthesis in callus cultures of saffron *{Crocus sativus* L.). *Biotechnol. App. Biochem.* 12: 336-340.

Sung, Z.R. 1976. Turbidimetric measurement of plant cell culture growth. *Plant Physiol.* 57: 460-462.

Suvarnalatha, G. Nagin Chand, Ravishankar, G. A. and Venkataraman, L. V. 1993. Computer-aided modeling and optimization for capsaicinoid production by immobilized *Capsicum frutescens* cells. *Enzyme Microbial Technol.* 15: 710-715.

Suvarnalatha, G.,Narayan, M.S., Ravishankar, G. A.and Venkataraman, L.V. 1994. Flavour production in plant cell cultures of basmathi rice *(Oryza sativa* L.). /. *Sci. Food. Agric.* 66: 439-442.

Swanson, S. M.,Mahady, G. B. and Beecher, C.W.W. 1992. Stevioside biosynthesis by callus, root, shoot and rooted-shoot cultures *in vitro. Plant cell Tiss. Org. Cult.* 28: 151-157.

Takayama, S., Misawa, M.,Ko, K. and Misato, T. 1977. Effect of cultural conditions on the growth of *Agrostemma githago* cells in suspension culture and the concomitant production of an anti-plant virus substance. *Physiol. Plant.* 41: 313-320.

Takeda, J. 1988. Light induced synthesis of anthocyanin in carrot cells in suspension. 1. The factors affecting anthocyanin production. *J. Expt. Bot.* 39: 1065-1077.

Tal, B. Rokem, J. S. and Goldberg, I. 1983. Factors affecting growth and product formation in plant cells grown in continuous culture. *Plant Cell Rep. 2:* 219-222.

Tanaka, H. 1981, Technological problems in cultivation of plant cells at high density. *Biotechnol. Bioeng.* 23: 1203-1218.

Tanaka, H. 1982. Oxygen transfer in broths of plant cells at high densities. *Biotechnol. Bioeng.* 24: 425-442.

Tanaka, H., Semba, H., Jitsufuchi, T. and Harada, H. 1988. The effect of physical stress on plant cells in suspension cultures. *Biotechnol. Lett.* 10: 485-490.

Tanaka, H., Aoyagi, H. and Jitsufuchi, T. 1992. Turbidimetric measurement of cell biomass of plant cell suspensions. *J. Ferment. Bioeng.* 73: 130-134.

Taticek, R., Moo-Yong, M.,Legge, R. L. 1990. Effect of bioreactor configuration on substrate uptake by cell suspension cultures of plant *Eschscholtzia californica. Appl. Microbiol. Biotechnol.* 33: 280-286.

Taya, M.,Hegglin, M.,Prenosil, J. E. and Bourne, J. R. 1989a. On-line monitoring of cell growth in plant tissue cultures by conductometry. *Enzyme Microbial Technol.* **11:** 170-176.

Taya, M., Yoyama, A., Kondo, O. Kobayashi, T. and Matsui, C. 1989b. Growth characteristics of plant hairy roots and their cultures inbioreactors. *J. Chem. Eng.* 22: 84-89.

Thirumalachar, D. K. 1967. Variability for capsaicin content in chilli. *Curr. Sci.* 36: 269-270.

Thorn, M., Maretzki, A., Komor, E. and Sakai, W. S. 1981. Nutrient uptake and accumulation by sugar cane cell cultures in relation to the growth cycle. *Plant Cell Tiss. Org. Cult.* 1: 3-14.

Tiburcio, A. F., Gendy, C. A. and Tran, T. V. K. 1989. Morphogenesis in tobacco subepidermal cells: Putrescine as a marker of root differentiation. *Plant Cell Tiss. Org. Cult.* 19: 43-54.

Toivonen, L.,Ojala, M. and Kauppinen, V. 1990. Indole alkaloid production by hairy root cultures of *Catharanthus roseus:* growth kinetics and fermentation. *Biotechnol. Lett.* 12: 519-524.

Treat, W. J., Engler, C. R. and Soltes, E. J. 1989. Culture of photomixotropic soybean and pine in a modified fermentor using a novel impeller. *Biotechnol. Bioeng.* 34: 1191-1202.

Tremouillaux-Guiller, J.,Andreu, F., Creche, J.,Chenieux, J. C. and Rideau, M. 1987. Variability in tissue culture of *Choisya ternata.* Alkaloid accumulation in protoclones and aggregate clones obtained from established lines. *Plant Cell Rep.* 6: 375- 378.

