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Production of Secondary Metabolites Using Plant Cell Cultures

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Abstract Plant cell cultures represent a potential source of valuable secondary metabolites which can be used as food additives, nutraceuticals, and pharmaceuticals. The synthesis of phytochemicals by the cell cultures in contrast to these in plants is independent of environmental conditions and quality fluctuations. In many cases, the chemical synthesis of metabolites is not possible or economically feasible. Moreover, the natural food additives are better accepted by consumers in contrast to those which are artificially produced.

In this chapter, the process for obtaining the secondary metabolites from plant cell cultures is represented as a multi-stage strategy, and each link should be described

according to specifications of cell cultures or products. For the establishing of high-producing and fast-growing cell lines, the parent plants should be selected. The expression of synthetic pathways can be influenced by environmental conditions, the supply of precursors, and the application of elicitors, and it can be altered by special treatments such as biotransformation and immobilization. The efficiency of bioprocessing can be increased by the simplification of methods for product recovery, based on the principle of continuous product release into the cultivation media. This can be induced through influencing membrane permeability by chemical or physical factors, e.g., high electric field pulses.

The combined research in the fields of establishment of in vitro cultures, targeting of metabolite synthesis, and development of technologies for product recovery can exploit the potential of plant cells as sources of secondary metabolites.

Keywords Biotransformation · Elicitors · Exudation · Food additives · Immobilization · Membrane permeabilisation · Plant cell culture

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BA	Benzyladenine
DMSO	Dimethylsulfoxide
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
FW	Fresh weight
IAA	Indole-3-acetic acid
NAA	1-Naphthalene acetic acid

1 Introduction

Plant cell culture systems represent a potential source of valuable secondary metabolites which can be used as food additives (flavors, fragrances, and colorants), nutraceuticals, and pharmaceuticals [65]. The problems related to obtaining of secondary metabolites from plants include environmental factors, political and labor instabilities in the producing countries, uncontrollable variations in the crop quality, inability of authorities to prevent crop adulteration, and losses in storage and handling. In many cases, the chemical synthesis of these is either extremely difficult or economically infeasible [42].

The production of useful and valuable secondary metabolites from cell cultures is an attractive proposal. Cell culture technology was developed as a possible tool to both study and produce plant secondary metabolites. The evolving importance of the secondary metabolites has resulted in a high level of interest in the possibility of altering their production through improving cultivation technology [65]. During the past four decades, research has concentrated on the use of plant cell cultures, particularly in Japan, Germany,

and the USA, for the commercial production of a wide range of secondary metabolites, in the same way as bacteria and fungi have been used for antibiotic or amino-acid production [40]. For example, there has been tremendous success in the production of shikonin from cell cultures of *Lithospermum erythrorhizon*, berberine from *Coptis japonica* [36], and sanguinarine from *Papaver somniferum* [11].

This chapter reviews the recent advances in the optimization of environmental factors for metabolite production by plant cell culture, new developments in plant cell bioprocesses, and emerging research on phytochemical recovery.

2
Plant Cell Culture Technique

2.1
Plant Secondary Metabolites Produced by Cell Cultures

Plants form an important part of our everyday diet, and their nutritional values have been intensively studied for decades. Over 80% of the approximately 30 000 known natural products are of plant origin [45], which is estimated to be nearly fourfold greater than that in the microbial kingdom.

For centuries, humans has been used plants as a source of carbohydrates, proteins, and fats for food and shelter. In addition to essential primary metabolites, higher plants synthesize a wide variety of secondary metabolites.

Plant secondary metabolites can be defined as compounds that have no recognized role in the maintenance of fundamental life processes in plants, but they do have an important role in the interaction of the plant with its environment. They mostly have an ecological role as attractants of pollinating insects or in defence mechanisms against predators. The distribution of secondary metabolites in plants is far more restricted than that of primary metabolites; a compound is often only found in a few species, or even within a few varieties within a species. The production of these compounds is often low (less than 1% DW), and it depends greatly on plant species and plant's physiological and developmental stage [42]. Moreover, secondary metabolites often accumulate in the plant in specialized cells or organs.

2.2
Application of Plant Cell Cultures

Many plants containing high-value compounds are difficult to cultivate [49]. At the same time, the chemical synthesis of plant-derived compounds is often not economically feasible because of their highly complex structures and specific stereo-chemical characteristics. The production of valuable secondary

metabolites in plant cell cultures is an attractive alternative to the extraction of the whole plant material.

Plant cell cultures were first established in the late 1930s. However, it was only in 1956 that Pfizer Inc. filed the first patent for the production of metabolites by cell cultures [50]. Larger quantities of visnagin and diosgenin were isolated from cell cultures than from the whole plant [5]. In 1978, Zenk (1978) demonstrated the outstanding metabolic capacities of plant cells and highlighted the spontaneous variability of plant cell biosynthetic capacity. This natural variability is exploited to identify high-yielding cultures for use on an industrial scale [4]. Since the late 1970s, research and development in this area has seen a high increase in the number of filed patent applications. In 1983, shikonin was produced by plant cell cultures on an industrial scale for the first time by Mitsui Petrochemical Industries Ltd. [20].

Currently, plant cell culture has direct commercial applications as well as value in basic research into cell biology, genetics, and biochemistry.

The application of plant cell culture has three main aspects [65]:

1. **breeding and genetics:**
   - **micropropagation** – using meristem and shoot culture to produce large numbers of identical individuals;
   - **selection** – screening of cells, rather than plants, for advantageous characters;
   - crossing distantly related species by **protoplast fusion** and regeneration of the novel hybrid;
   - production of dihaploid plants from **haploid cultures** to achieve homozygous lines more rapidly in breeding programs;
   - **transformation**, followed by either short-term testing of genetic constructs or regeneration of transgenic plants;
   - **removal of viruses** by propagation from meristematic tissues;
2. **model system** for study of plant cell genetics, physiology, biochemistry, and pathology;
3. **production of secondary metabolites** – growth in liquid culture as a source of products.

This chapter reviews the recent advances in metabolite production by plant cell cultures.

Application for Production of Secondary Metabolites

When compared with the intact plant, cultured plant cells often produce different quantities with different profiles of secondary metabolites and these quantitative and qualitative features may change with time [59].

As shown in Table 1, some metabolites in plant cell cultures can be accumulated with a higher titer compared with those in the parent plants,

Table 1 Product yield from plant cell cultures compared with the parent plants

Product	Plant	Yield (% DW) Culture	Plant	Culture/ plant	Refs.
Ajmalicine	*Catharanthus roseus*	1.0	0.3	3.3	Lee and Shuler 2000
Anthraquinones	*Morinda citrifolia*	18	2.2	8	Zenk 1977
Berberine	*Coptis japonica*	13	2	3.3	Fujita and Tabata 1987
Caffeic acid	*Vanilla planifolia*	0.02	0.05	4	Knorr et al. 1993
Ginsenoside	*Panax ginseng*	27	4.5	6	Matsubara et al. 1989
Nicotine	*Nicotiana tabacum*	3.4	2.0	1.7	Mantell et al. 1983
Rosmarinic acid	*Coleus blumei*	27	3	9	Petersen and Simmond 2003
Shikonin	*Lithospermum erythrorhizon*	20	1.5	13.5	Kim and Chang 1990
Ubiquinone-10	*Nicotiana tabacum*	0.036	0.003	12	Fujita and Tabata 1987

suggesting that the production of plant-specific metabolites by plant cell culture instead of whole plant cultivation possesses definite potential [65].

Kim and Chang (1990) showed that shikonin by *Lithospermum erythrorhizon* was accumulated in higher levels in cultured cells than in the intact plants. Similar results were shown by Petersen and Simmonds (2003) in the production of rosmarinic acid by *Coleus bluemei*. Higher quantities of berberine have been obtained from growing cells of *Coptis japonica* [20]. This plant accumulates significant amounts of berberine in its roots in four to six years; similar concentrations could be obtained in four weeks using tissue culture. Hara et al. have isolated a cell line of *Coptis japonica* that contained 13% DW of berberine. This culture produced about 1500 mg l^{-1} of this antibacterial alkaloid in 14 days. There are a number of examples of cultured cells producing metabolites not observed in the plant. Thus, *Lithospermum erythrorhizon* cultures have been observed to synthesize rosmarinic acid [57].

2.2.1 Food Additives from the Plant Cell Cultures

The reason for the use of metabolites synthesized by the plant cell cultures as food additives is not only that they are difficult or impossible to synthesize chemically, but consumers also more easily accept a natural product than an artificially produced one [50]. Food additives contribute to making food-

stuffs palatable and attractive by enhancing or improving their flavor, color, and texture. Food technologies try to respond to these criteria especially with regard to the texture, taste, and aroma of the foodstuff. The need to have the same taste and aroma in order to suit the consumer tastes makes it compulsory to use additional natural or artificial aromas.

Since the late 1950s, many food additives have been questioned mainly by national and international regulatory authorities about their safety for long-term use and consumption. At the same time, the consumer associations, aware of the inclusion of additives in foodstuffs, have been exerting pressure on governmental bodies to have chemical or artificial additives replaced by natural additives from plant tissues, or additives synthesized by plant cell cultures [52]. The most valuable food additives that can be obtained from the plant cell cultures are food colorants (anthocyanins and betalaines), flavors (saffron and vanillin), sweeteners (steviosides), pungent food additives (capsaicin), and anti-bacterial food preservatives (thio-

Table 2 Food additives from plant cell cultures

Product type	Plant species	Refs.
Colours		
Anthocyanins	*Vitis vinifera*	Curtin et al. 2003
	Aralia cordata	Sakamoto et al. 1994
	Perilla frutescens	Zhong 2001
Betalaines	*Beta vulgaris*	Trejo-Tapia et al. 2007
	Chenopodium rubrum	Knorr et al. 1993
Crocin	*Crocus sativus*	Chen et al. 2003
Carotenoids	*Lycopersicon esculentum*	Rhodes et al. 1991
Anthraquinones	*Cinchon. ledgeriana*	Rhodes et al. 1991
	Morinda citrifolia	Zenk 1977
Naphthoquinones	*Lithospermum erythrorhizon*	Kim and Chang 1990
Flavours		
Vanillin	*Vanilla planifolia*	Dornenburg and Knorr 1996
Garlic	*Allium sativum*	Rhodes et al. 1991
Onion	*Allium cepa*	Rhodes et al. 1991
Coffee flavour	*Coffea arabica*	Kurata et al. 1998
Cocoa flavour	*Theobromo cacao*	Rao and Ravishankar 1999
Pungent food additive		
Capsaicin	*Capsicum frutescens*	Rhodes et al. 1991
	Capsicum annuum	Johnson and Ravishankar 1996
Sweeteners		
Stevioside	*Stevia rebaudiana*	Rao and Ravishankar 1999
Glycyrrhizin	*Glycyrrhiza glabra*	Rao and Ravishankar 1999
Thaumatin	*Thaumatococcus danielli*	Rao and Ravishankar 1999

phene). Some food additives obtained from plant cell cultures are listed in Table 2.

