Chapter 1

Introduction and Review of Literature
INTRODUCTION

The word 'tumour' (neoplasm) is a lesion resulting from the autonomous or relatively autonomous abnormal growth of cells, which persists after the initiating stimulus has been removed. Tumours can result from the neoplastic transformation of any nucleated cell in the body, although some cell types are more prone to tumour formation than others, the transformed cells are called neoplastic cells. By transformation involving a series of genetic alterations, cells escape permanently from normal growth regulatory mechanisms. The neoplastic cells in tumours designated malignant, possess additional potentiality, lethal abnormal characteristics enabling them to invade and to metastasize or spread to other tissues.

Malignant neoplasmas - those that invade and spread and therefore of greater clinical importance - develop in approximately 25% of the human population. The individual risk increases with age. The mortality rate is high, despite modern therapy, so that cancer accounts for about one fifth of all deaths in developed countries. However, the mortality rate varies considerably between specific tumour types.

Two important genetic mechanisms leading to tumour growth are loss or inactivation of recessive inhibitory genes i.e. tumour suppressor genes, enhanced or abnormal expression of dominant stimulatory genes i.e. oncogenes. An oncogene is an altered form of normal cellular gene called a protooncogene. It encodes a regulating protein with dominant transforming properties. Protooncogenes and oncogenes are classified primarily according to their functional role and position in pathways of signal transduction and sub-categorized as growth factors, receptors, non-receptors, tyrosine kinases, GTP binding proteins, serine/ threonine kinases or nuclear proteins and transcription factors. It is a very diverse group of genes and the
product of these genes negatively regulate the growth of cancer cells. More than a dozen suppressor genes have been cloned and characterized and several more have been localized in the genome (Holywood et al, 1995). These genes encode proteins that negatively regulate the growth of cells and just as for protooncogenes function, at a variety of levels in signal transduction and cell cycle.

Cancer treatment traditionally involves three modalities of therapy: Surgery, radiotherapy and chemotherapy (Berger, 1986). The purpose of treating cancer with chemotherapeutic agents is to prevent cancer cells from multiplications, invasion and metastasis in the host (Skeel, 1999). The primary effect of chemotherapeutic agents is on tumour cell growth and multiplication. Since cell growth and multiplication is characteristic of normal as well as cancer cells, an agent that has marked growth-inhibiting or controlling effect on cancer cells and a minimal or no toxic effect on the host are most effective. In majority of chemotherapeutic regimens, the drugs are capable of not only inhibiting but also completely eradicating the neoplastic (tumour) cells while sufficiently preserving the normal cells (Baserga, 1981).

The design of drug treatment regimens is based on a number of considerations. These include prior empirical knowledge of the responsiveness of the pathologic category of tumour to specific drugs and knowledge of synergistic or antagonistic action of drug combinations in their cytotoxic activity as well as mechanisms of acquired resistance to the drugs and the knowledge of the drugs' pharmacokinetic behavior and of patterns of normal organ toxicity.

Now a days numerous compounds are used as chemotherapeutic agents. Antifolates, 5-flourouracil, hydroxyurea, antimicrotubule
agents, alkylating agents, platinum analogues, antibiotics, anthracyclines, plant alkaloids -are certain classes of chemotherapeutic agents used in cancer chemotherapy.

From the perspective of drug lead finding, two factors critically distinguish natural products from synthetic chemicals, namely, molecular diversity and biological functionality. It is universally recognized that molecular diversity among natural products far outweighs that of today's combinatorial libraries based on scaffolds, which, despite considerable advances, are still relatively limited in scope.

Our dependence on plants for natural products is expected to continue because some compounds are difficult to synthesize due to their structural complexity. Secondary metabolites can be applied as starting compounds for further chemical modification. During the last 30 years, there has been an increasing interest among scientists to produce high value natural plant products by cell culture that can overcome many of the problems associated with industrial production of these phytochemicals by extraction from field grown plants. In cultures, factory-type production of these phytochemicals can be carried out throughout the year, unaffected by the season. The risk of crop failure due to natural hazards and the danger of extinction of some species due to their mass extraction from natural populations are eliminated.

Cell culture system provides means for de novo synthesis of natural products, at the same time they also serve as factories for bioconversion of low value compounds to high value products. Moreover, some novel compounds produced in cell cultures are not produced in intact plants. For example Ueda et al (1981) isolated
tarennosid from *Gardenia jasminoides*, which was absent in the intact plant.

Since the early 1950s when the concept of tissue culture production of natural compounds was conceived, many technological advances have been shown to produce higher amounts of the products than the intact plant from which they are derived. Ulbrich *et al* (1985) produced rosmarinic acid from *Coleus blumei* cultures (23% of dry weight) which was several times higher than the amount present in the intact plant (3% of dry weight). Of the various plant products produced by plant tissue cultures, pharmaceuticals have received maximum attention. The two countries which have made substantial contributions to this field of research are Japan and Germany.

Various important strategies are used to optimize product yield from plant tissue cultures, namely, improvement of culture conditions, selection of high yielding cell lines, elicitation, immobilization, hairy root cultures, biotransformation etc. The productivity of cell lines is greatly influenced by the culture conditions. In general growth and production of secondary metabolites are inversely related, both in whole plants and cell cultures.

As early as 1950, a National Cancer Institute screening programme designed to identify promising new natural products, discovered that extracts derived from the *Camptotheca acuminata* tree were cytotoxic to cancer cells. Later the active principle was identified as camptothecin (Wall and Wani, 1968). Early biochemical pharmacology studies showed that camptothecin damaged DNA (Horowitz and Horowitz, 1973), ultimately leading to inhibition of DNA and RNA synthesis. However, the mechanism underlying these drug actions remained obscure. Nonetheless, because of promising
preclinical activity, the drug entered clinical trials in the early 1970s, under National Cancer Institute Sponsorship.

In phase I studies, responses were observed in patients with colorectal, stomach, small bowel and non-small cell lung cancers as well as melanoma. Important advances in the 1980s led to a resurgence of interest in the camptothecins. First was the discovery that camptothecin had a unique molecular target, topoisomerase I, a key nuclear enzyme responsible for relaxing torsionally strained DNA (Ihsiang et al, 1985). Currently, the camptothecins are the only well characterized inhibitors of topoisomerase I. Investigations into the mode of anti-tumour action identified camptothecin as an inducer of DNA strand breaks in mammalian cells.

The elucidation of the novel mechanism of action led to successful attempts to develop more soluble, less toxic topoisomerase I inhibitors from camptothecin with even greater preclinical activity. Interest increased further with the discovery that camptothecin derivatives, such as irinotecan (CPT-11) and topotecan, have clinical anticancer activity. Four camptothecin analogues are currently undergoing clinical evaluation, including irinotecan, topotecan, 9-amino camptothecin and GG211.

The stem wood and the bark of C. acuminata contained camptothecin. In Peoples Republic of China, camptothecin is widely used in the treatment of cancer.

Later Govindachari et al (1972) reported the presence of camptothecin in another plant, Nothapodytes foetida, a member of the family Icacinaceae. Roja and Heble (1994) reported the presence of 9-methoxy camptothecin in the same plant. The amount of camptothecin in the bark of this plant was 0.08% of dry weight.
In 1976, Tafur et al. reported the presence of camptothecin and 10-methoxy camptothecin from *Ophiorrhiza mungos*. But they didn't do any quantitative determination. Since then many other species of *Ophiorrhiza* plants were screened for the production of camptothecin.

In *Ervatamea hayneana* (Apocynaceae) (Gunasekera et al., 1979) and *Merrilliodendron megacarpum* (Arisawa et al., 1981) also, the presence of camptothecin was detected.

The biotechnological production of camptothecin demanded more attention during the last 20 years. Callus and cell suspension cultures of *Nothapodytes foetida* was established and the methods for improving production were carried out. Similarly, *in vitro* culture techniques are well established in the case of certain *Ophiorrhiza* species. Saito et al. (2001) reported camptothecin production from hairy root cultures of *Ophiorrhiza pumila*. They reported the exudation of camptothecin into the medium.

In 2002, Sudo et al. designed a bioreactor for the production of camptothecin. They established a scale-up culture of the hairy roots using a 3 l bioreactor for a feasible production of camptothecin. They obtained $8.7 \pm 1.3$-mg/l camptothecin from 8-week culture grown in the 3l reactor.

The present study attempts improvement of camptothecin production from *Nothapodytes foetida* cultures and establishment of an *in vitro* system for the production of camptothecin from *Ophiorrhiza mungos*. 
REVIEW OF LITERATURE

Cancer causes death of six million people within one year globally. In India, the cancer registry data estimated that half a million new cancer cases are reported per year in the country. The formation of cancer is a multistage process in which multiple genetic alterations occur usually over the span of years to derail sufficiently the control of cell growth, division and differentiation. As in cancer predisposing syndromes, these genetic alterations are acquired in the form of chromosomal translocations, deletions, inversions, amplification and point mutations. Cancer arises from a stepwise accumulation of genetic changes that liberates neoplastic cells from the homeostatic mechanisms that govern normal cell proliferation. The recent application of transcriptional profiling to cancer had documented changes in the expression of thousands of genes, as normal cells undergo transformation into their neoplastic derivatives (Golub, 1999; Poron, 2000)

Normal cells require mitogenic signals before they can move from a quiescent state into an active proliferative state. These signals are transmitted into the cell by transmembrane receptors that bind distinctive classes of signaling molecules: diffusible growth factors, extra cellular matrix components and cell-to-cell adhesion/ interaction molecules. No type of normal cell can proliferate in the absence of such stimulatory signals. Many of the oncogenes in the cancer catalogue act by mimicking normal growth signaling in one way or another (Hanahan and Weinberg, 2000).