Trinder, P. 1969. Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. /. *Clin. Pathol.* 22: 158-161.

Tulecke, W. and Nickell, L. G. 1960. Methods, problems and results of growing plant cells under submerged culture. *Trans. New York Acad. Sci.* 22: 196-204.

Ulbrich, B., Weisner, W. and Arens. H. 1985. Large scale production of rosemarinic acid from plant cultures of *Coleus blumei.* In: *Secondary Metabolism of Plant Cell Culture* (B. Deus- Newmann, W. Barz and E. Reinhard Eds.) Springer-Verlag: Berlin, pp 293-303.

van Gulik, W. M., Jeijer, J. J., ten Hoopen, H. J. G., Luyben, K. C. A. M., Libbenga, K. R. 1989. Growth of *Catharanthus roseus* cell supension culture in a modified chemostat under glucose limiting conditions. *Appl. Microbiol. Biotechnol.* 30:270-275.

van Gulik, W. M., ten Hoopen, H, J. G., Heijnen, J. J. 1993. A structured model describing carbon and phosphate limited growth of *Catharanthus roseus* plant cell suspensions in batch and chemostat culture. *Biotechnol. Bioeng.* 41: 771-780.

Vaush, R.,Ratilsky, M. B., Keren, Z. M. and Robenfield, B. 1986. In: VIInternational Congress of Plant Tissue and Cell Culture (D. A. Somers, B. G. Gengenbach, D. D. Biesboer, W. P. Hackett and C. E. Green Eds.) IAPTC: USA. pp 116.

Vega, F. A., Ramos, M. F., Fernandez, M., Santesteban, M. and Galar, A. 1987. Pharmacological activity of anthocyanins. *Cienc. Ind. Farm.* 6:205-208.

Wagner, F., Vogelmann, H. 1977. Cultivation of plant tissue cultures in bioreactors and formation of secondary metabolites. In: *Plant Tissue Culture and its Biotechnological Applications* (W. Barz, E. Reinhard and M.M. Zenk Eds.) Springer-Verlag: New York, pp 245-252.

Wangsamuth, R. and Doran, P.M. 1994. Foaming and cell floatation in suspended plant cell cultures and the effect of chemical antifoams. *Biotechnol. Bioeng.* 44: 481-488.

Warren, G. S. 1992. The cell biology of plant culture systems, *la: Plant Biotechnology* (M.W. Fowler, G. S. Warren and M. Moo- Young Eds.) Pergamon Press: Oxford, pp 1-18.

Weatherburn, M.W. 1967. Phenol-hypochlorite reaction for determination ofammonia. /. *Anal. Chem.* 39: 971-974.

White, P. R. 1934. Potentially unlimited growth of excised tomato root tips in a liquid medium. *Plant Physiol.* 9: 585-600.

White, P. R. 1963. *The Cultivation of Animal and Plant Cells.* Ronald Press: New York.

Wickremesinhe, E. R. M. and Arteca, R. N. 1994. *Taxus* cell suspension cultures: Optimizing growth and production of taxol. *J. Plant Physiol.* 144: 183-188.

Wilkinson, M., Sweeny, J. G. and lacobucci, A. 1977. High pressure liquid chromatography of anthocyanidins. /. *Chromatog.* **132:** 349- 351.

Williams, P. D.and Mavituna, F. 1992. Immobilized plant cells In: *Plant Biotechnology* (M. W. Fowler, G. S. Warren and M. Moo- Young Eds.) Pergamon Press: Oxford, pp 63-78.

Wilson, S.B., King, P.J. and Street, M.E. 1971. Studies on the growth in cultured plant cells. XII. A versatile system for the large scale batch or continuous culture of plant cell suspensions. *J. Exp. Bot.* **21:** 460-462.

Wink, M. 1988. Plant breeding: importance of plant secondary metabolites for protection against pathogens and herbivores. *Theor. Appl. Gen.* 75: 225-233.

Wiznsama, R., Verpoorte, R., Harkes, P. A. A., Vanvliet, T. B., Tenhoopen, H. J. G. and Baerheim, S. 1986. The influence of initial sucrose and nitrate concentration on the growth of *Cinchona ledgeriana* cell suspension cultures and the production of alkaloids and anthraquinones. *Plant Cell Tiss. Org. Cult.* 7: 21-29.

Yamakawa, T.,Kato, S., Ishida, K.,Kodama, T. and Monida, Y. 1983. Production of anthocyanins by *Vitis* cells in suspension cultures. *Agri. Bíol. Chem.* 47: 2185-2191.