Aromas and Fragrances

Natural aromas are a mixture of numerous compounds; more than 500 have been identified in roasted coffee beans and 200 in apples. Natural aromas are susceptible to the conservation processes of foodstuffs, such as sterilization, pasteurization, freezing, etc. Some aromas are altered by enzymatic or chemical reactions and usually disappear if stored for a long period. This is why their substitutes have been sought since the end of the 19th century. Artificial aromas used to be manufactured from coal or oil derivatives, and they used to be added in very low concentrations (10^{-6}–10^{-9}). The present trends are either to produce synthetic molecules, which are identical to natural molecules, or to use plant cell cultures [65]. Aromas from the cell cultures have an advantage of a constant composition and are independent on the season. Thus, the characteristic aromas of cocoa and coffee have been produced by cell cultures of *Tlaeobroma cacao* and *Coffea arabica*, respectively [33].

Pigments

The use of additional pigments was strongly criticized by the associations of consumers in the 1970s, because most of the colors are produced by chemical synthesis and they are unrelated to any naturally occurring material. The biotechnological methods used for producing natural food colorants consist of growing higher plant cells [65].

1. **Shikonin compounds**, such as shikonin and its derivatives acetyl and isobutyl shikonin, accumulated in roots of *Lithospermum erythrorhizon*. Because of a shortage of this plant, the mass cultivation of *Lithospermum erythrorhizon* cells to produce shikonin compounds has been successfully established [25].
2. **Anthocyanins** are the large group of water-soluble pigments responsible for many of the bright colors in flowers and fruit. They change color over the pH range due to the existence of four pH-dependent forms: at low pH they are red and at pH over six they turn blue. They are commonly used in acidic solutions in order to impart a red color to soft drinks, sugar confectionary, jams, and bakery toppings. Pure anthocyanins are priced at \$2000 kg^{-1}, but crude materials (grape pomaces and waste from juice and wine industries) are rather inexpensive [10]. Many researchers describe the production of anthocyanins using cultured cells of various plant species; most of them seem to use an anthocyanin-producing cell line as a model system for secondary product production because of their color, which allows production to be easily visualized.

3. **Crocin**, the main pigment of *Crocus sativus* stigma, is used extensively as yellow food colorant. Commercial production of saffron pigment is restricted by its high price and limited availability. As a geophyte, saffron grows slowly and propagates only by vegetative production through the formation of daughter corms. It takes 200 000 flowers and over 400 h of hand labor to produce 1 kg of saffron stigma. A plant tissue culture method offers a great potential for crocin production [8].
4. **Madders** are red colorants from *Rubia tinctorum*, a perennial plant from the coastal regions of the Mediterranean, and its roots have been used as red dyes in Western Europe. The major components in the pigment are alizarin, purpurine, and its glycoside, ruberythric acid. Pure alizarin is an orange crystal and is soluble in boiling water and in other solvents. Alizarin shows a yellow color in acidic to neutral pH and tends to be reddish with increasing pH. It is highly resistant to heat and light, which is favorable to the food industry. Through the selection of high-producing cell lines and elicitor application, yellow-pigment-producing cells of *Rubia tinctorum* were obtained [61].

2.2.2 Pharmaceuticals from Plant Cell Cultures

Higher plants are a rich source of bioactive constituents used in **pharmaceutical industry**. Some of the plant-derived natural products include drugs, such as morphine, codeine, cocaine, quinine, anti-cancer *Catharanthus* alkaloids, belladonna alkaloids, colchicines, phytostigminine, pilocarpine, reserpine, and steroids, such as diosgenin, digoxin, and digitoxin [42].

Table 3 Plant-derived pharmaceuticals of importance

Product	Use	Plant species	Cost US $ kg^{-1}
Ajmalicine	Antihypertensive	*Catharanthus roseus*	37 000
Ajmaline	Antimalarial	*Rauvolfia serpentine*	75 000
Camptothecin	Antitumour	*Camptotheca acuminata*	432 000
Codeine	Sedative	*Papaver somniferum*	17 000
Colchicine	Antitumour	*Colchium autumnale*	35 000
Ellipticine	Antitumour	*Orchrosia elliptica*	240 000
Morphine	Sedative	*Papaver somniferum*	340 000
Shikonin	Antibacterial	*Lithospermum erythrorhizon*	4500
Taxol	Anticancer	*Taxus brevifolia*	600 000
Vinblastine	Antileukemic	*Catharanthus roseus*	1 000 000
Vincristine	Antileukemic	*Catharanthus roseus*	2 000 000

Rao and Ravishankar (2002)

Plant-derived drugs represent a huge market value. According to Rao and Ravishankar (2002), worldwide, 121 clinically useful prescription drugs are derived from plants. Furthermore, 12% of drugs considered as basic and essential by the WHO are exclusively derived from flowering plants [49].

The surveys of plant medicinal usage in the USA have shown an increase from just about 3% of the population in 1991 to over 37% in 1998 [46]. Prescription drugs containing phytochemicals were valued at more than US$30 billion in 2002 in the USA [48]. 75% of the world's population relies on plants for traditional medicine. Some plant-derived pharmaceuticals are listed in Table 3.

An example of a high-value drug produced from plant cell cultures is paclitaxel, an anti-cancer drug originally extracted from the bark of 50-year-old Pacific yew trees, *Taxus brevifolia* [58].

2.2.3 Advantages and Disadvantages of Plant Cell Cultures

The advantages of plant cell cultures over the conventional production are as follows:

1. it is independent of geographical and seasonal variations and environmental factors – the synthesis of bioactive secondary metabolites runs in controlled environments and the negative biological influences that affect secondary metabolites production in nature are eliminated (microorganisms and insects);
2. it offers a defined production system which ensures the continuous supply of products, uniform quality, and yield;
3. it is possible to select cell lines with higher production of secondary metabolites;
4. it is possible to produce novel compounds that are not normally found in parent plant;
5. it allows the efficient downstream production;
6. plant cell can perform stereo- and regio-specific biotransformations for the production of novel compounds from cheap precursors;
7. with automatization of cell growth control and regulation of metabolic processes, cost price can decrease and productivity increase.

There are a number of successfully established and commercialized plant cell cultures producing a high amount of secondary metabolites (Table 4).

However, this technology is still being developed and despite the advantages, there is a variety of problems to be overcome before it can be adopted for the production of useful plant secondary metabolites.

Table 4 High yields of secondary products

Product	Plant species	Yield (% DW)	Refs.
Anthocyanins	*Perilla frutescens*	8.9	Zhong 2001
Anthraquinones	*Morinda citrifolia*	18.0	Zenk 1977
	Coleus blumei	21.4	Petersen and Simmond 2003
Sanguinarine	*Papaver somniferum*	2.5	Dicosmo and Misawa 1995
Serpentine	*Catharanthus roseus*	2.2	Moreno et al. 1995
Shikonin	*Lithospermum erythrorhizon*	13.5	Kim and Chang 1990

Obstacles for the Cell Cultures

In theory, it is anticipated that cell cultures will be suitable for industrial production of useful plant chemicals in a manner similar to that of microbial fermentation. Nevertheless, there are some significant differences between microbial and plant cell cultures that must be considered when attempting to apply plant cell cultures to the available technology.

Generally, the problems with the plant cell cultures can be classified as biological (slow growth rate, physiological heterogeneity, genetic instability, low metabolite content, product secretion) and operational (wall adhesion, light requirement, mixing, shear sensitivity, aseptic condition) [65].

Table 5 shows a comparison of some of the characteristics of plant and microbial cultures of relevance to fermentation. In particular, it serves to demonstrate some of the problems that can be encountered with plant cell

Table 5 Characteristics of microbial and plant cell relevant to fermentation

Characteristics	Plant cell	Microoorgnism
Size		
Diameter (μm)	40–200	1–10
Volume (μm^3)	$> 10^5$	1–50
Inoculum	5–10	≤ 1
Growth	aggregates	single cells
Cultivation time	2–3 weeks	2–10 days
Duplication time, hrs	15–120	0.3–6
Oxygen consumption, O h^{-1} g^{-1}	≤ 5	50
Water content (%)	> 90	80
Product accumulation	mostly intracellular (vacuole)	mostly extracellular (medium)
Requirements for acepticity	high	low

Dörnerburg and Knorr 1997

cultures. The large size of the plant cell contributes to its comparatively high doubling time, which thus prolongs the time required for a successful fermentation run. The vacuole is the major site of product accumulation and, since product secretion is uncommon, the high metabolite yields seen in microorganisms that secrete product cannot be expected. There is some ongoing research on membrane permeabilization of plant cells, which may serve to relieve the constraints of product inhibition by facilitation of leakage into the extracellular medium (See Sect. 4.3 "Membrane Permeabilisation") [17]. Thus, there are some considerable hurdles which need to be overcome at the biochemical level.

3 Strategies to Increase Secondary Metabolite Production

The objective of the food industry is to develop techniques to allow production of secondary products from the plant cell culture which would be less expensive than extraction of the whole plant grown under natural conditions and less expensive than the synthesis of the product. Confronted with having to increase the amount of secondary metabolites in plant cell cultures, the need for biochemical and molecular research on the secondary metabolism of plants has been frequently emphasized [12]. The research in this area could lead to the successful manipulation of secondary metabolism and could significantly increase the amounts of the compounds. It should be possible to achieve the synthesis of a wide range of compounds, such as alkaloids, flavonoids, terpenes, steroids, glycosides, etc., using plant-cell-culture technology.

The strategy for obtaining the secondary metabolites from the plant cell cultures can be represented as a multi-stage process (Fig. 1). Each link may be optimized separately or in combination with other processes or treatments.