Cancer cells can also switch the types of extra cellular matrix receptor (integrins) they express, favouring ones that transmit progrowth signals. (Giancotti and Ruoslathi, 1999). Both ligand-activated growth factor (GF) receptors and progrowth integrins

Within a normal tissue, multiple antiproliferative signals operate to maintain cellular quiescence and tissue homeostasis, these signals include both soluble growth inhibitors and immobilized inhibitors embedded in the extra cellular matrix and on the surface of nearby cells. Antigrowth signals can block proliferation by two distinct mechanisms. Cells may be forced out of the active proliferation cycle into the quiescent (G0) state from which they may re emerge on some future occasion when extra cellular signals permit. Cancer cells evade these anti proliferative signals to prosper.

Programmed cell death is a major source of cell attrition. Acquired resistance toward apoptosis is a hallmark of almost all types of cancer. Besides this they exhibit limitless replicative potential. Disabling pRb and p53 tumour suppressor proteins enable cancer cells to continue multiplying for additional generations. There are different strategies used in cancer treatment - surgery, radiation and chemotherapy. The physical removal of tumour mass is the foundation of surgery. Radiotherapy and chemotherapy are exposure to toxic ionizing radiation or cytotoxic chemicals respectively to destroy cancer cells without having to find and remove them.

Chemotherapeutic agents can be classified into two groups based on their source/ origin- non-plant derived anticancer agents and plant derived anticancer agents. Non - plant derived anticancer agents include alkylating agents, antimetabolites, antitumour antibiotics, enzymes etc. Plant derived anticancer agents include various secondary metabolites.
1. Importance of Natural products in anticancer therapy.

The drug discovery process increasingly requires the availability of a large number of compounds. Basically there are two different approaches for drug discovery— one is screening and the other is to try to improve the efficacy of traditional medicine and to resolve the mode of action.

High -throughput screening has important role in drug discovery. However this technique requires a large number of compounds to be effective: these cannot be supplied by radical organic synthesis and hence two other sources - combinational chemistry and chemo diversity from nature are used. Chemo diversity in nature offers a valuable source, like secondary metabolites.

Nature is an attractive source of new therapeutic compounds as a tremendous chemical diversity is found in millions of species of plants, animals, marine organisms and microorganisms. The development of novel agents from natural sources presents obstacles that are not usually met when one deals with synthetic compounds. For instance, there may be difficulties in accessing the source of samples, identification and isolation of the active compound in the sample and problems in synthesizing the necessary amount of the compound of interest.

An analysis of the number of chemotherapeutic agents and their sources indicates that over 60% of approved drugs are derived from natural compounds. (Cragg et al.1997).

1.1 Anti-tumour compounds from plants.

One of the most significant chemotherapeutic agents is isolated from the periwinkle *Catharanthus roseus*. The introduction of *vinca*
alkaloid vincristine was responsible for an increase in the cure rate of Hodgkin's disease and some forms of leukemia. Vincristine inhibits microtubule assembly inducing tubulin self association into coiled spiral aggregates (Noble, R.L. 1990).

Another example of a highly active agent derived from a natural product is etoposide, which has produced high cure rate in testicular cancer when used in combination with bleomycin (Williams et al. 1987). Etoposide is an epipodophyllotoxin derived from Podophyllum peltatum and Podophyllum emodi. It has also significant activity against small cell lung carcinoma. It is a topoisomerase II inhibitor, stabilizing enzyme-DNA cleavable complexes leading to DNA breaks (Liu, 1989).

The Taxanes - paclitaxel and docetaxel-show impressive antitumour activity against breast, ovarian and other tumour types. Paclitaxel stabilizes microtubules, leading to mitotic arrest. Flavopiridol is one of the most important plant based agents currently in development, representing the first cyclin dependent kinase inhibitor to enter the clinic (Kelland, 2000). Flavopiridol is a synthetic flavone derived from the plant alkaloid rohitukine which was isolated from the leaves and stems of Amoora rohituka and later from Dysoxylum binectariferum. The mechanism of action of flavopiridol involves interfering with the phosphorylation of cyclin dependent kinases, hampering their activation and blocking cell cycle progression at growth phase I (G1) or G2.

A number of additional plant derived agents are currently under investigation. Homoharringtonine is an alkaloid isolated from the Chinese tree Cephalotaxus harringtonia and has shown efficiency against various leukemia. The mechanism of action is the inhibition of protein synthesis, blocking cell-cycle progression etc. 4-Ipomoeaanol is a pneumotoxic furan derivative isolated from the sweet potato Ipomoea
butatas (Rehm and Devor, 1993), and has been under clinical evaluation as a lung cancer specific antineoplastic agent. This compound is converted to DNA binding metabolites upon metabolic activation by Cytochrome p450 enzymes that are present in cells of the lung. Similarly, β-lapachone is a DNA topoisomerase I inhibitor that induces cell-cycle decay at G1 or S (synthesis) phase before inducing either apoptotic or necrotic cell death in a variety of human carcinoma cells, including ovary, colon, lung, prostate and breast.

Camptothecin, an indole alkaloid has shown significant antitumour activity against colorectal and ovarian cancer respectively (Bertino Jr., 1997). These compounds were initially obtained from the bark and wood of Camptotheca acuminata (Nyssaceae) and act by inhibiting topoisomerase I.

1.1.1 Camptothecin

The camptothecin analogues are a promising family of anticancer agents. It is a naturally occurring alkaloid found in the bark and wood of Chinese tree, Camptotheca acuminata, isolated by Wall et al in 1968 (Fig: 1.1).

Camptothecin has a unique molecular target, topoisomerase I, a key nuclear enzyme responsible for relaxing torsionally strained DNA (Hsiang et al, 1985). Currently camptothecins are the only well characterized inhibitors of topoisomerase I.

All the camptothecins have a basic five-ring structure with a chiral centre located at C-20 in the terminal lactone ring. The topoisomerase I inhibitory activity of these agents is stereo-specific, with the naturally occurring S-isomer of CPT being a much more potent inhibitor than the R-isomer. Substitutions at C-9 or C-10 can enhance water solubility without interfering with drug activity. In general, substitutions at C-12 decrease activity.
Topoisomerase I, the target enzyme of the camptothecins is a 100 Kda protein composed of 765 amino acids (D'Arpa et al, 1988). Expression of topoisomerase I is found in nearly all-mammalian cells at a high copy number, estimated at about 106 per cell. Unwinding of the DNA helix is essential for DNA replication or RNA transcription (Liu, 1989). This unwinding generates a torsional strain in the DNA resulting from super coiling of the helix above and below the region of ongoing nucleic acid synthesis. Topoisomerase I relax both positively and negatively super coiled DNA and allow these functions to proceed in an orderly fashion. Unlike other topoisomerases, topoisomerase I is constitutively expressed at relatively constant levels throughout the cell cycle, even in cells that are not actively dividing (Potmesil, 1994). Thus the inhibitors of topoisomerase I, such as the camptothecins, may potentially be active in tumours, that have low growth fractions and are resistant to other anticancer agents.

1.1.2 Topoisomerase- mechanism of action.

Topopisomerase I preferentially bind to super coiled double stranded DNA and cleaves the phosphodiester bond resulting in a single stranded nick. During this process, the topoisomerase I enzyme is temporarily bound by a covalent bond between a tyrosine residue at position 723 and the 3' end of the single stranded DNA (Tsao et al, 1994). This normally short lived intermediate has been called cleavable complex, and once it has been formed, free rotation of DNA molecule can occur about the remaining intact phosphodiester bond, allowing for the relaxation of the torsional strain in the DNA. Finally, religation of the strand break restores the integrity of the double stranded DNA, and the enzyme dissociates from the now relaxed double helix (Plate 1).
1.1.3 Mechanism of action of the camptothecin

In the presence of camptothecins, the topoisomerase I enzymatic reaction is altered, resulting in a drug induced stabilization of the cleavable complex. (Hsiang et al, 1985). Camptothecins interact non-covalently with the DNA bound topoisomerase I and inhibit the religation step of the reaction. Consequently, there is accumulation of stabilized cleavable complexes and a persistence of single stranded DNA breaks. However, this DNA damage alone is not toxic to the cell, because the lesions are highly reversible and can be repaired rapidly once the drug is removed. Instead, ongoing DNA synthesis is required in order to convert these stabilized cleavable complexes into more lethal DNA damage. Irreversible damage to the DNA occurs only when a DNA replication fork enters a cleavable complex, resulting in the formation of a complete double stranded break in DNA (Tsao et al, 1994) (Plate 1).

Although the camptothecin can clearly produce irreversible DNA damage in the presence of ongoing DNA synthesis, the events responsible for cell death have not been elucidated fully. The camptothecins can cause cell-cycle arrest, typically in the G2 phase. Camptothecin-induced DNA damage correlates with altered activity of the p34cdc2 / cyclin B complex, which has been tightly linked to regulation of the G 2 to M- phase transition in the cell cycle. The camptothecin cytotoxicity also has been associated with the endonucleolytic degradation of DNA, resulting in a pattern of DNA fragmentation similar to that described for apoptosis (Kharbanda et al, 1991).

