Suman Chandra
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Biotechnology for Medicinal Plants

Micropropagation and Improvement

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ISBN 978-3-642-29973-5 ISBN 978-3-642-29974-2 (eBook)
DOI 10.1007/978-3-642-29974-2
Springer Heidelberg New York Dordrecht London

Library of Congress Control Number: 2012942923

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Suman Chandra and Hemant Lata dedicate this book to their parents and children Rishi and Riddhi for their love and support

Foreword

Plants are by far the most abundant and cost effective renewable resource of medicines since ancient times. Even today, the World Health Organization (WHO) estimates that up to 80 % of the world's population still relies mainly on traditional remedies such as herbs in one form or another for their medicine. The scientific foundation for the emergence of pharmacology in the eighteenth and nineteenth centuries was based largely on plant-derived substances. With the introduction of new botanical therapeutic agents, plants are also poised to continue with a key role in the era of modern medicine. Worldwide, the beneficial impact of plant biotechnology has been almost exclusively on medicinal plants. Plant biotechnology has been successfully employed for the large-scale production of phytopharmaceuticals, perfumes, colors, flavors, and biopesticides. Recently, we have begun to realize the ability to better exploit plant-derived bioactives as a result of dramatic advances in metabolic engineering, biochemical genomics, chemical and molecular characterization, and pharmaceutical screening. Furthermore, plant biotechnology acts as a core research tool in the basic research for plant biology and a practical tool for plant improvement.

This volume is a unique overview of medicinal plant biotechnology, describing the whole spectrum of biotechnological tools from cell-culture techniques, to the biosynthesis and accumulation of pharmaceutical compounds in the plants, genetic transformation, metabolic engineering, metabolomics, processes for the production of authenticated plant raw materials and quality control of materials for their safe and efficacious use.

Dr. Suman Chandra and Dr. Hemant Lata, along with Dr. Ajit Varma, have envisaged this special volume that aims to review the state of the art for medicinal plant biotechnology. I have been privileged to see the exemplary work of Drs. Chandra and Lata in our own program using many of these approaches, and to see their aspirations and their maturation in applying this expertise in our own highly interdisciplinary program. They are great team players and gave themselves unselfishly to this task. I am very pleased to see them working with Dr. Varma in bringing together these chapters by eminent scientists from across the globe. Their dedicated work on this project is evident in the quality, comprehensiveness,

and cohesiveness of this volume. This work will be useful for researchers in the pharmaceutical and biotechnological industries, medicinal chemists, biochemists, botanists, molecular biologists, academicians and students alike. I congratulate the editors and authors for their endeavors and wish the book all success.

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Preface

Plant-based medicines are used in all civilizations and cultures, and have been one of the indispensable lines of defense in maintaining health and combating diseases worldwide. With the increasing demand of medicinal plants at the global level for use in pharmaceuticals and dietary supplements, biotechnology has emerged as a powerful tool for their conservation and improvement. By adopting techniques such as in vitro propagation and genetic transformation, biotechnology plays an important role in multiplication and genetic enhancement of medicinal plants. The rapid advances in plant genomics, transcriptomics, proteonomics, and the recent emergence of metabolomics along with molecular biology and analytical chemistry will greatly facilitate and enhance the metabolic engineering of medicinal plants.

The purpose of "Biotechnology for Medicinal Plants: Micropropagation and Improvement" is to present in a single volume the comprehensive knowledge and the experience of renowned researchers and scientists in the field of medicinal plant biotechnology. The book provides an overview of modern plant biotechnology and discusses the potential applications of plant biotechnology in the improvement of medicinal plants. Each chapter is independently written by experts in their field of endeavor ranging from micropropagation protocols, advantages of different biotechnological tools in the plant micropropagation, the biosynthesis of useful secondary metabolites, the metabolomics, the transcriptomics, the metabolomic engineering, the bioinformatics, to the quality control of phytopharmaceuticals.

The book begins with introductory chapters on the overview of downstream processes of plant cell and tissue culture (Chap. 1, Yesil-Celiktas and Vardar-Sukan) and the *Agrobacterium rhizogenes*-mediated transformation in medicinal plants (Chap. 2, Roychowdhury et al.) followed by the biotechnology of *Scutellaria* propagation (Chap. 3, Joshee et al.), microbial endophytes (Chap. 4, Rathod et al.), *Cannabis* propagation for the production of phytocannabinoids (Chap. 5, Chandra et al.), and the micropropagation of medicinal *Epilobium* species (Chap. 6, Constantin et al.). In the next few chapters the discussion is focused on the advantages and amplification of photoelicitation (Chap. 7, Matsuura et al.), microspore culture

(Chap. 8, Ferrie), and cellular heterogeneity (Chap. 9, Patil and Roberts) in plant cell and tissue culture.

Hairy root cultures are a useful means for studying the biochemical and gene expression profiles of the biosynthetic pathways, and for metabolic engineering of medicinal plants. Chapter 10 (Sheludko and Gerasymenko) outlines the biosynthetic potential of hairy roots for the production of novel natural products, while in Chap. 11 (Bunsupa et al.) focus is laid on the molecular biology and biotechnology of quinolizidine alkaloid biosynthesis in leguminosaeLeguminosae plants.

Chapter 12 (Aravindaram and Yang), summarizes the various analytical techniques most frequently used in phytomedicinal research and the potential as well as the limitations of various approaches are discussed.