1. The initial step of this technology includes the selection of **parent plant** according to its molecular and biochemical characteristics, particularly regarding the high contents of the desired metabolites. In theory, any part obtained from any plant species can be employed to induce callus tissue; however, successful production of callus depends upon plant species and their qualities. Dicotyledons are rather amenable for callus tissue induction as, compared to monocotyledons, the calluses of woody plants generally grow slowly. Stems, leaves, roots, flowers, seeds, and any other parts of plants are used, but younger and fresh explants are preferable explant materials.
2. Afterwards, the **selection of cell line** becomes important. It includes the establishment of **high-producing** and **fast-growing** in-vitro cultures. It is possible to identify cell lines that can produce amounts of compounds equal or even higher than in the plant from which they derive [11]. More-

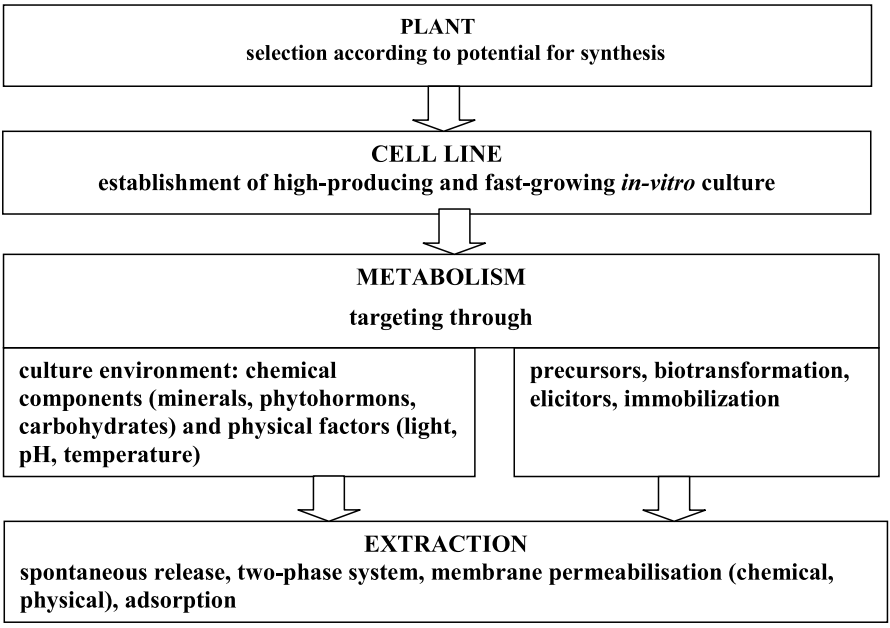

Fig. 1 Scheme of plant secondary metabolite production in cell cultures

over, increase of metabolite levels using **mutants** is possible, and selection of suitable analogues for this purpose could be an important factor in order to produce a variety of products. Maximization of the production and accumulation of secondary metabolites by plant cultured cells requires production of new genotypes through **protoplast fusion** or **genetic engineering**; however, this presupposes the identification of the genes encoding key enzymes of secondary metabolic pathways and their expression. Use of mutagens increases the variability which already exists in living cells. Furthermore, new molecules, which have previously not been found in plants, can be produced by cell cultures.

3. **Targeting metabolism.** A number of chemical and physical factors strongly affect the production of secondary metabolites. The expression of many secondary metabolite pathways is easily altered by external factors such as environmental conditions (chemical and physical) and special treatments (precursors, elicitors).
   (a) Plant cell **culture medium** includes inorganic components, organics, and phytohormones. Changing of medium components (concentration, proportion, and form) is a very powerful way of enhancing the culture efficiency of plant cell cultures. Thus, high auxin level stimulates cell growth, but often negatively influences secondary metabolite production [63].
   Physical conditions, such as light, temperature, and medium pH, have

also been examined for their effect upon secondary metabolite accumulation in many types of cultures.

(b) **Special treatments** include feeding with precursors, application of elicitors, biotransformation, and immobilization.

The concept of feeding with **precursors** is based upon the idea that supply with compounds which are intermediate or at the beginning of biosynthetic route gives a good chance of increasing the yield of the final product.

The production of the desired metabolites is often limited by the lack of particular precursors; **biotransformation** using an exogenous supply of biosynthetic precursors may improve the accumulation of compounds. Biotransformation is a process through which the functional groups of compounds are modified by cell cultures to chemically different product [24]. Plant cells can transform natural or artificial compounds introduced into the cultures through a variety of reactions, such as hydrogenation, dehydrogenation, isomerisation, glycosylation, and hydroxylation.

Plants and plant cells in vitro show physiological and morphological responses to microbial, physical, or chemical factors, which are known as **elicitors**. Since the secondary metabolites protect plants from the environmental changes, the way to induce their synthesis is to apply unfavorable factors, i.e., simulate pathogen attack, herbivores, heavy metals, etc. Elicitation is a process of induced or enhanced synthesis of secondary metabolites by the plants to ensure their survival, persistence, and competitiveness. Biotic and abiotic elicitors are used to stimulate secondary metabolite product formation in plant cell cultures.

Cell **immobilization** can result in much higher concentrations of the plant cells because of the certain grade of cell specialization while hundreds or thousands of them are immobilized in one aggregate. Most of the research in this area has utilized hydrocolloidal gels, such as alginate and carrageenan, which were used to entrap the plant cells into a gel matrix while allowing easy access of substrates.

4. Perhaps the most efficient bioprocessing concepts for the production of phytochemicals result in **spontaneous release into medium** where they can be more easily recovered. One of the most fruitful areas of research for the production of lower-value products may be the study of methods to induce product leakage from cells that normally accumulate the product. A study of the intracellular compartments in which synthesis of chemicals occurs may also be necessary, since the substances are transported to the vacuole for accumulation. Thus, an alternative consideration is prevention of vacuolar accumulation and, consequently, enhancement of substances released into the medium.

Plants often have sites of synthesis and storage of the secondary metabolites in separate cells or organs. Inhibition of metabolic enzymes as well as inhibition of membrane transport can be eliminated by the accumulation of synthesized products in a second phase introduced into the aqueous medium or **two-stage** system.

3.1 Selection According to Molecular and Biochemical Characteristics

Screening and selection of plant species and cultivars rich in useful metabolites are the strategies for enhancement of secondary metabolite content in plant cell cultures. Plants with high contents of the desired products should be used for callus induction to obtain high-producing cell lines.

3.1.1 Plant Genotype and Cultivar

Genetic potential is one of the most important factors influencing the biochemical status of plants and plant cell cultures. Environmental and physiological factors may modify the expression of genes participating in phytochemical synthesis, but the genetic background is the major determinant. The diversity of genetic potential toward secondary metabolite profile can be shown on the example of the plants from the family *Brassicaceae*. It comprises roughly 350 genera and 3500 species and includes vegetables, ornamental species, and oil cultures. The glucosinolate profiles and levels vary extensively and are usually represented by six to ten individual glucosinolates (Table 6).

The comparison of the profiles of main glucosinolates in *Brassica* vegetables shows that the general content and distribution of sub-classes of glucosinolates is unique for each species. For example, the main glucosinolate in *Brassica juncea* seed is dominated by progoitrin, *Brassica oleracea* seeds

Table 6 Distribution profile of glucosinolates in *Brassicaceae*

Species	Glucosinolates (mg 100 g^{-1} FW)	% of total glucosinolates		
		Aliphatic	Aromatic	Indole
Brassica rapa L. var. *rapa*	21–340	42	30	18
Brassica rapa L. var. *rapa teltoviensis*	790–890	29	46	25
Brassica oleracea L. var. *capitata alba*	26–275	47	33	20
Brassica oleracea L. var. *italica*	40–340	47	9	44
Brassica oleracea L. var. *botrytis*	14–208	47	14	39

Schreiner M, 2005

contain mainly gluconapoleiferin, whereas *Brassica napus* seeds contain gluconapoleiferin, gluconapin, and glucobrassicanapin [55].

3.1.2 Obtaining of Fast-Growing and High-Productive Cell Lines

Plant cell cultures are sometimes characterized with inherent genetic and epigenetic instability. Variability between cells often leads to gradual reduction in productivity and can be attributed to genetic changes by mutation in the culture, or epigenetic changes caused by physiological conditions. These undesirable changes can be reversed by the screening for a desired cell population from the heterogeneous ones, typically presented in plant cell cultures [17].

Cell cloning methods provide a promising way of selecting cell lines yielding increased levels of product. The physiological characteristics of individual plant cells are not always uniform. For example, pigment-producing cell aggregates typically consist of producing cells and non-producing cells. The heterogeneity in the biochemical activity existing within a population of cells has been exploited to obtain highly productive cell lines. This is similar to monocolony isolation of bacteria.

For example, by cell cloning using cell aggregates of *Coptis japonica*, Matsubara et al. (1989) obtained strain that grew faster and produced a higher amount of berberin. During the three weeks of cultivation, the selected cell line of *Coptis japonica* produced sixfold higher amount of berberine, particularly 1.2 $g\,l^{-1}$, as primary callus. The selected strain was very stable, producing a high level of berberin even after 27 generations. As shown in Table 7, a strain of *Euphorbia milli* accumulated about sevenfold the level of anthocyanins produced by the parent culture after 24 selections [40].

In cultures of *Lithospermum erythrorhizon*, extensive screening of a number of clones resulted in a 13-fold to 20-fold increase in shikonin produc-

Table 7 Influence of cell cloning on productivity of plant cell cultures

Products	Plants	Factors (increase of production)	Refs.
Anthocyanins	*Vitis vinifera*	2.3–4	Curtin et al. 2003
	Euphorbia milli	7	Mulabagal and Tsay 2004
Berberine	*Coptis japonica*	2–6	Matsubara et al. 1989
Biotin	*Lavendula vera*	9–10	Misawa 1985
Shikonin	*Lithospermum erythrorhizon*	7–20	Kim and Chang 1990
Ubiquinone-10	*Nicotiana tabacum*	15–180	Dicosmo and Misawa 1995

tion [25]. *Lavendula vera* cells grown in the light accumulated a high level of free biotin [38]. To select a high-producing cell line, pimelic acid, a precursor of biotin, was used. The level of biotin accumulated by a selected cell line was 0.9 $\mu g\,l^{-1}$, which was ten times the amount found in the leaves.

Japan Tobacco Inc. isolated a number of strains of *Nicotiana tabacum* producing high levels of ubiquinone-10 [11]. After the 13th recloning, a strain was selected from approximately 4000 cell clones tested. When *Nicotiana tabacum* BY-2, a parent strain used for the cloning, was isolated, the titer for ubiquinone-10 was only 0.36 $mg\,g^{-1}$ DW; therefore, the level was increased by selection until 5.2 $mg\,g^{-1}$, which corresponded to 180 times the amount produced by the parent plant.