Topoisomerase I cleavage is not a random event and the single stranded nicks appears with increased frequency at specific sequence sites in the DNA. Camptothecins does not stabilize all topoisomerase I
PLATE-1

Mechanism of DNA strand breakage and religation by Topoisomerase I

Superscoiled duplex DNA

Action of Camptothecin to stabilize the DNA topoisomerase I cleavable complex, resulting in DNA damage

CPT

DNA replication fork

3' 5'

4

3'

Interaction between the cleavable complex and DNA replication fork.
cleavable complex equally. Instead, enhanced stabilization of cleavage sites by camptothecin occurs, when a guanine residue is immediately 3’ to the phosphodiester bond normally cleaved by the enzyme. In the absence of the drug, topoisomerase I has no specific base preference at this location, suggesting that only a subset of the total topoisomerase I cleavage site is stabilized by camptothecin.

1.2 Plant Biotechnology for the production of alkaloids

In order to survive, plants have developed sophisticated mechanisms including an elaborate chemical arsenal of toxic substances, such as terpenes and alkaloids that inhibit the growth of other plants and make them unattractive to predators. Several plant-derived compounds are currently successfully employed in cancer treatment.

The term 'secondary metabolite' covers a wide range of chemically dissimilar compounds- having non-essential role for the survival of the individual plant cell, but, in contrast, where the in vivo role of these compounds has been ascertained, it is clear that they are often essential for the survival of the plant as a whole. Secondary metabolites are involved in the interaction between plants, plants and organisms etc.

1.2.1 Description of alkaloids

Of all known natural products, about 20% (i.e. About 16000) are classified as alkaloids. Most secondary metabolites are derived from just a few building blocks: the acetate C2 unit (polyketides), the phenyl alanine/tyrosine derived C9 unit (phenyl propanoids), the isopentenyl diphosphate C5 unit and some amino acids. For many of these alkaloids biological activities have been reported; but presently only 30
are commercialized. Most of these are medicines, but some are used as flavouring, poison and model compounds for pharmacological studies. The volume of production of these alkaloids is very much limited. Alkaloids such as quinine, quinidine etc. have a yearly production of 300 to 500 metric tons, ajmalicine about 3600 kg and compounds like vincristine, vinblastine in the kilogram range only (Veltkamp et al, 1985).

These chemicals are now produced by extraction from plant material that is cultivated in or sometimes collected from the wild. The main problems associated with this are—variable qualities and quantities of the plant material, plants that need to grow several years before they are ready for harvesting (e.g. \textit{Cinchona} bark) and over collection of endangered species (e.g. \textit{Taxus brevifolia}). Alternative methods like synthesis of natural products has been tried but the technique was not economically feasible.

Semi synthesis starting with more readily available precursors has been successful in some cases (e.g.: -the coupling of catheranthine and vindolin for the production of 3,4 anhydro vinblasin).

Plant biotechnology provides an alternative method for production of this economically important secondary metabolites. The major possibilities are—production by plant cell cultures, isolated plant enzymes production by means of genetically engineered plants or plant cell cultures and production of novel compounds.

1.2.1.1. Production of alkaloids by means of plant cell cultures.

Plant secondary metabolite biosynthetic pathways are very complicated and regulated by a variety of mechanisms. So as in the case of production of a primary metabolite like a protein, we cannot
introduce the entire interconnected pathways into a microorganism by genetic manipulation.

Plant cells are totipotent which means that each cell carries all the genetic information of all plant functions, including the biosynthesis of secondary metabolites, theoretically.

1.2.1.2. Bioconversion of available precursors.

Bioconversion is carried out by the conversion of a readily available precursor, probably by means of stereo specific reactions. For bioconversions, either plant cells (e.g.: production of L-DOPA from tyrosine by immobilized cells of Mucuna prurence (Pras, 1988) or isolated enzymes from the plant itself are used. Bioconversion with plant enzymes seems to offer great potential for biotechnological applications.

1.2.1.3. Production by genetically engineered plants or plant cell cultures

By unraveling the biosynthetic pathways and the regulation thereof on the level of enzymes and genes, it might become possible to identify genes, which could be subjected for genetic engineering. Various possibilities are - combining genes of secondary metabolism with other promoter genes, adding further copies of an already present gene to increase enzyme production, suppressing genes by antisense DNA (eg: blocking competitive pathways or blocking catabolism) and introducing parts of a pathway into another plant that is already capable of performing part of the synthesis. For example tryptophan decarboxylase gene is transferred from Catheranthus roseus into tobacco resulting in a plant producing significant amount of tryptamine (Goddijn, 1991).
1.3. Strategies to improve product yield from plant tissue culture

The production of alkaloids in plant cell cultures is a result of enormously complex set of interactions between cellular and extracellular compartments. The plant cell culture is a dynamic system in which the smallest change can have large and sometimes fatal consequences and hence provides many opportunities for manipulation to improve product yield.

1.3.1. Screening and Selection

Based on phytochemical data, a plant is selected and cultures are initiated from various types of tissues like leaf, anther, root, stem etc. From these several types of cultures like callus, cell suspension, shoot, root etc can be regenerated. Plant cells continuously grow and divide in cultures. The cultured cell lines are heterogeneous in their ability to produce useful compounds. They are mixtures of producing and non-producing cell lines for any one compound. Every cell line has its own unique characteristics and certain strategies for production improvement will work only with that special cell line.

To obtain a highly producing cell line, screening or selection is done. Unlike screening, selection is an active process, which deliberately favours only the survival of the wanted variant while the wild type cell does not survive. (Berline and Sasse, 1985.)

1.3.1.1 Screening

The methodology for screening includes two main strategies: cell aggregate cloning and single cell cloning.

**Cell aggregate cloning:** In the selection of high yielding plant cells, single cell cloning can be used to select the first most promising specific cells. But with an established cell line that produces relatively large
amounts of a secondary metabolite it is better to clone small cell aggregates of 10-100 cells when specific cells are desired (Yamamoto et al, 1982). Yamada and Saito (1981) repeated cell cloning using cell aggregates of *Coptis japonica* and obtained a strain, which grew, fastest and produced a higher amount of berberine and cultivated the strain in a 14l bioreactor.

**Single cell cloning:** Theoretically, single cell cloning should be the best method for isolating cells that yield large amounts of useful compounds. Practically, however, many clones started from a single cell are heterogeneous in their ability to produce useful compounds. Another important step is to determine the relative amount of useful compound present in a small cell aggregate clone. When pigments or compounds that fluoresce are desired, cells with high yields can be selected by sight alone or with the aid of a fluorescence microscope. Visual screening facilitated by the colour of the alkaloid, yielded highly productive cell lines of berberine (Sato and Yamada, 1984).

Screening is performed at different levels- species, specimens, organ, cell culture, single cells or protoplasts. In a comparison of 458 cell lines of *C. roseus*, which were all initiated from excised anthers and grown under identical conditions, several different production profiles, with respect to the presence of different types of indole alkaloids were obtained. Productivity varied from non-producing (32% of the cell lines) up to 1.5 total alkaloid per cell dry weight (Kurz et al 1985). A strain of *Euphorbia mili* was recognized to accumulate about 7 times higher amounts of anthocyanins than that of the parent strain after 24 selections (Yamamoto et al, 1982). The strong fluorescent properties of serpentine allowed determination of its concentration in individual
cells by flow cytometry and subsequent sorting of cells with high contents yielded a highly productive cell line (Brown et al, 1984).

1.3.1.2 Selection

Selection pressure on a plant cell population can be applied by the addition of selective chemicals to the medium and / or by the creation of selective growth conditions. Selective chemical agents like specific enzyme inhibitors can be added to the medium to obtain cells with increased enzyme activity. 4- methyl tryptophan was used for the selection of C.roseus cells with high tryptophan decarboxylase (TDC) activity, as only high producing cell lines can detoxify 4-methyl tryptophan and survive in the selection media. These cells with high TDC activity will have higher levels of tryptamine (Sasse et al, 1983).

Selection by changed environmental conditions was used for obtaining photomixotrophic and photoautotrophic cells. This yielded leaf like cells with well developed chloroplasts. Lupine cells of this type were able to produce sparteine and lupanine(Wink and Hartmann, 1980)

1.3.2. Influence of growth phases

The accumulation of most secondary metabolites in cultured cell is maximal during the stationary phase of growth, for example, the accumulation of anthocyanin in suspension cultures of Daucus carota (Noe et al,1980) and Catheranthus roseus (Hall and Yeoman,1986) and the accumulation of DOPA in callus cultures of Sizolobium hassjoo. However, Berlin et al,(1986) reported peaks of accumulation of betalins during the logarithmic phase in suspension culture of Chenopodium rubrum.

There was no obvious lag phase in batch cultures of hairy roots of Artemisia annua L. The artemisinin content decreased slowly during
the exponential phase, increased while the growth rate slowed down and remained consistent after the growth started (Liu et al, 1998).