Yamakawa, T.,Onomichi, K. Kodama, T. and Minoda, Y. 1985. Application of feeder layer method for improved colony formation of grape cells and protoplasts at low cell density. *Agric. Bíol. Chem.* 49: 3583-3585.

Yamamoto, Y., Mizuguchi, R. and Yamada, Y. 1982. Selection of a high and stable pigment-producing strain in cultured *Euphorbia míllii* cells. *Theoretical Appl. Gen.* 61: 113-116.

Yamamoto, Y. 1984. In: *A Manual for Plant Cell Culture* (Y. Yamada Ed.) Kodansha Scientific: Tokyo, pp 45.

Yamamoto, A. and Yamada, Y. 1987. Selection of reserpine producing cell strain using UV light and optimization of reserpine production in the selected cell strain. *Plant Cell Tiss. Org. Cult.* 8: 125-133.

Yeoman, M. M.,Midzybrodzka, M. B. Lindsey,K. and Mc Lauchlan, W. R. 1980. The synthetic potential of cultured plant cells. In: *Plant Cell Cultures:* Results and Perspectives (F. Sala, B. Parisi, R. Cella and O. CifferiEds.) Elsevier North Holland Pub.: Amsterdam, pp 327-343.

Yu, S. and Doran, P. M. 1994. Oxygen requirements and mass transfer in hairy-root culture. *Biotechnol. Bioeng.* 44: 880-887.

Zamski, E. and Wyse, R. E. 1985. Stereospecificity of glucose carrier in sugar beet suspension cells. *Plant Physiol.* 78: 291-295.

Zenk, M. H.,E1-Shagi, H. and Ulbrich, B. 1977a. Production ofrosmarinic acid by cell suspension cultures of *Coleus blumeí. Naturwíssenschafteur.* 64: 585-586.

Zenk, M. H.,Shagi, H. EL.Arens, H.,Stockigt, J., Weiler, E. W. and Deus, B. 1977b. Formation of indole alkaloids serpentine and ajmalicine in cell suspension cultures of *Catharanthus roseus.* In: *Plant Tissue Culture and its Biotechnological Application* (W. Barz, E. Reinhard and M. H. Zenk Eds.) Springer-Verlag: Berlin, pp 27-43.

Zenk, M. H. El-Shagi, H. and Schulte, U. 1985. Anthraquinone production by cell suspension cultures of *Morinda citrifolia. Planta Med.* Suppl.: 79-101.

Zink, M. W. and Veliky, L A . 1979. Acid phosphotases of *Ipomea* sp. *in vitro:* I. Influence of pH and inorganic phosphates on the formation of phosphatases. *Can. J. Bot.* 57: 739 -753.

Zhong, J. J., Seki, T.,Kinoshita, S. and Yoshida, T. 1991. Effect of light irradiation on anthocyanin production by suspended cultures of *Perilla frutescens. Biotechnol. Bioeng.* 38: 653-658.

Zhong, J. J., Seki, T.,Kinoshita, S. and Yoshida, T. 1992. Rheological characteristics of cell suspension and cell cultures of *Perilla frutescens. Biotechnol. Bioeng.* 40: 1256-1262.

Zhong, J. J., Yoshida, M., Fujiyama, K., Seki, T. and Yoshida, T. 1993a. Enhancement of anthocyanin production by *Perilla frutescens* cells in a stirred bioreactor with internal light irradiation. /. *Ferment. Bioeng.* 75: 299-303.

Zhong, J.J., Fijiyama, K.,Seki, T. and Yoshida, T. 1993b. On-line monitoring of cell concentration of *Perilla frutescens* in a bioreactor. *Biotechnol. Bioeng.* 42:542-546.

Zhong, J. J. 1995. Recent advances in cell cultures of *Taxus* spp. for production of natural anticancer drug taxol. *Plant Tiss. Cult. Biotechnol.* 1: 75-80.

Zhong, J. J. and Yoshida, T. 1995. High-density cultivation of *Perilla frutescens* cell suspensions for anthocyanin production: Effects of sucrose concentration and inoculum size. *Enzyme Microbial Technol.* 17: 1073-1079.

PUBLICATIONS

R. Madhusudhan, S.Ramachandra Rao **and** G.A. Ravishankar 1995. Osmolarity as a measure of growth of plant cells in suspension culture. *Enzyme and Microbial Technology* **17:989-991.**

R. Madhusudhan and G.A. Ravishankar 1996. Gradient of aπthocyanin in cell aggregates of *Daucus carota* in suspension culture. *Biotechnology Letters* 18: 1253 - 56.