Cell cloning is a very useful technique to increase the level of secondary metabolites. However, it is not obvious why cultures contain both high- and low-yielding cells. Kim and Chang (1990) indicated that the lack of specific enzymes represents the most important reaction for the inability of plant cell cultures to produce secondary metabolites.

Protoplast fusion. Maximization of the production and accumulation of secondary metabolites by plant cultured cells requires production of new genotypes through protoplast fusion, but this presupposes the identification of the genes encoding key enzymes of secondary metabolic pathways and their expression once introduced in the plant cells. This suggests that use of mutagens to increase the variability already exists in living cells.

Since most cultured cells occur as aggregates, selection of high-producing but aggregated cell lines of *Lithospermum erythrorhizon* is not effective and is labor intensive. The Mitsui group prepared protoplasts from the cultured cells with appropriate enzymes and selected high shikonin-compound-producing protoplasts using a cell sorter [46]. The selected protoplasts were generated to cell lines and cultivated in suspension. From 48 cell lines, they obtained a cell line having 1.8-fold the productivity of the parent line. The cell line showed stable production of shikonin compounds.

Sakamoto et al. (1994) reported about the visual selection of *Euphorbia millii*. This procedure was repeated 28 times and one of the cells was determined to produce 1.32% DW anthocyanins in the cells. The levels of the pigments in flowers and leaves were 0.28% and less than 0.01%, respectively.

Use of mutagens. Mutation strategies have been employed in order to obtain overproducing cell lines [46]. In this method, a large population of cells is exposed to a toxic (or cytotoxic) inhibitor or environmental stress, and only cells that are able to resist the selection procedures will grow. For example, *p*-fluorophenylalanine, an analogue of phenylalanine, was extensively used to select high-yielding cell lines with respect to phenolics. Increased capsaicin in *p*-fluorophenylalanine cell lines of *Capsicum annuum* was reported.

In the fermentation industry, induction of genetic mutant strains of microorganisms is used extensively to produce a variety of products, includ-

ing amino acids, nucleotides, and antibiotics. However, mutagenesis has limited applicability to plant cell cultures, because of their diploid genetics: the chance of obtaining a double mutation in a target gene is less than 10^{-6} [63]. Although, in principle, haploid plants can be produced from another cultures, in practice, haploid cell cultures tend to revert to the diploid state. This makes the chance of isolating over-producing cells from mutagen treatment of haploid cells very low. Furthermore, biosynthetic pathways of many secondary metabolites and their regulation mechanisms in higher plants are not always precisely understood; therefore, it is also difficult to know what kind of mutants should be induced in order to increase product synthesis.

However, Berlin et al. (1981) induced *p*-fluorophenylalanine-resistant cell lines of tobacco cell cultures and found that, out of 31 resistant cell lines, five lines of *Nicotiana tabacum* and five lines of *Nicotiana glauca* accumulated higher levels of phenolics. The resistant strain of *Nicotiana tabacum* produced six to ten times higher levels of cinnamoyl putrescine than that of the parent strain.

Generally, plant cells accumulate their metabolites intracellularly, which is disadvantageous in commercial production because the amount of released compounds is usually low. Induction of a mutant having altered permeability could be important. *Thuja occidentalis* excreted monoterpenoids, but the levels in the medium were only 5% of those in the plant. However, *Macleya microcarpa* cells excreted nearly all the alkaloids detectable in the culture flask [65]. After three days of cultivation of *Tinospora rumphii* cells, 0.57 mg (5.3% DW) of isoquinoline alkaloids have been found in cells and 0.50 mg in culture filtrate, and, after seven days, 0.50 mg of the alkaloids were accumulated in the cells and 1.02 mg in filtrate.

3.2 Targeting Metabolism

A number of chemical and physical factors, such as media components' pH, temperature, and light, affect production of secondary metabolites in plant cell cultures [40]. Manipulation of growing conditions is one of the most fundamental approaches for optimization of culture productivity.

3.2.1 Culture Environment

3.2.1.1 Chemical Parameters

Plant cell culture media include inorganic components (macroelements and microelements), organics (sacharose), and phytohormones.

To cultivate the callus and cells in suspension, various kinds of media (inorganic salt media) have been designed. Agar or its substitutes are added to the media to prepare solid medium.

Many media have been developed and modified and nutrient compositions of some typical media are described in Table 8.

One of the most commonly used media for plant tissue cultures is that developed by Murashige and Skoog (MS) [14]. The significant feature of the MS medium is its very high concentration of nitrate (NO_3^-), potassium (K^+), and ammonia (NH_4^+) (see Table 8). Many researchers are also using the B5 medium established by Gamborg. The levels of inorganic nutrients in the B5 medium are lower than in the MS medium.

The effects of the medium employed in various processes have been reported, e.g., effects of calcium and phosphate in the cultivation of *Coffea arabica* suspended cells, phosphate effects on sapogenin steroid production in suspension cultures of *Agave amanuensis* [56], and phosphate and sucrose in nicotine production by *Nicotiana tabacum* cell cultures [35].

Inorganic Components

Zenk et al. (1978) tested various basal media for the production of serpentine, indole alkaloids by *Catharanthus roseus* suspension, as summarized in Table 9. The results indicate that the amount of serpentine depends on the composition of the basal medium used. Among them, Murashige–Skoog's (MS) formulation was recognized to be the most suitable for the production of this particular alkaloid.

Nitrogen. Plant tissue culture media, such as MS, LS, or B5, have both nitrate (NO_3^-) and ammonium (NH_4^+) as sources of nitrogen. For example, nitrogen source is very important for plant suspension cultures of *Holarrhena antidysenterica* for accumulation of alkaloids [65], in cell suspensions of *Vitis vinifera* for anthocyanin formation, and in shikonin production by *Lithospermum erythrorhizon* cell cultures [25].

The ratio of NH_4^+/NO_3^- and overall levels of total nitrogen have been shown to markedly affect the production of secondary plant products. The reduced levels of NH_4^+ and increased levels of NO_3^- promoted the production of shikonin and betacyanins, whereas higher ratios of NH_4^+ to NO_3^- increased the production of berberine and ubiquinone [14]. Reduced levels of total nitrogen improved the production of capsaicin in *Capsicum frutescens*, anthraquinones in *Morinda citrifolia*, and anthocyanins in *Vitis* species [54, 64].

Phosphate concentration in the medium has a great effect on the production of secondary metabolites in plant cell cultures. Higher level of phosphate enhanced the cell growth, whereas it had a negative influence on secondary product accumulation.

Medium limited in phosphate either induces or stimulates both the product and the levels of key enzymes leading to the product. Reduced phosphate

Table 8 Media for plant cell cultures ($mg\,l^{-1}$)

Components	Murashige–Skoog	White	Gamborg	Nitsch	Schenk–Hildebrandt	Knop
$(NH_4)_2SO_4$	–	–	134	–	–	–
$MgSO_{4\times}7H_2O$	370	720	500	250	400	250
Na_2SO_4	–	200	–	–	–	–
KCl	–	65	–	1500	–	–
$CaCl_{2\times}2H_2O$	440	–	150	25	200	–
$NaNO_3$	–	–	–	–	–	–
KNO_3	1900	80	3000	2000	2500	250
$Ca(NO_3)_{2\times}4H_2O$	–	300	–	–	–	1000
NH_4NO_3	1650	–	–	–	–	–
$NaH_2PO_{4\times}H_2O$	–	16.5	150	250	–	–
$NH_4H_2PO_4$	–	–	–	–	300	–
KH_2PO_4	170	–	–	–	–	250
$FeSO_{4\times}7H_2$	27.8	–	27.8	–	15	–
Na_2EDTA	37.3	–	37.3	–	20	–
$MnSO_{4\times}4H_2O$	22.3	7	10	3	10	–
$ZnSO_{4\times}7H_2O$	8.6	3	2	0.5	0.1	–
$CuSO_{4\times}5H_2O$	0.025	–	0.025	0.025	0.2	–
H_2SO_4	–	–	–	0.5	–	–
$Fe_2(SO_4)_3$	–	2.5	–	–	–	–
$NiCl_{2\times}6H_2O$	–	–	–	–	–	–
$CoCl_{2\times}6H_2O$	0.025	–	0.025	–	0.1	–
$AlCl_3$	–	–	–	–	–	–
$FeCl_{3\times}6H_2O$	–	–	–	–	–	–
$FeC_6O_5H_{7\times}5H_2O$	–	–	–	10	–	–
KI	0.83	0.75	0.75	0.5	1.0	–
H_3BO_3	6.2	1.5	3	0.5	5	–
$Na_2M_0O_{4\times}2H_2O$	0.25	–	0.25	0.25	0.1	–
Sucrose	30 000	20 000	20 000	50 000–36 000	30 000	–
Glucose	–	–			–	–
Myo-inositol	100	–	100	–	1000	–
Nicotinic acid	0.5	0.5	1.0	–	0.5	–
Pyridoxine HCl	0.5	0.1	1.0	–	0.5	–
Thiamine HCl	0.1–1	0.1	10	1	5	–
Ca–pantothenate	–	1	–	–	–	–
Biotin	–	–	–	–	–	–
Glycine	2	3	–	–	–	–
Cysteine HCl	–	1	–	10	–	–
Folic acid	–	–	–	–	–	–
Glutamine	–	–	–	–	–	–

Gamborg and Phillips, 1995

Table 9 Effects of different media on growth and serpentine production in cell suspension cultures of *Catharanthus roseus*

Basal medium	Cell yield g DW l^{-1}	Serpentine mg l^{-1}	Serpentine, % DW
Blaydes	7.6	4.4	0.06
Gamborg – B5; + 2,4-D (l mg l^{-1})	4.6	0.5	0.01
Gamborg + 2,4 D (2 mg l^{-1})	5.2	0	0
Gamborg + NAA (1.86 mg l^{-1})	7.6	1.2	0.02
Gamborg	5.1	0	0
Heller + IAA (0.175 mg l^{-1}); BA (1.13 mg l^{-1})	5.4	6.6	0.12
Linsmaier and Skoog	9.3	0	0
Murashige and Skoog	8.9	10.4	0.12
Nitsch and Nitsch	2.3	2.0	0.09
Velicky and Martin	5.0	0	0
White	0.8	0	0

Zenk, 1978

levels induced the production of ajmalicine and phenolics in *Catharanthus roseus* and nicotin in *Nicotiana tabacum* [35]. In contrast, increased phosphate was shown to stimulate synthesis of digitoxin in *Digitalis purpurea* and betacyanin in *Chenopodium rubrum* [5].