1.3.3. Differentiation and culture type.

The formation in tissue cultures of several types of indole alkaloids was shown to be inseparably connected with morphological differentiation of the cells. For Catharanthus roseus, a time course study has been made of the formation of alkaloids in seedlings, during the first stage of development (De Luca et al, 1986). It was demonstrated that the formation of vindoline, and thus the synthesis of vinblastine, was connected to morphological differentiation. Similarly in Cinchona, seedlings, alkaloid formation is abundantly expressed, but (Aerts et al, 1990) in suspension cultures productivity is very low, if any. Also, the formation of Iboga indole alkaloids in Tabernanthera tissue cultures proved to be dependent on morphological differentiation (Sierra et al, 1991).

On the other hand cell cultures are capable of producing alkaloids, which had not yet been detected in the plant. Recently it was reported that a total of 85 novel compounds, including 23 alkaloids, have so far been isolated from 30 different plant cell cultures (Ruyter et al, 1989). In certain cases, cell suspension cultures yielded secondary metabolites at optimum levels. For example, Nabila et al (2003) reported the presence of rosmarinic acid in cell suspension cultures of Salvia fruticosa (5.1 mg/100mg DW), which was higher than the amount present in the root cultures of the same plant (2.62 mg/100mg DW). Similarly, anthraquinone production (2.9 g/l) was obtained from cell suspension cultures of Morinda elliptica (Abdullah et al, 1998).

1.3.4 Organogenesis

The development of a certain level of differentiation is considered to be important in the successful production of
phytochemicals by cell cultures. Propagation of differentiated tissues as root and shoot cultures offer an alternative when the desired compound is not formed in suspension-cultured cells. In *Digitalis purpurea* cultures, Hagimori (1980) showed stimulation of digitalis cardenolides production by organ dedifferentiation in callus tissues. A similar phenomena was also found in rotenone formation using *Derris elliptica* (Kodama, 1980) and morphinane alkaloid production using *Papaver somniferum*.

A very high concentration of ajmalicine (0.166% of DW) was obtained from *Catharanthus roseus* shoot cultures grown in MS medium (Satdive et al, 2003). Shoot cultures of *Withania somnifera* accumulated 0.04% of DW of withaferin A and 0.06% of DW of withanolide D (Ray and Jha, 2001) and the production increased with the number of shoots induced. Phatak and Heble (2002) reported that multiple shoot cultures of *Mentha arvensis* showed high level of pulegone.

In shoot cultures of *Catharanthus roseus* up to 2.6 µg of 3,4-anhydro vinblastine per gram fresh weight was detected. (Endo et al, 1987). Shoot cultures of *Cinchona ledgeriana* produced 3.5 mg of alkaloids (quinine and quinidine) per gram tissue. (Chung and Staba, 1987). Root cultures of this species were also able to produce these alkaloids; the productivity was increased 5 times by feeding tryptophan, a precursor of the alkaloids, to the culture (Hay et al, 1987). Alkaloids were also detected in root cultures of *Hyoscyamus niger* (Hashimoto and Yamada, 1983).

1.3.5 Hairy root culture

The use of *Agrobacterium rhizogens* has been recurring attention recently in secondary metabolism research. It inserts the Ri-plasmid into wounded tissue, causing the growth of very fine adventitious roots, so-called 'hairy roots'. These roots can be cultured in hormone
free medium and there are several examples of enhanced accumulation of secondary products, relative to non-transformed tissue (Flores, 1986; Yoshikawa and Furuya, 1987). Flores (1986) reported that all hairy root clones of *Hyoscyamus* plants grew faster than ordinary root cultures and produced the similar levels of tropane alkaloids to that accumulated in the intact plants.

For most alkaloid producing plants; hairy root cultures, have been initiated: *Hyoscyamus, Datura, Atropa, Nicotiana, Catheranthus, Cinchona and Peganum*. In general the alkaloid contents found in the hairy roots are similar to those found in normal plant roots. Another interesting aspect of hairy root cultures is that, with *A. rhizogenes* other new genes can also be introduced. Because of their rapid growth and the potential of genetic engineering, hairy roots, or transformed cells have been shown to be less sensitive to optimization procedures such as medium optimization (Berlin *et al*, 1990) and elicitation (Eilert *et al*, 1987). The biotechnological application of hairy root cultures is promising for a number of reasons- stable high level production, auxin independent growth, the stability for adaptation to fermentor systems.

Liu *et al* (1998) reported high artemisinin content in hairy roots of *Artemisia annua*. According to Deyu Xie *et al* (2000) 0.54 % (mg/g DW) artemisinin was obtained from hairy root cultures of *Artemisia annua*. Hairy root cultures of *Tropaeolum majus* produced glucotropaeolin in higher amounts compared to callus and cell suspension cultures (Marzena and Henryk, 1999).

*Psoralea* hairy root cultures (Bougaud *et al*, 1999) produced higher amount of daidzein compared to callus cultures. Nisit *et al* (1998) reported that hairy roots of *Solanum aviculare forst* produced 6.2 mg/g/l solasodine which was higher than that produced by callus (1.4 mg/g/l) and cell suspension (0.7 mg/g/l) cultures. The hairy roots exhibit
genetic stability. Cytogenetic analysis of the transformed root cultures of *Datura stramonium* showed great chromosome stability, which was not the case with normal root cultures. (Ana Maria Baiza et al, 1998). *Hyssopus officinalis* transformed roots were grown in Woody Plant Medium and the cultures produced rosmarinic acid (8.03% of DW) and lithospermic acid B (3.89% of DW) (Murakami et al, 1998).

1.3.6. Culture conditions

The environment of the selected plant cells or organ cultures should provide optimum conditions for the cells (or organs) to express their genetic information concerning secondary metabolite formation, resulting in optimum levels of secondary product. Optimization of environmental conditions is a matter of trial and error, because fundamental knowledge on the regulation of alkaloid formation is lacking. Conditions, which have been reported to influence the productivity of the culture, are - composition of the culture medium, light, temperature, bioreactor type and aeration.

1.3.6.1 Medium composition

The standardization of optimum production media include - standardization of the concentrations of various macro and micro elements (or in other words selection of the best basal medium suitable for production), standardization of concentrations of various growth regulators, various organic undefined supplements like coconut water, activated charcoal etc.

The effects of sucrose, phosphate, nitrogen and many other nutrients on indole alkaloid production have been extensively studied. At present, it is accepted that nitrogen and phosphate, promote growth and inhibit alkaloid production (Knobloch et al, 1980). The effect of nitrogen on alkaloid production is dependent on the amount of carbon available to the cells which makes the C/N ratio an important factor to
be taken into consideration. By the determination of the cellular C/N ratio, Rho and Andre (1991) identified three distinct growth phases - an active growth phase, an accumulation phase and a biomass decline phase (endogenous metabolism).

The optimum concentration of elements in a medium differs considerably in the case of cell, root and shoot cultures. Optimal concentration for maximal root growth was obtained for phosphate (0.56mM NaH$_2$PO$_4$.H$_2$O) and nitrate (12.97 mM KNO$_3$) respectively. *Rheum ribes* cell suspension cultures produced maximum amount of anthraquinones in a medium containing NO$_3$ and NH$_4$ in the ratio1:1 (Farzami and Ghorbanli, 2002). In the case of *Lavendula spica*, a combination of 2.5mM (PO4)$^{3-}$, 14.1 mM NO$_3$, 1 mM Fe $^{2+}$, 30 g/l sucrose promoted a 7-fold enhancement on the productivity of the blue pigment in comparison to the control medium. Among all these, phosphate exerted the higher beneficial effect and Fe $^{2+}$ showed to be essential for the accumulation of the pigment (Gabriela et al, 2003).

1.3.6.1.1 Carbon source

Sucrose and glucose are the preferred carbon source for plant tissue cultures. The concentration of carbon source affects cell growth and yield of secondary metabolites in many cases. The maximum yield of rosmarinic acid produced by cell suspension cultures of *Salvia officinalis* was 3-5 g/l, when 5% of sucrose was used, but it was 0.7 g/l in the medium containing 3% sucrose. 100 gm /l sucrose had a positive effect on salidoside synthesis from compact callus aggregates of *Rhodiola sachaliensis* (Xu et al, 1999).

Scagg et al (1990) obtained improved biomass and alkaloid production in batch cultures of *C.roseus* with increase of sucrose up to 6%. High concentrations of sucrose increased biomass accumulation, but caused callusing of the roots (Toivonen et al, 1989).
Higher sucrose concentrations decreased cell growth regardless of the hormonal composition and combination. 5% sucrose was more suitable for increase in total anthocyanin content. At higher sucrose levels, anthocyanin content was high due to the cessation of cell growth. The presence of a high concentration of sucrose in the culture medium could induce a considerable increase in the carbohydrate content of the cells, as was shown in _Catheranthus roseus_ cells (Schlatmann _et al_., 1994). A sucrose concentration of 9.9% and 8% greatly enhanced anthocyanin production in _Vitis_ cell cultures (Yamakawa _et al_., 1983) and a 5% sucrose concentration enhanced anthocyanin accumulation in _Populus_ (Matsumoto _et al_., 1970) and _Euphorbia milli_ (Yamamoto _et al_., 1989) suspension cultures.

1.3.6.1.2 Growth regulators.

Plant growth regulators or phytohormones affect growth and differentiation and thus affect secondary metabolism of cultured cells. The effect varies with the type and the quantity of phytohormones applied. Some important phytohormones are auxins, cytokinins, abscisic acid, gibberellins and ethylene.