Potassium ion (K^+) serves as a major contributor to osmotic potential, a specific requirement for protein synthesis, and an activator for particular enzyme systems [65]. Higher K^+ concentration caused slower cell growth. More of soluble sugar was stored within the cells under K^+ deficiency.

Microelements are required in trace amounts for plant growth and development, and have many diverse roles [21]. Manganese, iodine, copper, cobalt, boron, molybdenum, iron, and zinc usually comprise the microelements, although other elements, such as nickel and aluminum, are frequently found in some formulations. Iron is usually added as iron sulphate, although iron citrate can also be used. Ethylenediaminetetraacetic acid (EDTA) is used in conjunction with the iron sulphate. EDTA complexes allow slow and continuous release of iron into the medium. Uncomplexed iron can precipitate out of the medium as ferric oxide.

Plant cell cultures are usually grown heterotrophically using simple sugars as **carbon source** and inorganic supply of other nutrients. The level of sucrose affected the productivity of secondary metabolites in cultures [35]. **Sucrose or glucose** at 2 to 4% are suitable carbon sources, which are added to the basal medium. Fructose, maltose, and other sugars also support the growth of various plant cells. The choice of the most suitable carbon source and its optimal concentration depend on the plant species and products.

In many cases, the concentration of the carbon source affects cell growth and yield of secondary metabolites. Sucrose concentrations of 2.5% and 7.5%

in *Coleus blumei* media resulted in rosmarinic acid yields of 0.8 and 3.3 $g\,l^{-1}$, respectively [38].

Carbon source was also found to be a significant factor in plant cell metabolism, which affected the accumulation of alkaloids by suspension cultures of *Holarrhena antidysenterica* and of shikonin by *Lithospermum erythrorhizon* cell cultures [65]. For indole alkaloid accumulation in cell culture as of *Catharanthus roseus*, 8% (w/v) sucrose was found to be optimal in the tested concentration range of 4–12% [27]. Yields of benzophenanthridine alkaloids from suspension cultures of *Eschscholtzia californica* were increased tenfold to 150 $mg\,l^{-1}$ by increasing the sucrose concentration to 8% [5]. The osmotic stress created by sucrose alone and with other osmotic agents was found to regulate anthocyanin production in *Vitis vinifera* [13] and nicotine accumulation in suspension cultures of *Nicotiana tabacum* [35]. However, higher concentrations of sucrose at 5% reduced the anthocyanin production in cell suspension cultures of *Aralia cordata*, where 3% favored the anthocyanin accumulation [54].

Vitamins, such as thiamine (vitamin B1) and myo-inositol, are considered essential for the culture of plant cells in vitro. However, other vitamins are often added to plant cell culture media. Thus, MS medium includes myo-inositol, nicotinic acid, pyridoxine HCl, and thiamine HCl [14].

Amino acids are also commonly included in the organic supplement. The most frequent are glycine (arginine, asparagine, aspartic acid, alanine, glutamic acid, glutamine, and proline are also used), but in many cases their inclusion is not essential [21]. Amino acids provide a source of NO_3^- and, like NH_4^+, uptake causes acidification of the medium. Other supplements include casamino acid, peptone, yeast extracts, malt extracts, and coconut milk. Coconut milk is also known as a supplier of growth regulators.

Gelling agents. Media for plant cell culture in vitro can be used in either liquid or solid forms, depending on the type of culture being grown. For any culture types that require to be grown on the surface of the medium, it must be solidified or gelled. Agar, produced from seaweed, is the most common type of gelling agent, and is ideal for routine applications. However, because it is a natural product, the agar quality can vary from supplier to supplier and from batch to batch.

Growth regulators concentration is often a crucial factor in secondary product accumulation [63]. Phytohormones or growth regulators are required to induce callus tissues and to promote the growth of many cell lines. Since each plant species requires different kinds and levels of phytohormones for callus induction, its growth, and metabolites production, it is important to select the most appropriate growth regulators and to determine their optimal concentrations.

Auxins and cytokinins have shown the most remarkable effects on growth and productivity of plant metabolites. The type and concentration of auxin or

cytokinin or the auxin/cytokinin ratio dramatically alter both the growth and the product formation in cultured plant cells.

1. **Auxins** are generally used in plant cell culture at a concentration range between 0.1 to 50 μM. An increase of auxin levels in the medium stimulates dedifferentiation of the cells, cell division, and callus formation and growth. They are reported to diminish the level of secondary metabolites. That is the reason why auxins are commonly added to the medium for callus induction. However, for production of metabolites, they are added at a low concentration. As an auxin, 2,4-D or NAA is frequently used. The growth regulator 2,4-D has been shown to inhibit the production of secondary metabolites in a large number of cases. The elimination of 2,4-D or its replacement by NAA or IAA enhanced the production of anthocyanins in suspension of *Daucus carota*, nicotine in *Nicotiana tabacum* [35], shikonin in *Lithospermum erythrorhizon*, and of anthraquinones in *Morinda citrifolia* [58, 64]. However, stimulation by 2,4-D has been observed in carotenoid biosynthesis, in suspensions of *Daucus carota* [35], and in anthocyanin production in *Oxalis linearis* [37].
2. **Cytokinins** are used in plant cell culture at a concentration range of 0.1 to 10 μM. They promote cell division and modulate callus initiation and growth. Cytokinins have different effects depending on the type of metabolite and species concerned. Kinetin stimulated the production of anthocyanin in *Haplopappus gracilus*, but it inhibited the formation of anthocyanins in *Populus* cell cultures [46].
3. **Gibberellins** are represented by over 90 forms, but gibberelic acid is the most commonly used for plant cell cultures. Dicosmo and Misawa (1995) reported that the growth of *Taxus cuspidata* callus was significantly promoted by the addition of gibberellic acid into the solid medium. However, gibberellic acid suppresses production of anthocyanins in a number of cultures [54].

3.2.1.2 Physical Factors

Physical factors, such as light, temperature, and medium pH, effect secondary metabolite accumulation in many types of cultures.

Temperature. A temperature range of 17–25 °C is normally used for the induction of callus tissues and growth of cultured cell suspension [46]. However, each plant species as well as its cell culture may favor a different temperature. When the temperature was maintained at 19 °C, biotransformation of digitoxin to digoxin was favored, whereas 32 °C was optimal for the purpureaglycoside-A formation in *Digitalis lanata* cell cultures. A higher yield of ubiquinone in *Nicotiana tabacum* cell cultures has been observed at 32 °C when compared to 24 °C. Courtois and Guren (1980) reported a 12-fold

higher production of alkaloids in cell cultures of *Catharanthus roseus* at 16 °C as compared to the normal 27 °C.

Light. The spectral quality, intensity, and period of light irradiation may affect plant cell cultures. Sakamoto et al. (1994) demonstrated the stimulatory effect of light irradiation on the formation of compounds such as anthocyanins, vindoline, catharanthine, and caffeine in cell suspension cultures. Thus, the accumulation of anthocyanin was strongly stimulated by light in cell cultures of *Daucus carota* and *Vitis vinifera* [10]. Mulder-Krieger et al. (1988) found that illumination affected the composition of sesquiterpenes in callus cultures of *Marticaria chamomilla*. Illumination of *Coffea arabica* cell suspensions enhanced caffeine biosynthesis by a factor of ten [33].

Medium pH. The medium pH is usually adjusted between five and six, and pH extremes are avoided. In medium, hydrogen ion concentration changes during the culture growth. The medium pH decreases during ammonia assimilation and increases during nitrate uptake [46].

Osmotic pressure. Accumulation of anthocyanins was enhanced by a high osmotic potential in *Vitis vinifera* cell suspension cultures [14]. Addition of sucrose or mannitol in the medium enhanced the osmotic pressure and the level of anthocyanins accumulated in *Vitis vinifera* culture was increased to 1.5 times and reached 55 $\mu g\,cell^{-1}$.

3.2.2 Treatments

3.2.2.1 Precursor Feeding

Precursor feeding has been an obvious and popular approach to increasing secondary metabolite production in plant cell cultures. The concept is based upon the idea that any compound, which is an intermediate, in or at the beginning of a secondary metabolite biosynthetic route, stands a good chance of increasing the yield of the final product. Attempts to induce or increase the production of plant secondary metabolites, by supplying precursor or intermediate compounds, have been effective in many cases. Feeding ferulic acid to cultures of *Vanilla planifolia* resulted in an increase in vanillin accumulation [53]. Similarly, anthocyanin synthesis in *Daucus carota* was restored by the addition of a dihydroquarcetin (naringen). Furthermore, addition of geraniol to *Catharanthus roseus* cell cultures led to accumulation of nerol and citronellol [34]. Fontanel and Tabata (1987) reported that an addition of 500 mM tropic acid to the medium of *Scopolia japonica* increased the amount of alkaloids by up to 14 times.

In many cases, amino acids have been used as inexpensive precursors of secondary metabolites. Amino acids have been added to cell suspension culture media for production of tropane alkaloids, indole alkaloids, etc. Phenyl-

alanine is one of the biosynthetic precursors of rosmarinic acid [44], and its addition to *Salvia officialis* suspension cultures stimulated the production of rosmarinic acid and it shortened the production time as well.

Use of the distant precursor, phenylalanine, and a near precursor, such as isocapric acid, resulted in enhanced capsaicin content in cell cultures of *Capsicum frutescens* [24]. The addition of leucine led to enhancement of volatile monoterpenes in cultures of *Perilla frutescens* [34].

3.2.2.2 Elicitation

Secondary metabolites represent the adaptations of plants to environmental stress, or they may serve as defensive, protective, or offensive chemicals against microorganisms, insects, and higher herbivorous predators. When infected by pathogenic microorganism, plants respond with rapid activation of various spatially and temporally regulated defense reactions. These responses include oxidative cross-linking of cell wall proteins, production of phytoalexins, hydrolytic enzymes, and incrustation of cell wall proteins with phenolics, and, finally, hypersensitive death of plant cell. Microbial invasion of plants induce the synthesis of anti-microbial secondary metabolites in the same way as stress factors, such as UV-irradiation, osmotic shock, fatty acids, inorganic salts, and heavy metal ions, induce the synthesis of secondary metabolites in plants. Plant cells in vitro show physiological and morphological responses to microbial, physical, or chemical factors, which are known as elicitors.

Elicitor may be defined as a substance which, when introduced in small concentrations to a living cell system, initiates or improves the biosynthesis of specific compounds. Elicitation is the induced or enhanced biosynthesis of metabolites due to the addition of trace amounts of elicitors [42].

Production of many valuable secondary metabolites using various elicitors was reported [40]. In many cases elicitors used in cell culture are methyl jasmonate, salicylic acid, chitosan, and heavy metals.

Classification of Elicitors

The type and structure of elicitors varies greatly. Depending of their origin, they are classified as biotic or abiotic.

Biotic stress can be caused by bacterial, viral, or fungal attack, as well as by **biotic elicitors**. They include:

- Enzymes, cell wall fragments of microorganisms, polysaccharides derived from microorganisms (chitin or glucans), and glycoproteins;
- phytochemicals produced by plants in response to physical damage, fungi or bacteria attack, polysaccharides derived from plant cell walls (pectin

or cellulose), fragments of pectin formed by action of microorganisms on plant cell wall [66];
- chitosan, glucans, salicylic acid, methyl jasmonate (formed by the action of plant on microbial cell walls) [17].

Abiotic elicitors are the substances of non-biological origin. The causes of the abiotic stress can be of chemical or physical nature; among them are:

- Chemicals such as inorganic salts, heavy metals, some chemicals that disturb membrane integrity,
- physical factors like mechanical wounding, ultraviolet irradiation, high salinity, high or low osmolarity, extreme temperature (freezing, thawing), high pressure.

Elicitation and Production of Secondary Metabolite by Plant Cell Cultures

Table 10 illustrates different plant species producing various secondary metabolites on elicitation.

For example, sodium orthovanadate and vanadyl sulphate induced the accumulation of isoflavone glucosides in *Vigna angularis* cultures and indole alkaloid accumulation in *Catharanthus roseus* cultures, respectively ([42].

Most of the strategies employing fungal elicitors utilize undefined mixtures, such as autoclaved fungal homogenate or fungal culture filtrates. With the consideration of several parameters, such as elicitor specificity and concentration, duration of contact, and quality of cell wall materials, substantial enhancement of product accumulation has been reported.

Microbial infections of intact plants often elicit the synthesis of specific secondary metabolites. The best understood systems are those of fungal pathogens, in which case the regulatory molecules have been identified as glucan polymers, glycoproteins, and low molecular weight organic acids (Table 11).

Table 10 Abiotic elicitors and production of secondary metabolites

Abiotic elicitor	Product	Cell culture	Refs.
High electric field pulses	Amaranthin	*Chenopodium ruburum*	Knorr et al. 1993
High hydrostatic pressure	Amaranthin	*Chenopodium ruburum*	Knorr et al. 1993
	Anthraquinones	*Morinda citrifolia*	Döernenburg and Knorr 1997
Metal ions: Cu^{2+}, Cd^{2+}, Al^{3+}, Zn^{2+}, Cu^{2+}, Va^{2+}	Isoflavonoids	*Vigna algularis*	Namdeo 2007
Ultrasound	Saponins	*Panax ginseng*	Hu et al. 2003
	Anthraquinones	*Morinda citrifolia*	Dörnerburg and Knorr 1997

Table 11 Biotic elicitors and production of secondary metabolites

Biotic elicitor	Product	Cell culture	Refs.
Agaropectin	Shikonin	*Lithospermum erythrorhizon*	Namdeo 2007
Chitosan	Anthraquinones	*Rubia tinctorum*	Vasconsuelo et al. 2004
	Antraquinones	*Morinda citrifolia*	Dornenburg and Knorr 1997
Fungal elicitor	Acridone expoxide	*Ruta gravelones*	Namdeo 2007
	Antraquinones	*Morinda citrifolia*	Dornenburg and Knorr 1997
	Codeine, morphine	*Papaver somniferum*	Dicosmo and Misawa 1995
	Taxol	*Taxus* sp.	Wang et al. 2003
	Rosmarinic acid	*Coleus blumei*	Szabo et al. 1999
	Sanquinarine	*Papaver somniferum*	Dicosmo and Misawa 1995
Jasmonic acid	Anthocyans	*Viti vinifera*	Curtin et al. 2003
Methyl jasmonate	Capsidiol, nicotine	*Nicotiana tabacum*	Namdeo 2007
	Rosmarinic acid	*Coleus blumei*	Szabo et al. 1999
	Taxol	*Taxus* sp.	Tabata 2006
Salicylic acid	Azadirachtin	*Azadirachta indica*	Namdeo 2007
Yeast elicitor	Antraquinones	*Morinda citrifolia*	Dornenburg and Knorr 1997
	Rosmarinic acid	*Coleus blumei*	Petersen and Simmond 2003

Dicosmo and Misawa (1995) described that a cell line of *Papaver somniferm* synthesized and accumulated sanguinarine, a quaternary benzophenanthridine alkaloid when exposed to a homogenate of the fungas *Botrytis*. A portion of the sanguinarine was released into the culture medium. Treatment of *Papaver somniferum* cell suspensions with a homogenate of *Botrytis mycelium* resulted in accumulation of 3% DW of sanguinarine.

The enhancement of production of secondary metabolites after elicitation is compared with that of the control, as shown in Table 12.

In addition, the content of rosmarinic acid in cultured cells of *Lithospermum erythrorhizon* increased after addition of yeast extract: a maximum was reached in 24 h [44]. When the plant cells were treated with yeast extract, on the 6th day of the cultivation, the level of rosmarinic acid increased 2.5 times.

However, the use of microbial elicitors may not be economical since an elicitor-producing microorganism should be cultivated separately from culti-

Table 12 Comparison of production of secondary metabolite after elicitation

Cells culture	Elicitor	Products	Product concentration			Refs.
			Control	Elicitation		
Catharanthus roseus	*Pythium* sp.	Ajmalicine	0	400	$\mu g\, l^{-1}$	Asada and Shuler 1989
Morinda citrifolia	Chitin	Anthra-quinones	3	7	$\mu g\, g^{-1}$ FW	Dornenburg and Knorr 1997
Rubia tinctorum	Chitosan Sp-cAMPS Forskolin	Anthra-quinone	58	128 69.3 56.9	$\mu mol\, g^{-1}$ FW	Vasconsuelo et al. 2004
Papaver bracteatum	Dendryphion	Sanguin-arine	50	450	$\mu g\, g^{-1}$ FW	Dicosmo and Misawa 1995
Vitis vinifera	Jasmonic acid	Antho-cyanins	9.2	20.7	$mg\, g^{-1}$ DW	Curtin et al. 2003

vation of plant cells. The fermentation cost for an elicitor-producing microorganism is not always low.

Characteristics of Elicitors

Several parameters, such as elicitor concentration and selectivity, duration of elicitor exposure, age of culture, cell line, growth regulation, nutrient composition, quality of cell wall materials, and substantial enhancement of product accumulation have been reported.

Elicitor concentration. Namdeo (2007) reported higher accumulation of ajmalicine in *Catharanthus* roseus cultures when treated with different concentrations of elicitor extracts of *Trichoderma viride*, *Aspergillum niger*, and *Fusarium moniliforme*. Ajmalicine accumulation was higher in cells elicited with higher concentration (5.0%) of elicitor extracts compared to lower concentration (0.5%). However, further increasing of the concentration up to 10.0% adversely affected the accumulation of ajmalicine. High dosage of elicitor has been reported to induce hypersensitive response leading to cell death, whereas an optimum level was required for induction.

Duration of elicitor exposure. Cells of *Catharanthus* roseus exposed with elicitor extracts of *Trichoderma viride* for 24, 48, 72, and 96 h were examined. About threefold increase in ajmalicine production by *Catharanthus* roseus cells elicited with extracts of *Trichoderma viride* for 48 h [2]. However, further increasing exposure time resulted in decrease in ajmalicine content.

Age of culture. *Catharanthus* roseus cells of 20-day-old cultures showed higher yields of ajmalicine on elicitation. Highest ajmalicine ($166\ \mu g^{-1}$ DW)

was accumulated in 20-day-old cells elicited with extracts of *Trichoderma viride* [42].

Apart from these characteristics, the efficiency of elicitation also depends on elicitor specificity, cell line or clones of microbial elicitor used, presence of growth regulators, and the environmental conditions.

3.2.3 Immobilization and Application of Immobilized Cells

Immobilization has been characterized as a technique that confines a catalytically active enzyme or cell and prevents its entry into the mobile phase, which carries the substrate and product [30]. Immobilization of plant cells has distinct advantages as biocatalyst over the immobilized enzyme system. Immobilizing cells in a gel, which is permeable to the molecules of the nutrient medium or on polymers (with a view to preserving their metabolic capacity and to using them several times), has the advantage of extending the production time of cells (over six months) and of making the cells catalyse the same reaction almost indefinitely.

The use of immobilized cells should bypass the direct extraction of the compounds from the biomass as the products arise in the medium itself. Immobilized cells can carry out multi-enzyme operations; by selecting highly biosynthetic cells, catalytic activity can be enhanced; there is no need to provide co-factors since cells produce them themselves.

Immobilized plant cells can be used for single and multi-step biotransformations of precursors to desired products as well as for the de-novo biosynthesis of secondary metabolites (see Sect. 3.2.4 "Biotransformation").

Immobilization of plant cells is considered to be of importance in research and development in plant cell cultures, because of the potential benefits that could be provided [30]:

- The extended viability of cells in the stationary (and producing) stage, enabling maintenance of biomass over a prolonged time period;
- simplified downstream processing (if products are secreted);
- promotion of differentiation, linked with enhanced secondary metabolism;
- reducing the risk of contamination;
- reduced shear sensitivity (especially with entrapped cells);
- promotion of secondary metabolite secretion, in some cases;
- minimization of fluid viscosity increase, which in cell suspension causes mixing and aeration problems.

An immobilization system, which could maintain viable cells over an extended period of time and release the bulk of the product into the extracellular medium in a stable form, could dramatically reduce the costs of phytochemicals production in plant cell culture. However, an immobilized system also has the problems described below:

- Immobilization is normally limited to cases where production is decoupled from cell growth;
- initial biomass must be grown in suspension;
- secretion of product into the extracellularly medium is imperative;
- where secretion occurs, there may be problems of extracellular degradation of the products;
- when gel entrapment is used, the gel matrix introduces an additional diffusion barrier.

Biocatalysts can be immobilized by confinement within a porous membrane (gel entrapment, membrane reactor, interfacial membrane) or by attachment to a solid surface (inner surface of a porous structure, outer surface of a carrier by adsorption or covalent bond) [30].