Cytokinins were found to stimulate alkaloid biosynthesis in some tumourous cell lines of _C. roseus_ (Kodja _et al_., 1989). The effects observed were dependent on the cell line, the nature and the concentration of the cytokinin and the growth phase at which the cells were treated (Decendit _et al_., 1994). The response to cytokinin is dependent on the plasma membrane calcium influx with the involvement of calmodulins for alkaloid accumulation in suspension cultures of _C. roseus_ (Kodja _et al_., 1989, Merillon _et al_., 1991). _Duboisia myoporoides_ callus induced with 10 µM BA and 1µM NAA produced tropane alkaloids (Khanam _et al_., 2000).
In general, an increase of auxin levels such as 2,4-D in the medium stimulates differentiation of the cells and consequently diminishes the level of secondary metabolites. In suspension cultured cells of *C. roseus*, the addition of 2,4-dichlorophenoxy acetic acid (2,4-D) and other auxins caused a suppression of transcription of the genes encoding the enzyme tryptophan decarboxylase (*tdc*) and strictosidine synthase (*sss*) (Goddijn *et al.*, 1992; Pasquali *et al.*, 1992). The *tdc* mRNA levels were highly induced after the transfer of cells cultured on the medium containing 1-Naphthalene acetic acid (NAA; 10mM) and Kinetin (1mM) to hormone free medium. The addition of NAA, indole acetic acid (IAA) or 2,4-D to these cultures in hormone free medium caused a rapid transcriptional down regulation of *tdc* and *sss*. However, the effect was less pronounced for *sss* than for *tdc*, probably due to a higher stability of the *sss* mRNA. However production of L-Dopa by *Mucuna pruriens* (Brain, 1976), ubiquinone by *N- tabacum* (Ikeda *et al.* 1976) and diosgenin by *Dioscorea deltoidea* (Staba and Koul 1971) were stimulated by high levels of 2,4-D.

Low concentration of naphthalene acetic acid (0.025 mg/l) enhanced the growth of the roots, but inhibited the production of artemisinin. The growth and artemisinin production in hairy root cultures were greatly promoted by the addition of Gibberellins (GA3) to the medium. Its optimum concentration was 4.8mg/l (Satdive *et al.*, 2003).

1.3.6.2 pH of the culture medium

The alkaloids produced by suspension-cultured cells of *C. roseus* are accumulated inside specialized acidic vacuoles (Neumann *et al.*, 1983). The alkaloid productivity and the storage capacity can be influenced by the size of the pH gradient between the medium and vacuoles. Shifting the medium pH between low and high values is
used to release intra cellular alkaloids into the medium (Payne et al, 1988b; Asada And Shuler, 1989). Transformed roots of *Brugmansia candida* cultures accumulated scopalamine and hyoscyamine at pH 5.5. Lowering the pH to 3.5 and 4.5 reduced the accumulation of both alkaloids in the roots, but at a pH of 4.5, their release increased significantly (Sandra and Ana, 1999). Transient modifications of the medium pH values have been applied to immobilized cultures of *C.roseus* in order to release indole alkaloids. Jardin et al (1999) were able to obtain 100% release of the alkaloids produced by *C.roseus* immobilized cultures into the medium by periodical addition of acid or base solutions, without apparent cell lysis or decrease in the culture viability.

The initial pH of the culture media is generally adjusted to 5.5-6. For *Catheranthus roseus* cultures higher production was reported at pH 5.5(Doller et al, 1976). In the cultures of *Hyoscyamus nubicus* a 7-fold increase in alkaloid production was found on media with an initial pH of 3.5 (Koul et al, 1976).

1.3.6.3 Light

Light was found to stimulate serpentine production in *Catheranthus roseus* cultures (Knobloch et al, 1982), but an inhibitory effect on alkaloid production was observed in cultures of *Cinchona ledgeriana* (Payne et al, 1987) and *Scoporia parviflora* (Tabata et al, 1972). Drapeae et al (1987a) found a higher rate of accumulation of serpentine in cultures exposed to light for 15 hr per day instead of 24 hr. Catheranthine production was completely repressed in the absence of light. In large-scale fermentations the enhancement of product formation by light will be difficult because of technical and economical constraints.
Light favours chloroplast development in tissue cultures. Loyola-Vargas et al (1992) observed a rapid greening of dark brown \textit{C.roseus} calluses when transferred to white light. The increase of chlorophyll in these calli was similar to serpentine accumulation, suggesting a correlation between chlorophyll accumulation and the synthesis of serpentine. Calli exposed to red or blue light had a constant ajmalicine content and their serpentine content was always lower than that observed under white light.

1.3.6.4. Temperature

Culture temperature influences both growth and productivity. By lowering the culture temperature of \textit{C.roseus} cells from $27^\circ$C to $16^\circ$C a strong reduction in growth rate and a strong increase in alkaloid content was observed (Courtois et al, 1980). At a culture temperature of $38^\circ$C the alkaloid content was strongly reduced.

1.3.6.5 Cell density

Cell density affects productivity directly: the more producing cells per litre, the more products per litre. High cell densities require specific precautions with respect to oxygen and nutrient supply. By adjusting aeration (oxygen added to the aeration gas) and stirring, \textit{Coptis japonica} cells were cultured at densities of up to 75 mg/l (dry weight) in a culture tank filled with a hollow paddle type stirrer (Matsubara et al, 1989). The highest yield of berberine, 3.5 g/l was produced intracellularly in 5.5 g/l of the cell mass.

1.3.6.6 Aeration

Aeration of suspension cultures grown in bioreactors needs to be optimized with respect to dissolved oxygen and carbon dioxide levels. High aeration rates may lead to extensive (toxic) concentrations of dissolved oxygen, increased shear forces and sub critical levels of key
volatiles such as gaseous hormones (ethylene) and carbon di oxide (Hegarty et al, 1986).

In shake flasks the dissolved oxygen and CO₂ levels are strongly determined by the type of the closure of the flask. A cotton wool plug is highly permeable. Two layers of aluminium foil rapidly result in oxygen-limited growth of the culture. Silicone foam stoppers combine a good and reproducible gas exchange with low evaporation (Schripsema et al, 1990).

In *C.roseus* cell suspension cultures oxygen limitation caused growth suppression. Leckie et al (1991a) observed that by increasing the initial oxygen mass transfer coefficient (KLa) in batch cultures of *C.roseus*, it was possible to shorten the time that cells accumulate serpentine without altering the final yield. The increased availability of dissolved oxygen stimulated the oxidative metabolism responsible for the conversion of ajmalicine to serpentine. Recently, it has been demonstrated that high cell density cultures of *C.roseus* cultivated at high dissolved oxygen (DO) concentration had higher ajmalicine content than those cultivated at a lower DO concentration (Schlatmann et al 1994).

The type of closure can significantly alter the headspace gas composition of shake flask cultures. Depending on the permeability of the shake flask closure, gaseous compounds produced by the culture can accumulate in the headspace of a shake flask. In *C.roseus* cultures with limited gas exchange, ethylene and carbon di oxide accumulated and ajmalicine production was inhibited (Lee and Shuler, 1991).

The oxygen supply in bioreactors is dependent on the type of bioreactor. Stirred bioreactors are aerated in order to supply oxygen, and agitation is needed to improve mixing and mass transfer characteristics. On the other hand, agitation and forced aeration in this
kind of bio-reactor can lead to the removal of gaseous compounds produced by the cultures, like CO₂ and ethylene. Schlatman et al (1993) produced in a stirred bioreactor, the gas regime observed in shake flask by recirculating a large part of the exhaust gas back into the bioreactor.

1.3.7 Elicitation

The accumulation of secondary products by plant cell cultures can be enhanced by stress factors such as osmotic shock, addition of inorganic salts, heavy metal ions, fungal homogenates and UV irradiation. Increased alkaloid production is obtained by increased osmotic stress induced by the addition of osmotic agent like mannitol (Rudge and Morris, 1986), NaCl (Smith et al, 1987) etc. As a response to stress, the enzymes of the biosynthetic pathways other than the primary metabolism are induced, resulting in an accumulation of secondary products. These stress agents are normally referred as elicitors considering their capacity to induce the biosynthesis of stress metabolites or particularly the biosynthesis of phytoalexins. Homogenates of microorganisms may contain actual elicitor molecules, such as oligosaccharides or fatty acid derivatives. Other elicitors like cellulase, metal ions and UV light, release endogenous elicitors from the plant cell wall.

The best understood systems of microbial elicitors are those of fungal pathogens, in which case the regulatory molecules have been identified as glucan polymers, glycoprotein and low molecular weight organic acids.(Di Cosmo and Tallevi1985).Fungal elicitation has been an effective technique for enhancement of levels of secondary metabolites(Eilert et al, 1986) and as a tool to study metabolism (Srinivasan et al,1996). Most of the strategies employing fungal elicitation utilize fairly undefined mixtures, such as autoclaved fungal homogenates (Yoshikawa et al, 1993 b) or the fungal culture filtrate.
Effect of fungal homogenates and other elicitors on *C. roseus* cultures have been studied, but only the accumulation of ajmalicine and catheranthine was monitored (Sim *et al.*, 1994; Vazquez-Flota *et al.*, 1994). Examples of microbial elicitor induction include psoralen production in *Parsely* (Tietjen, 1983), diosgenin production in Mexican yam, and many others. Eilert and Eolters (1989) added autoclaved culture homogenate of yeast, *Rhodotorula rubra* into suspension cultures of *Ruta graveolens* and found that S-adenosyl L-methionine anthranilic acid N-methyl transferase was elicited.