Physical entrapment in a porous matrix is the most flexible and most popular approach employed for whole cell immobilization. A process designed for the efficient entrapment of whole cells should allow for the following:

- High retention of cell viability (biological activity of the entrapped cells should not be impaired by the immobilization conditions);
- porosity of the formed gel should be uniform and controllable (free exchanges of substrates, products, co-factors, and gases is essential for efficient performance of the immobilized cells);
- gel should retain good mechanical, chemical, and biological stability (it should not be easily degraded by enzymes, solvents, pressure changes, or shearing forces);
- gel should be composed of reasonably priced components.

Various immobilization methods have been developed (entrapment, adsorption, and covalent coupling). The most widely used technique involves the entrapment of cells in some kind of gel or combination of gels which are allowed to polymerize around them. Brodelius and Pedersen (1993) as well as Alfermann and Petersen (1995) described the entrapment of viable cells of *Catharanthus roseus*, *Morinda citrifolia*, and *Digitalis lanata* in calcium alginate gel and this technique has received much attention. Calcium alginate, agar, agarose, gelatin, carrageenan, and polyacrylamide can be used as matrix [25]. However, gels of alginate are most widely used because of their simplicity and relative lack of toxicity. The other alternative supports are polyurethane foam and hollow-fibre membranes. Table 13 gives a number of examples of the systems of immobilization, which have been used with plant cells together with the associated plant species and their products.

Work with *Catharanthus roseus* showed that agar, agarose, and carageenan were all suitable immobilization matrices, suitable for the maintenance of cell viability; but alginate was superior in terms of ajmalicine production [7].

Adsorption immobilization has been successfully used with a number of plant species. *Capsicum frutescens* cells immobilized on polyurethane foam produced 50 times as much capsaicin as suspension cells [24]. In general,

Table 13 Immobilized plant cell systems used for production of secondary metabolites

Immobilization method	Plant species	Substrate/ precursor	Product	Refs.
Biotransformation				
Agarose	*Catharanthus roseus*	Cathenamine	Ajmalicine	Asada and Shuler 1989
Alginate	*Digitalis lanata*	Digitoxin	Digoxin	Alfermann and Petersen 1995
Polyurethane foam	*Papaver somniferum*	Codeinone	Codeine	Dicosmo and Misawa 1995
Synthesis from precursors				
Alginate	*Nicotiana tabacum*	Phenylalanine	Caffeoyl putrescine	Berlin et al. 1981
Alginate, agarose	*Catharanthus roseus*	Tryptamine, secologanin	Ajmalicine	Brodelius and Pedersen 1993
	Lithospermum erythrorhizon		Shikonin	Kim and Chang 1990
Polyurethane foam	*Capsicum frutescens*	Isocapric acid	Capsaicin	Brodelius and Pedersen 1993
De novo synthesis				
Alginate	*Morinda citrifolia*		Anthraquinon	Dornenburg and Knorr 1997
Aginate, agarose	*Catharanthus roseus*		Ajmalicine	Brodelius and Pedersen 1993
Hollow fibres	*Glycine max*		Phenolics	Brodelius and Pedersen 1993
Polyurethane foam	*Capsicum frutescens*		Capsaicin	Johnson and Ravishankar 1996

it appears that mild immobilization either through gel entrapment or surface adsorption enhances productivity and prolongs the viability of cultured cells.

Immobilized cells can also be used as biocatalysts for biotransformations (see Table 14 and Sect. 3.2.4 "Biotransformation"). Such a system compares favorably with the use of freely suspended cells since, in the case of immobilization, the catalyst is theoretically reusable and the product is easily separated from the biomass.

Immobilization can have a dramatic impact on cellular physiology and secondary product formation. The cell culture responses are summarized in Table 14.

Dicosmo and Misawa (1995) found that glass fibres could be used as a carrier of plant cells to produce useful plant metabolites. *Papaver somniforum* cells were immobilized on fabric of loosely woven polyester fibres arranged in a spi-

Table 14 Effects of immobilization on secondary metabolite production in cell cultures

Type of immobilization	Plant species	Product	Fold change	Refs.
Foam	*Capsicum frutescens*	Capsaicin	> 100	Johnson and Ravishankar 1996
Calcium alginate	*Lithospermum erythrorhizon*	Shikonin	2.5	Kim and Chang 1990
Natural glass	*Papaver somniferum*	Saquinarine	2	Dicosmo and Misawa 1995
Gel	*Coffea arabica*	Methylxanthin	13	Brodelius and Pedersen 1993
	Capsicum frutescens	Capsaicin	> 100	Johnson and Ravishankar 1996
	Chenopodium rubrum	Betacyanin		Knorr and Berlin 1987

ral configuration on stainless steel support frame to produce sanguinarine, an antibiotic in oral hygiene. The yield was 3.6 mg g^{-1} FW by immobilized cells and was more than twice as much as by suspension cells.

Polyurethane-immobilized *Capsicum frutescens*-cell-fed capsaicin precursors produced this metabolite at levels of up to 100 times those of non-fed cultures. *Capsicum frutescens* cells immobilized on polyurethane released capsaicin entirely into the medium, although other species immobilized by the same method retained the product intracellularly [24].

Many metabolites still appear to accumulate in the cell vacuoles and it is therefore important to further gain information on how these metabolites may be released into the culture medium. *Chenopodium rubrum* cells, immobilized in alginate beads, secreted the red betacyanin pigment amaranthin into the medium [31]. However, the pigment was subsequently degraded; chitosan and DMSO permitted further product release into the extracellular medium, but this was also accompanied by product degradation.

3.2.4 Biotransformation and its Advantages

Biotransformation can be defined as a process through which organic compounds can be modified by cell cultures resulting in chemically different products. There are two main reasons to choose plant cells for biotransformation purposes: Firstly, these cells are generally able to catalyze the reactions stereospecifically, resulting in chirally pure products. Secondly, they can perform regio-specific modifications that are not easily carried out by chemical synthesis or by microorganisms [46]. These reactions include reduction, oxidation, hydroxylation, acetylation, esterification, glucosylation,

isomerization, methylation, demethylation, epoxidation, etc. [1]. The presence of biotransformation potential in plant cells is a necessary condition for practical application.

Advantages of biotransformation include enhancement in the productivity of the desired compound and the production of novel compounds. Importantly, the studies on biotransformation lead to basic information to elucidate the biosynthetic pathway, and catalysis can be carried out under mild conditions, thus reducing undesired by-products, energy, safety, and costs. The range of flavor metabolites and pharmaceuticals produced by plant cell cultures through biotransformation is shown in Table 15.

The conversion of monoterpenes was studied with *Mentha* species by Dornenburg and Knorr (1997). Suspension cultures of *Mentha canadensis* and *Mentha piperita* were able to synthesize limonene as well as oxygenated, acetylated, or glucosilated monoterpenes. However, the yields of these compounds were low and monoterpene glucosides were accumulated in higher amounts than free monoterpenes. *Mentha* suspension cultures metabolized exogenous monoterpene ketones and monoterpene alcohols within 24 h, and glucosilation occurred. Glucosilation was a detoxification mechanism of phytotoxic compounds by plant cells, and it resulted in accumulation of glucosilated and water-soluble products. Otherwise, the exogenously applied toxic monoterpenes were degraded and metabolized by cell cultures without special accumulation sites. Exogenous terpenes have been shown to be rapidly metabolized by cell suspension cultures to form biotransformation and degradation products. It was concluded that the plant cell culture processes for acceptable product yields can be conceivable if the desired product can be accumulated in a nonpolar organic phase or adsorbed.

For a successful process, the following prerequisites must be met (Knorr 1987): the culture must have the necessary enzymes; the substrate or precursor must not be toxic to the culture; the substrate must reach the cellular compartment of the cell; and the rate of product formation must be faster than its further metabolism.

Table 15 Biotransformation of flavor compounds by plant cell culture systems

Plant species	Substrate	Product	Refs.
Coffea arabica	Vanillin	Vanillin glucosides	Johnson and Ravishankar 1996
	Capsaicin	Capsaicin glucoside	Johnson and Ravishankar 1996
Mentha spp.	Pulegone	Isomenthone	Dornenburg and Knorr 1997
	Menthol	Neomenthol	
Papaver bracteatum	Linalyl acetate	Linalool, geraniol	Dicosmo and Misawa 1995
Vanilla planifolia	Ferulic acid	Vanillin	Romagnoli and Knorr 1988

4
Release and Product Recovery

4.1
Exudation

Many compounds, synthesized by plants, are stored in the vacuole. Enhancing transfer of compounds from the vacuole to the culture medium would be very useful in terms of costs for product recovery. This could include the development of additional chemical or environmental agents to induce such exudation. It may also be possible to recover the substances secreted into the medium. For this method, it could be necessary to examine the physiological mechanisms of metabolite release from the plant cells.

Lee and Shuler (2000) showed that the accumulation of indole alkaloids in *Catharantus roseus* vacuoles has been attributed to an ion-trap mechanism, whereby the basic indole alkaloids are trapped in the acidic vacuole due to their positive charge at low pH, preventing diffusion across the tonoplast. Kim et al. (2004) reported that almost all of the taxol produced by *Taxus brevifolia* cell cultures was detected in the culture filtrate.

4.2
Two-Stage Systems

The sites of synthesis and storage of secondary compounds in plant cells often take place in separated compartments. The accumulation of secondary metabolites in cell cultures is most likely associated with the presence of highly specialized structures containing secretory and accumulatory elements, such as oil glands, glandular trichomes, or a glandular epidermis [17]. Encapsulation of cytotoxic compounds also serves as a self-protection mechanism of intact plants. In undifferentiated callus or suspension cultures, these accumulation sites are missing. This is probably the reason for the low yields of such compounds reached in these plant cell cultures.

A low accumulation of secondary compounds in cell cultures in a number of cases may not be due to a lack of key biosynthetic enzymes, but rather due to feedback inhibition, enzymatic or non-enzymatic degradation of the product in the medium, or volatility of substances produced. In such cases, it should be possible to increase the net production by the addition of an artificial site for product accumulation, for example, by use of second solid or liquid phase introduced into the aqueous medium.

The use of in situ product removal of metabolites has a number of key potential advantages beyond promoting secretion. The removal and sequestering of the product in a non-biological compartment may increase its total production [46].

The addition of an artificial site for the accumulation of secondary metabolites can be an effective tool for increasing biosynthetic pathways in plant cell cultures. If the formation of a product is subject to feedback inhibition or intracellular degradation, the removal and sequestering of the product in an artificial compartment may increase total metabolite production. Table 16 summarizes several examples of two-phase or adsorption cultures.