Mizukami (1992) reported a transient increase in rosmarinic acid content in cultured *Lithospermum erythrorhizon* after addition of yeast extract (YE) to the suspension cultures. A maximum was reached in 24 hrs. When plant cells were treated with YE on the 6th day of cultivation, the level of rosmarinic acid increased 2.5 times and the activity of phenylalanine lyase in the cells rapidly increased before synthesis of rosmarinic acid.

The microbial and endogenous elicitors are able to bind (largely unknown) receptors. Following receptor binding a set of reactions are induced, in which ethylene, cAMP and Ca$^{2+}$ play a role and which eventually leads to *de novo* biosynthesis of the phytoalexins. After the addition of *Pythium aphanidermatum* filtrate to a *C. roseus* cell line, Seitz *et al.* (1989) found, besides the induction of the alkaloid pathway, an increase in the accumulation of phenolic compounds correlated with an induction of phenyl ammonia lyase activity (PAL). The maximum tryptamine accumulation occurred earlier than the elicitor mediated induction of tryptophan decarboxylase (TDC) activity.

Smith *et al.* (1987a) studied the effect of sodium chloride and sorbitol on catheranthine accumulation. The addition of NaCl at a concentration of 1.7 g/l to 5-day-old cultures increased the intracellular
accumulation of catheranthine by about 90% of the control value. Similar experiments with sorbitol at a concentration of 0.2M promoted an increase in catheranthine accumulation of about 35% of the control values, while the addition of potassium chloride resulted in an increase of catheranthine concentration of about 200%.

Backer-Royer et al (1990) studied the effect of some heavy metals on alkaloid production by *C. roseus* cultures. After adaptation for a number of subcultures of the cells to a concentration of 200μM copper and cadmium, the total indole alkaloid accumulation was increased.

The production of dimeric alkaloids by shoot cultures of *C. roseus* could be induced by irradiation with near UV light. The levels of vinblastin and leucrosine increased in cultures under irradiation with near UV light(370nm), whereas the levels of catheranthine and vindoline, precursors of dimeric alkaloids, decreased.

There are only a limited number of alkaloids whose production can be induced by elicitors, the effects are cell line specific and often transient. Elicitors have been found for the induction of biosynthesis of indole alkaloids (e.g. : - ajmalicine in Catheranthus *roseus* cultures (Eilert et al, 1986)). When the alkaloids are excreted in the medium after elicitation, the biomass can be recycled and re-elicitated. Successful re-elicitation procedures have been developed for the production of sanguinarine and ajmalicine in *Papaver* and *Catheranthus* cell cultures respectively (Kurz et al, 1987). Although the mechanism by which elicitors increase the productivity of secondary metabolite has not been elucidated, their stimulatory activity is quite significant if an appropriate elicitor is chosen to stimulate the synthesis of a particular compound. The use of specific elicitor components to determine their effects on several indole alkaloids in the pathway may lead to more precise strategies to enhance synthesis of desired alkaloids. In addition
to elicitor specificity, elicitor dosage and timing of harvest after elicitation are important factors in studying the enhancement of alkaloid levels. High dosage of elicitors has been known to induce hypersensitive response (Collinge and Susarenko, 1987; Roewer et al, 1992).

A dramatic increase in cell growth and hypericin production was observed after exposure to jasmonic acid in the case of Hypericum perforatum (Walker et al, 2002). The plant floral scent Methyl Jasmonate (MeJa) has been identified as a vital cellular regulator that mediates diverse developmental processes and defense response against biotic and abiotic stresses (Cheong and Choi, 2003). A jasmonate inducible AP2/ERF domain factor ORAC3, activated gene expression with a jasmonate responsive promoter element (JERE) in the promoter of the terpenoid indole alkaloid biosynthetic gene strictosidine synthase (van der Fits and Memelink, 2001).

The combined elicitors of malate and sodium alginate resulted in high catharanidine yield in shake flask cultures of Catharanthus roseus (Zhao et al, 2001). Methyl jasmonate elicitation yielded up to a 7-fold increase in total valepotriate content in transformed root cultures of Valerianella locusta (Nisit et al, 2002).

1.3.8. Precursor feeding

Addition of appropriate precursors or related compounds to the culture media sometimes stimulates secondary metabolite production. For C. roseus cultures, for example, it was found that increased indole alkaloid production was obtained after feeding with L-tryptophan, tryptamine, secologanin, loganin, loganic acid or shikimic acid (Zenk et al, 1985). Phenylalanine addition to Salvia officinalis cultures stimulated the production of rosmarinic acid (Ellis and Towers, 1970) and shortened the production time as well. The level of anticancer
compound- tripdiolide, produced by *T. wilfordii* cultured cells was increased by addition of 100 µg/l farnesol, which is dephosphorylated farnesyl phosphate, and an intermediate in the biosynthesis of terpenoids (Misawa, 1985).

Moreno *et al* (1993b) studied the effect of feeding different terpenoid precursors on alkaloid production. The addition of secologanin, its precursor loganin and loganic acid increased the accumulation of ajmalicine and strictosidine. In all cases a decrease in tryptamine content was observed. The highest alkaloid accumulation was observed with loganin feeding. This effect might be due to the higher chemical stability of loganin. The addition of mevalonic acid, an early precursor of the terpenoid pathway, did not affect alkaloid production.

A tryptophan decarboxylase over-expressing transgenic cell line T22 of *C. roseus* accumulated increased levels of alkaloids by feeding loganin or secologanin. Tryptamine or Tryptophan alone had no effect on alkaloid accumulation (Whitmer *et al*, 2002). But, Zhao *et al* (2001) had reported increased ajmalicine and catheranthine production by compact callus cultures of *Catheranthus roseus* on feeding with succinic acid, tryptamine and tryptophan.

Similarly, cell suspension cultures of *Taebernaemontana elegens* produced serotonin after feeding with tryptamine and loganin (Lucumi *et al*, 2002).

1.3.9 Biotransformation

Instead of the addition of a particular compound as a precursor into the culture medium of plant cells, a suitable substrate compound may be bio-transformed to a desired product using plant cells. Biotransformation of β- methyl digoxin using *D. lanata* cells has been extensively investigated by Reinhard and alfermann(1982). Similarly
the conversion of (-) codeinone to (-) codeine was carried out by cells of *Papaver Somniferum* (Furuya *et al*, 1978).

Bioconversion rate can be optimized by using immobilized cells, cell free preparations or immobilized (or purified) enzymes. Furuya *et al* (1984) reported the reduction of codeinone to be more efficient with immobilized *P. somniferum* cells than with suspended cells. Strictosidine could be produced in large quantities using immobilized strictosidine synthase (Pfitzner *et al*, 1982).

1.3.10 Inducers.

Some bioregulators were studied for their effect on alkaloid production. Simpson & Kelly (1989) studied the effect of cytochrome p-450 inducers phenobarbital and β-naphthoflavone and cytochrome p-450 inhibitor ketoconazole on alkaloid production. *C. roseus* cultures, growing on a medium that supports alkaloid biosynthesis, in the presence of phenobarbital, showed higher serpentine accumulation, but no induction was observed in the presence of β-naphthoflavone. The inhibitor ketoconazole was found to inhibit serpentine accumulation in concentrations that have no effect on growth.

In the last years, much attention has been paid to the role of salicylic acid and jasmonates as signal molecules in the defense response (Malami and Klessig, 1992). Salicylic acid (90.1 mM) was found to have a weak induction effect on steady state mRNA’s, 8-24 h after the exposure of the cells (Pasquali *et al*, 1992). In cell suspension cultures of several plant species, jasmonates were able to induce the accumulation of secondary compounds and the induction of enzymes involved in their biosynthesis (Gundlach *et al*, 1992).

1.3.11 Immobilization

Fixation of plant cells in a matrix, for example, polyurethane foam or entrapment of the cells in calcium alginate beads provides an
artificial surrounding for the cells, which protects them from hydrodynamic stress. High cell densities inside the matrix also allow the cell-to-cell contact and communication. Immobilized biomass is easily separated from the medium, which is useful in production and biotransformation systems. The advantages of immobilization are - the extended viability of cells in the stationary (and producing) stage, enabling maintenance of biomass over a prolonged time period, simplified downstream processing, the promotion of differentiation, linked with enhanced secondary metabolism, higher cell density enabling a reduced bioreactor size, thereby reducing costs and the risk of contamination, reduced shear sensitivity (especially with entrapped cells), promotion of secondary metabolite secretion, in some cases, flow-through reactors can be used enabling greater flow rates. An immobilization system, which could maintain viable cells over an extended period of time and release the bulk of the product into the extra cellular medium in a stable form, could dramatically reduce the costs of phytochemicals production in plant cell cultures.

Large scale immobilized alkaloid production systems have been developed for *C. roseus* and *Thalictrum rugosum*, using glass fiber mats (Di cosmo, 1990). *Cinchona pubescens* cells were efficiently immobilized in a semi rigid matrix of polyurethane foam (Robins, 1986). Polyurethane foam was also used for biotransformation of codeinone to codeine (Corchete and Yeoman, 1989). Immobilization of *Coffea* cells in calcium alginate resulted in a 13 fold increase of purine alkaloid production (Haldimann and Brodelius, 1987).

1.3.12 Permeabilization

For several reasons in a plant cell biotechnological production, the release of product from the biomass could be of interest for process design. Efficient downstream processing of products is facilitated when
the product can be recovered from the medium. It is essential to
determine the quantity of the product of interest not only in the
biomass, but also in the medium. If spontaneous release of the product
is not accomplished, however, there still exist ways to force release of
the product. Knowledge of the mechanisms involved in product
storage is therefore essential. Product release brought about by
lowering the pH of the culture medium was shown in the case of
\textit{Cinchona ledgeriana}, \textit{Nicotiana rustica} (Robins \textit{et al.} 1988), and
\textit{Catheranthus roseus} (Majerus and parcilleux, 1986). Several studies have
been carried out on the stimulation of product excretion by extraction
of the culture broth with an inert organic solvent (Beiderbeck, 1982).
Adsorption of alkaloids from the culture broth with adsorbents, mostly
XAD resins, also showed promising results.

The technique used to facilitate the forced release of metabolites
is known as permeabilization. Various strategies have been used:
chemical permeabilization, electric permeabilization and iontophoteric
permeabilization (Brodelius \textit{et al}, 1990). Chemical permeabilization
comprises the use of, among others, organic solvents such as DMSO,
chloroform and surface active chemicals (as Triton X-100). Results
with the various techniques used are always achieved at the cost of cell
viability and this might hamper further applications (Nijkamp \textit{et al},
1990). A short treatment with Tween 20, combined with L-phenyl
alanine feeding amplified the level of hyoscyamine released into the
medium in the case of tropane alkaloid production from \textit{Datura innoxia}
transformed roots (Boitel-Conti \textit{et al}, 2000).

1.3.13 Biosynthesis, enzymology and regulation

At present, several enzymes involved in the indole alkaloid
biosynthetic pathway have been isolated and characterized and the
genes encoding some enzymes of the pathway have been cloned.
Scheme 1.1 Biosynthesis of Camptothecin

Photosynthesis → Hexoses and Monosaccharides → Polyphenol pyruvic acid

Pyruvic acid → Acetyl CoA → Acetoacetyl CoA → β-hydroxy-β-methyl-glutaryl CoA → Mevalonic Acid → 5-Phospho Mevalonic acid → 5-Pyrophospho Mevalonic acid → Isopentenyl pyrophosphate → Geranyl pyrophosphate → Geraniol → 10-hydroxy geraniol

Cinnamic acid

Acetyl CoA → Acetoacetyl CoA → β-hydroxy-β-methyl-glutaryl CoA → Mevalonic Acid → 5-Phospho Mevalonic acid → 5-Pyrophospho Mevalonic acid → Isopentenyl pyrophosphate → Geranyl pyrophosphate → Geraniol → 10-hydroxy geraniol

Camptothecin

Strictosidine

Tryptophan

Tryptamine

Secologanin

Loganin

Secologanic acid

Secologanic acid
The biosynthesis of the *Catharanthus* alkaloids arises from two different pathways. Tryptamine is formed by the decarboxylation of tryptophan by the enzyme tryptophan decarboxylase (TDC). Secologanin is formed through several enzymatic steps initiated after the hydroxylation of geraniol by the enzyme geraniol-10-hydroxylase (G10h). Strictosidine synthase (SSS) couples these two products to form 3α (S) strictosidine, the universal precursor of all monoterpenoid indole alkaloids (Kutchan, 1993).

Recently, anthranilate synthase (AS), an enzyme involved in the tryptophan biosynthesis, has been purified from *C. roseus* cultures (Poulsen *et al.*, 1993). This enzyme showed a similar induction pattern to TDC. The enzyme activity is highly induced after the transfer of cells to induction medium and by the addition of fungal elicitors (Moreno *et al.*, 1991).

Immunoscreening of a cDNA expression library resulted in the isolation of cDNA for both *tdc* and *sss* genes (De Luca *et al.*, 1989). It was also demonstrated that both genes are present as single copy genes in *C. roseus* genome (Pasquali, 1994). The regulations of expression of both genes have been extensively studied (McKnight *et al.*, 1990). The addition of elicitors rapidly induced transcription of *tdc* and *sss* genes (Pasquali *et al.*, 1992), which were shown to be coordinately regulated at transcriptional level.

1.3.14 Alkaloid storage compartment

In plants, the site of alkaloid biosynthesis is often separated from the site of storage, for example tropane alkaloids are produced in the root and stored in the leaves. This means that alkaloids need to be excreted from the biosynthetically active cells and then transported to and taken up by the storage cells. In undifferentiated tissues like cells in suspension culture, this transport mechanism is likely to be seriously
affected resulting in the leakage of compounds to the extra cellular medium.

Uptake of alkaloids by isolated vacuoles and transport mechanism over membranes has been studied in detail. Carrier mediated active transport over membranes (and thus a specific uptake) has been postulated for indole alkaloids (Deus-Neumann et al, 1984) and isoquinoline alkaloids (Deus-Neumann et al, 1986). On the other hand, a passive transport mechanism in which the neutral alkaloids freely diffuse through the membrane and the protonated alkaloids are stored in the acidic vacuole (on trap mechanism) has been advocated for indole alkaloids in the cells of *C.roseus*, (Neumann et al, 1983) and quinoline alkaloids in the cells of *Cinchona ledgeriana* (Parr et al, 1986).

Ajmalicine diffuse across the tonoplast, driven by the pH gradient between cytosol and vacuole. It is subsequently trapped inside the vacuole in the form of serpentine, which is formed via oxidation of ajmalicine by the basic vacuolar peroxidases (Brodelius, 1988).

Little is known about excretion of alkaloids. For the ion trap mechanism the pH difference over the membrane is the driving force for transport. A changed pH gradient caused by, for example, a low cellular pH, might thus cause excretion. Excretion of the alkaloids at low pH has been reported for *Nicotiana* and *Cinchona* (Robinsl, 1988).

Storage capacity in a cell culture is available inside the cells (e.g., vacuoles) or in the extra cellular compartment. The storage facilities of the latter can be remarkably improved by the addition of liquid organic phases, resins or other sorbents to the medium. Addition of XAD-7 to *Catharanthus roseus* cultures resulted in increased yields of indole alkaloids (Payne et al, 1988).

Increased yields of nicotine and anabasine were obtained by adding XAD-2 and XAD-4 resins to *Nicotiana* transformed
roots. (Rhodes et al, 1986). Robins et al (1986) reported that the addition of XAD-7 into suspension culture of Cinchona ledgeriana stimulated the production of anthraquinones to 15 times of the control value. Addition of activated charcoal in the medium stimulated the yield of coniferyl alcohol upto 60 fold in Maticaria chamomile culture (Wilson et al, 1990).

The number of storage cells limits the internal storage capacity. Excreted alkaloids which are dissolved in the medium are exposed to catalytic activities in the medium (e.g. peroxidase) and can thus be degraded; as was demonstrated for quinolizidine alkaloids in lupine cell cultures (Wink, 1985).

1.3.15 Genetic modification

Plant secondary metabolites are active targets for genetic engineering. Mahamoud and Croteau (2001) reported increased flux through the mono terpene pathway in mint plant, resulting in an increased essential oil yield. They exploited a gene encoding deoxyxylulose phosphate reductoisomerase (DXR), which converts DXP to 2-C-methyl erythritol -4-phosphate and constituted the first committed step in the DXPs pathway of terpenoid biosynthesis, by replacing the DXR promoter with a strong constitutive promoter.

Shintani and Della Penna (1998) increased expression of a gene encoding γ-tocopherol methyl transferase, the enzyme that converts γ-tocopherol (Low vitamin E activity) to α-tocopherol (high vitamin E activity) in Arabidopsis. Seeds of the transgenic plants inverted the ratio of the α to the γ form, thereby increasing 10 -fold the seed vitamin E activity.

Several techniques are now available to change the genetic information in plant cells: transformation with Agrobacterium species, direct DNA injection and protoplast fusion. Novel techniques in molecular biology have found rapid application in plant cell
biotechnology, as, for example, the use of antisense DNA. (Mol et al 1990; Zenk et al 1988).

It is well known that many secondary metabolites are restricted to a specific organ, tissue or cell type, which may be the site of synthesis, accumulation or both. For example, tropane alkaloid biosynthesis is known to be developmentally regulated and occurs in a specific cell type (Nakajima & Hashimoto, 1999; Suzuki et al, 1999). Similarly, hairy root cultures of the endangered species Atropa belladona (Zarate, 1999) display high accumulation of major tropane alkaloids, atropine and scopolamine, with atropine levels similar to intact non-transformed roots.

Knowledge concerning the genes involved in alkaloid biosynthesis can be exploited in several ways to obtain higher yields. Rate limiting steps in the biosynthesis may be overcome by increasing the concentration of the enzyme. For this approach knowledge of the rate limiting steps and characterization of the enzymes are prerequisites. Transgenic A. belladona plants have been obtained by introducing h6h gene from Hyoscyamus niger under the constitutive control of the CaMV 35S promoter following the A. tumifaciens method (Yun et al, 1992). The regenerated transgenic plants showed a dramatic change in alkaloid composition with scopolamine being the predominant alkaloid present in the aerial parts, and in the branched roots the highly efficient conversion of hyoscyamin into scopalamin was particularly enhanced.