Robbins and Rhodes (1986) reported that the addition of amberlite XAD-7 resin to *Chinchona ledgerina* cells stimulated the production of anthraquinones by 15 times, which was 539 $mg\,l^{-1}$, compared to a medium without adsorbent. The yields of ajmalicine and serpentine produced by *Catharantus roseus* were also increased by the addition of XAD-7 and the ratio between both alkaloids produced was changed [34]. It is of interest that production of these alkaloids, which accumulate inside cells, was affected by

Table 16 Adsorbents used for two-phase plant cell cultivation systems

Adsorbents	Cell cultures	Refs.
Activated charcoal	*Marticaria chamomilla*, *Nicotiana tabacum*, *Vanilla fragrans*	Dornenburg and Knorr 1997
β-Cyclodextrin	*Mucuna pruriens*, *Mentha canadensis*	Dornenburg and Knorr 1997
Miglyol	*Matricaria chamomilla*	Rao and Ravishankar 2002
	Mentha canadensis, *Thuja occidentalis*, *Valeriana wallichii*, *Vitis vinifera*	Dornenburg and Knorr 1997
	Pimella anisum	Mulder-Krieger et al. 1988
Polyethylenglycol	*Nicotiana tabacum*	Knorr et al. 1987
Polydimethylsiloxan	*Eschscholizia californica*	Dornenburg and Knorr 1997
RP-8	*Marticaria chamomilla*, *Mentha piperita*, *Pimella anisum*, *Valeriana wallichü*	Dornenburg and Knorr 1997
XAD-2	*Galium vernum*, *Thuja occidentalis*	Dornenburg and Knorr 1997
XAD-4	*Nicotiana rustica*, *Thuja occidenialis*	Dornenburg and Knorr 1997
	Vanilla fragrans	Knorr et al. 1985
XAD-7	*Catharanthus roseus*	Lee and Shuler 2000
	Chinchona ledgerina	Robbins and Rhodes 1986
	Vanilla fragrans	Dornenburg and Knorr 1997
Wofatite	*Galium vernum*	Dornenburg and Knorr 1997
Zeolith	*Nicotiana tabacum*	Dornenburg and Knorr 1997

the presence of resin. The addition of XAD-4 increased the vanilla flavor production in *Vanilla fragrans* suspension cultures [28]. A similar approach was conducted by the addition of charcoal: it led to up to 60-fold improvements in yields of coniferyl alcohol in *Marticaria chamomilla* culture and the addition of Miglyol or silica gel RP-8 stimulated ethanol production in cell cultures of *Pimpinella anisum* [41].

Two-phase systems even accumulate traces of secondary metabolites from the culture medium, thus avoiding any type of feedback inhibition. Another effect may be the enhancement of secondary metabolite release from the cultures or the initiation of a release of compounds normally stored within the cells. Secreted secondary metabolites may be protected from degradation in the culture medium because of excreted catabolic enzymes and acids. Evaporation of the product into the gas phase can be reduced by trapping flavor compounds in artificial accumulation sites. Desired plant products can then be removed selectively from the culture systems. The product can be concentrated by in situ recovery, and downstream purification may be reduced if product removal from the culture medium and cells is selective. Consequently, recovery and purification are generally simplified, thus reducing production costs.

4.3 Membrane Permeabilisation

In most cases, products formed by plant cell cultures are stored in vacuoles. In order to release the products from vacuoles of plant cells, two membrane barriers (plasma membrane and tonoplast) have to be penetrated. Cell permeabilization depends on the formation of pores in one or more of the membrane systems of the plant cell, enabling the passage of various molecules into and out of the cell [7]. Attempts have been made to permeabilize the plant cells transiently, to maintain the cell viability, and to have short time periods of increased mass transfer of substrate and metabolites to and from the cell.

Permeabilization of plant membranes for the release of secondary metabolites is often connected with the loss of viability of the plant cells treated with permeabilizing agents and methods. Various methods have been used to initiate product release from cultured plant cells. These methods include chemical treatments (e.g., with solution of high ionic strength, change of external pH, permeabilization with dimethylsulfoxide DMSO, chitosan) and physical treatments (e.g., high electric field pulses, ultrasonics, ultra-high pressure.) [17, 28].

4.3.1 Chemical Permeabilisation

Active uptake mechanisms have also been reported for indole alkaloids in *Catharantus roseus* vacuoles [39]. In terms of product release, it is pertinent

to note that in cell cultures, an efflux of alkaloids was observed under certain conditions, indicating equilibrium between the intracellular and extracellular compartments that could be perturbed by medium acidification with subsequent product release. The release of serpentine by *Catharantus roseus* cells was observed when the cells were filtered and resuspended in fresh or conditioned medium, and it was suggested that temporary membrane uncoupling was responsible for it.

According to Dornenburg and Knorr (1997), *Chenopodium rubrum* cells could be permeabilized by treatment with chitosan. This polycationic polysaccharide induces pore formation only in the plasmalemma of the plant cell cultures. The leakage caused by chitosan can be considered as leakage from cytosol. Long-term permeabilization with chitosan showed a time-dependent amaranthin release from Chenopodium rubrum cells into the culture medium. Brodelius and Pedersen (1993) tested five permeabilizing agents on three different species, and although product release was achieved, cell viability dropped in most cases. The exceptions were DMSO and Triton X-100, applied to *Catharantus roseus* cells.

Trejo-Tapia et al. (2007) reported that treatment of *Beta vulgaris* cell culture for 15 min with 0.7 mM Triton X-100 induced the release of 30% of betacyanines without loss of cell viability (70%). After this permeabilization treatment, *Beta vulgaris* cultures regrew normally, reaching a maximum biomass concentration of 48% higher than non-permeabilized cultures after 14 days of culture. In addition, maximum betacyanines concentration was only 25% lower than that of non-permeabilized cultures.

4.3.2 Physical Permeabilisation

Physical factors causing membrane permeabilisation include high electric field pulses, high hydrostatic pressure, ultrasound, etc.

Application of the high electric field pulses is based on the principle of development of membrane pores under external electric fields. Depending on electric field strength or pulse number, the pore formation can be reversible or irreversible. Application of high electric field pulses [32] led to high levels of cell permeabilization in cultures of *Chenopodium rubrum*, but at field strengths beyond 0.75 kV/cm and constant amount of ten pulses, cell viability approached zero values.

Knorr et al. (1993) have shown that treatment with high hydrostatic pressure of 50 MPa increased the production of amarantin and antraquinones in cell cultures of *Chenopodium ruburum* and *Morinda citrifolia*. It was found that pressure higher than 250 MPa causes the loss of cells viability, most likely because of permeabilisation of tonoplast.

It has been assumed that the pressure-dependent destruction of the tonoplast, the loss of compartmentation, and subsequent release of the content of

the vacuoles may have caused the pH change in the medium and the resultant cell death.

Knorr et al. (1993) concluded that application of electric field pulses or high pressure has only limited potential for cell permeabilization with concurrent retention of cell viability. However, both procedures could become effective tools for product recovery from plant cells and tissues with minimum effects on product composition.

5
Industrial Production of Useful Biochemicals by Plant Cell Cultures

With technological advancement in the future, plant cell culture will have a greater contribution to the market. For example, the current world market of raw materials of ginseng is about one billion US$ [65]; although cell-cultured *Panax ginseng* occupies less than 1% of the market, its share will increase greatly with enhancement of the culture productivity.

Mitsui Petrochemical Industries Ltd. provides the industrial-scale production of shikonin by *Lithospermurn erythrorhizon* cell cultures. The process involved two stages: plant cells are first grown in a 200-l bioreactor and the resulting biomass is then transferred into a second bioreactor, in which the composition of the culture medium favors the synthesis of shikonin. The productivity of cell cultures reaches $60\,mg\,g^{-1}$ per week, which is 1000 times higher than that of the plant roots, which required a longer time of five to seven years [50]. The success of shikonin production can be regarded to the selection of a cell line, which accumulates tenfold higher level of shikonin than roots of the mature plant. This achievement also results from selection of optimal growth conditions and production media. Thus, cell cultures have now become the major commercial source of shikonin.

The Bio-organic Division of the Bhabha Atomic Research Centre (India) is carrying out research on mass cultivation of selected cell lines of *Rauwolfia serpentina* (ajmaline, reserpine), *Papaver somniferum* (thebaine, codeine, and morphine), *Artemisia annua* (artemisinin), and other plant species [11].

6
Conclusions and Outlook

In recent years, the market for plant products has rapidly expanded, and this trend will continue because more and more people prefer to use natural products. It is widely recognized that cultured plant cells represent a potential source of valuable phytochemicals, but only a few cell cultures are commercially used as a stable and productive source of the secondary metabolites. Over the last few decades, many strategies, such as media manipulation,

phytohormone regulation, precursor feeding, plant cell immobilization, biotransformation, and bioconversion, have been applied for optimization of the synthesis of the desired products from the plant cell cultures in appreciable quantity and at competitive economic value. For plant cell culture techniques to become economically viable, it is important to develop methods that would allow for consistent generation of high yields of products from cultured cells.

The increased use of genetic tools and an emerging picture of the structure and regulation of pathways for secondary metabolism will provide the basis for the production of commercially acceptable levels of products.

Selection of productive cell lines can result in accumulation of products in higher levels in cell cultures as compared to plant tissues. In order to obtain yields in high concentrations for commercial exploitation, efforts are focused on the stimulation of biosynthetic activities of cultured cells using various methods. The introduction of the techniques of molecular biology and production of transgenic cultures can affect the expression and regulation of biosynthetic pathways.

Knowledge of biosynthetic pathways of desired compounds in plants and cell cultures opens new possibilities to regulate the production of phytochemicals by feeding with precursors, application of elicitors, etc.

Because of the complex and incompletely understood nature of plant cells in in-vitro cultures, case-by-case studies have been used to explain the problems occurring in the production of secondary metabolites from cultured plant cells. Production of secondary metabolites by plant cell cultures must be competitive with other conventional means of production, such as extraction from the field-grown plants, alternative chemical synthesis, microbial fermentation, or improvement of the plant itself through somaclonal variation, and genetic engineering.

The significant advance in optimization of plant cell cultures can be achieved, although at present there are two main obstacles: the lack of an adequate process monitoring and control system for plant cells and the heterogeneity and instability of the cells.

The combined efforts of experts of plant science, food technology, pharmacognosy, biochemistry, molecular biology, and fermentation technology can exploit the potential of plant cells for the production of plant secondary metabolites.

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