Recently the number of characterized enzymes involved in the biosynthesis of alkaloid has increased exponentially. The pathways leading to berberine (Zenk et al, 1988) and ajmalicine (Stockigt and Schuebel, 1988) have been fully characterized. Substantial progress has been made with scopolamine biosynthesis (Hashimoto et al, 1990) and
early steps in indole alkaloid biosynthesis (Kutchan et al, 1988; Goddijn, 1991)

After purification of an enzyme one is able to identify the gene encoding for this protein, which opens ways to genetic modification. Genes for various enzymes have been cloned, and several transgenic organisms have been obtained, for example, *E.Coli* strain producing strictosidine synthase (Kutchan, 1989) and tobacco plants containing tryptophan decarboxylase activity (Goddijn, 1991). In some cases microbial genes were also used for manipulation, for example, lysine decarboxylase (Herminghaus et al, 1990), Ornithine decarboxylase (Hamill et al, 1990) and cytochrome p450 (Saito et al, 1989).

Attempts at increasing flux by manipulating the activity of single enzyme have met with mixed success. Most efforts aimed at increasing flux through biochemical pathways have targeted slow steps, where enzyme concentration is theoretically limiting, or regulatory enzymes that catalyze irreversible reactions that are regulated by specific effector molecules. Over expression of the gene encoding phytoene synthase, which catalyses the first committed step in carotenoid biosynthesis has had very different outcomes depending on where and when the gene was expressed (Zarate et al, 1999).

Addition of novel genes is another approach to induce organisms to replicate heterologus cDNAs to express a foreign gene in a host organism. Single step enzymes, as well as part of or all of a complete pathway can be transferred from one organism to another.

Stricosidine β-D-glucosidase gene involved in the terpenoid indole alkaloid pathway of *C.roseus* has been introduced via *A.lumifaciens* and expressed in suspended tobacco cells (Zarate et al, 2001). Furthermore, genetic transformation of the recalcitrant *C.roseus* plant through particle bombardment has resulted in insertion of the
reporter gene (gus, gfp) (Zarate et al., 1999) suggesting that the insertion of the above mentioned terpenoid indole alkaloid genes could be usefully pursued.

Down regulation of specific genes is another approach. Comparative metabolic pathways can be blocked by means of transformation with antisense RNA, RNA transcribed in reverse orientation from mRNA, which prevents translation. A completely different approach to the control of metabolism is by the insertion of of regulatory gene or genes inducible by exogenous signals (e.g., elicitors, stress stimulates etc.). It is widely accepted that biosynthesis of many secondary metabolites in plants can be induced by the stress inducing molecule - Methyl Jasmonate. In C. roseus, the gene Orca3, a jasmonate responsive APETALA 2 (AP2) domain transcription factor has been isolated (Van der Fits & Memelink, 2000). Over expression of Orca 3 in C. roseus cultures resulted in enhanced expression of several biosynthetic genes tdc, str, sgd, cpr (cytochrome p450 reductase). dh4 (deacetoxy vindoline 4- hydroxylase) and dat (acetyl CoA 4-0-deacetylvindoline) were not induced, suggesting that these two latter genes were not controlled by Orca 3.

Constitutive expression of a tomato cDNA in tomato resulted in dwarfism and lower lycopene levels in the fruit (Fray et al., 1995). By contrast, significant increase in fruit carotenoids were obtained when the bacterial phytoene synthase gene crt B was expressed in a fruit specific manner (Hirschberg, 2001). The same crt B gene expression resulted in 50 fold increase of α and β carotene in canola seeds (Shewmaker et al., 1999). These results highlight the importance of adjoining aspects of metabolism when manipulating a metabolic pathway.
Ye et al. (2000) produced transgenic plants over expressing daffodil phytoene synthase gene (PSY), daffodil lycopene β cyclase gene (LCY-β) and the Erwinia phytoene desaturase gene Crt I. The resulting rice seeds accumulated high levels of β carotene and were known as 'golden rice' popularly.

Another recent development in this field is that, De Jesus and Weathers (2003) reported that tetraploid clones of Artemisia annua hairy roots produced six times more artemisinin, than the diploid parent.

1.3.16 Combination of treatments

Many of the treatments described already have only a limited value: they only can be used for specific cell lines, the effect is transient, or it causes cell death. At present much attention is paid to combined strategies, for example, elicitation of alginate-immobilized Catharanthus roseus cells led to a 45 fold increase of ajmalicine production (90mg/l), which was adsorbed from the medium by the resin XAD-7 (Flores, 1987).

1.3.17 Large-scale production

There are mainly three interrelated categories of problems hampering the development of industrial plant biotechnology: biological problems, economical problems and technological problems.

Routien and Nickell took the first patent in large-scale culture in 1956. In the past decade most work on large scale culture used various types of low-shear bioreactors (e.g.: airlift bioreactors), as plant cells were thought to be very sensitive to shear stress. But recently, Scragg et al. (1990) showed that many plant cells are shear-tolerant and can be grown without any problem in stirred tank bioreactors. Large investments in all sorts of new reactors would be a major constraint for the commercialization of plant cell biotechnology.
1.3.17.1 Optimizing production

In order to achieve an industrial production one has to obtain a stable, high producing cell line of the plant of interest. For this two approaches are being used.

1. Screening and selection for high producing cell lines
2. Optimization of growth and production medium.

Also, understanding of biochemical pathways and their regulation; the effects of environmental conditions on growth and production need more investigation.

The productivity of plant cell cultures per amount of reactor volume per unit time has to be increased considerably in order to make most processes economically feasible. For the design of the process (production volume, process type, bioreactor size and type) detailed knowledge of both kinetics of growth and product formation and physical properties (rheology, shear sensitivity) is essential.

1.3.17.2 Process Design

Cell suspension cultures offer some advantages over the more highly organized structures. In cell suspension cultures, transport of nutrients, Oxygen, precursors and/or elicitors to the cells is not hampered by the limited diffusion, which may occur in relatively large tissue structures; and cell suspensions can be treated as cultures of microorganisms. An important disadvantage of cell suspension cultures is the low degree of cell differentiation, which is relatively low in the case of callus cultures but high in the case of root or shoot cultures. The disadvantage in this case is also the necessity of developing new technology.

Other important constraints determining the design of the process are the relation between the growth and product formation (growth associated or non growth associated), the potential of plant
material to be employed for de novo synthesis or for biotransformation and the occurrence of product release or accumulation.

Fermentation processes can be carried out in batch, fed batch or continuous mode. These different process types can be roughly characterized as follows. In the batch cultures, nutrients and inoculums are brought into the reactor, and after a period of time, the reactor contents are harvested and processed. Some characteristics of batch culture are limited process control, productivity loss as a result of cleaning and sterilization after each fermentation run, and large flexibility in the process. In fed batch culture, the nutrients are supplied to the reactor during the process. In a continuous culture, a continuous supply of nutrients and a continuous harvest of reactor contents (medium or medium plus biomass) can be achieved. Some characteristics are better process control, more equipment needed, greater equipment costs, contamination of the process has greater consequences, and relatively low flexibility.

Continuous culture without cell retention is only employable with growing biomass (non growing biomass will be washed out with the effluent flow). For alkaloid production this type of continuous culture is only suitable if alkaloid formation is growth associated, which greatly restricts application. Also, large cell aggregates or hairy roots are not easily transported, unless the size of the piping is large compared to the aggregate size. A continuous process with cell retention can be applied when product release can be achieved.

1.3.17.3 Bioreactors

Artemisia annua hairy roots were grown in liquid -phase bubble column and gas phase nutrient - mist reactors. In most cases, the bubble column reactor accumulated more biomass than the mist reactor, the highest final biomass concentration observed were 153g
DW/l in the bubble column reactor and 14. g DW/l. in the mist reactor. The average specific growth rate in the mist reactor was essentially constant and independent of the biomass concentration at the beginning of the mist mode (Kim et al, 2002)

1.3.17.4 Downstream processing

An essential part of a production process for alkaloids is product recovery. Down stream processing of alkaloids can be classified into two types- product stored in the biomass and product released from the biomass.

Recovery of alkaloid from biomass.

The problem of product recovery from biomass can be dealt with in two ways,

1. The biomass could be processed comparably to plant material in a classic alkaloid production process.

2. The biomass could be fractionated and after separation of solids and liquid, the liquid phase could be processed comparably to spent medium containing excreted alkaloids. In classic alkaloid recovery the alkaloids are extracted from the biomass.

In general, most alkaloids are basic, and this property is commonly used in most purification methods. There are mainly 3 types of extractants-

Water insoluble solvents:

Most alkaloids are commonly present in the plant as organic salts. In order to solubilise the alkaloids, the crude biomass extract is made basic by the addition of, potassium hydroxide, potassium carbonate or ammonia. At the resulting high pH, the alkaloids are mainly present in their neutral form. The neutral alkaloids are then easily extracted from the aqueous phase with a water insoluble organic solvent, for example, dichloromethane or chloroform.
Water soluble solvents: -

The alkaloids are extracted from the biomass with an alcohol, (e.g. methanol, ethanol or propanol). Most alkaloids, both salts and free bases are readily soluble in alcoholic solvents.

Acidified water: - The alkaloids are extracted from the biomass with acidified water. At a low pH, most alkaloids are protonated and readily soluble in aqueous solution. Subsequently, the extract is made basic and the neutral alkaloids are extracted from the aqueous phase with an organic solvent.