Free radicals are well known for their involvement in the pathogenesis of a large number of degenerative diseases such as cancer, cardiovascular diseases, neurological disorder and diabetes, in addition to their role in immunomodulation. Over the years, research on antioxidants present in medicinal plants has gained enormous popularity and emerged as an arena for providing potential therapeutic agents to prevent/quench free radical generation that may damage the biological system of the human body. Chapter 13 (Bhatt et al.), focuses on the antioxidants present in various important medicinal plants and their implication in the treatment of certain diseases.

Chapters 14–16 provide in-depth discussion on metabolic engineering in medicinal plants. In Chap. 14, Chow and Sato describe the metabolic engineeringmetabolic engineering and synthetic biology for the production of isoquinoline alkaloids, whereas in the following chapter (Chap. 15) Shoji and Hashimoto explore the role of Isoquinoline Alkaloids 'jasmonate-responsive transcription factors' as new tools for metabolic engineering and gene discovery in medicinal plants. In Chap. 16, Zarate et al. review methodologies and advances with the future directions of metabolic engineering of plant cellular metabolism.

Using *Hypericum* as a model, use of metabolomics and transcriptomics to analyze the regulation and biosynthesis of medicinal compounds is described in Chap. 17 (Crispin and Wurtele). Metabolomics, the comprehensive analysis of diverse metabolites, has greatly expanded metabolite fingerprinting and profiling as well as the selection and identification of marker metabolites in medicinal plants. The methodology typically employs multivariate analysis to statistically process the massive amount of analytical chemistry data, not only including the major, but also fingerprinting the minor metabolites, resulting from high-throughput and simultaneous metabolite analysis. Using the advances in computational science, Chap. 18 (Okada et al.) of this book focuses on multivariate analysis of analytical chemistry data and utility of the KNApSAcK family database to understand metabolic diversity in medicinal plants. This chapter also describes the effectiveness and potential of computational systems biology in medicinal plant research.

Authenticity and consistency in the quality of biomass are the key parameters for the safety and efficacy of any phytopharmaceutical. Somatic mutation is a common problem in micropropagation that can alter the genetics and the chemical profile of the propagated crops. The quality assurance of starting martial is

therefore a prerequisite. The concluding chapter (Chap. 19, Sucher et al.) of this book discusses the use of genomic and transcriptomic profiling of propagated plants for the quality control of plant-based drugs.

It has been a pleasure to edit this book, primarily due to the splendid cooperation of contributors in preparation of the manuscript according to the guidelines provided, strict adherence to time schedules and the richness of the material provided by them, for that we are highly thankful. We express our gratitude and heartfelt thanks to each author for their generous contribution of time and effort.

We also wish to thank Dr. Jutta Lindenborn and Dr. Christina Eckey at Springer Heidelberg, for their generous assistance and patience in finalizing the volume. Suman Chandra and Hemant Lata in particular express special gratitude to Prof. Dr. Mahmoud ElSohly for his guidance, constant encouragement, and kind support.

Oxford, MS, USA — Suman Chandra
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Chapter 1
Downstream Processes for Plant Cell and Tissue Culture

Ozlem Yesil-Celiktas and Fazilet Vardar-Sukan

1.1 Introduction

The first attempt to cultivate plant cells in vitro was almost a century ago. The aseptic culture of cells, tissues, organs, and their components under defined physical and chemical conditions in vitro, has become an important tool in both basic and applied sciences as well as in commercial applications (Thorpe 2007). The prospect of using such culturing techniques is for obtaining secondary metabolites, such as active compounds for pharmaceutics and cosmetics, hormones, enzymes, proteins, antigens, food additives and natural pesticides from the harvest of the cultured cells, or tissues. Industrial companies spend an increasing amount of resources on new product development and are anxious for getting good return on product development investments. Generally, product development is a risky activity as it is very resource demanding and the outcome is uncertain. The majority of all product development projects do not result in a marketable product and all innovations are far from being profitable Therefore, companies look for different ways to reduce the uncertainty and by doing so remain viable and competitive. The task of product development may be divided into two major sub-tasks. Considering the business mission of the companies and the target customers, one of the tasks is to decide what to develop in terms of product functions and properties. The other is to find good technical solutions to achieve the functions and properties. The first focuses on future customer requirements, demands, and market developments while the second deals with more technical issues (Sandell 1996) which will be the focus of this chapter.

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S. Chandra et al. (eds.), *Biotechnology for Medicinal Plants*,
DOI: 10.1007/978-3-642-29974-2_1,

The design and development of simplified and cost-effective separation processes to purify and isolate active compounds is a major challenge for commercialization of biotechnological industries. Various steps of separation processes used for the purification of active compounds are commonly known as downstream processes. Considering the sequence of a plant cell and tissue culture cultivation process, two major methodologies namely, cell culture studies and clonal propagation techniques are widely used. Cell culture studies begin with callus initiation using in vitro cultures for the purpose of determining the medium that best adapts for cultivation. When calli are obtained, they can undergo somaclonal variation, generally during several subcultures (Gurel 1989). When genetic stability is reached, callus lines need to be screened in order to evaluate the productivity of each cell line so that the best performing lines can be taken to cell suspensions. Various approaches can be used to increase the production of secondary metabolites in cell suspensions, but elicitation is generally one of the most successful. The next step is the bioreactor studies leading to a possible commercial production of secondary metabolites. This is a critical step as various problems might arise when scaling-up from shake flasks to bioreactors. Subsequently, the biomass is treated with a number of steps for recovery and purification purposes. Downstream operations used are solid–liquid separation processes such as centrifugation and filtration, followed by cell disruption techniques and concentration steps such as membrane processes, finally separation and purification processes such as various types of extraction and chromatographic techniques. Particularly, dilute bioreactor streams lead to high purification costs, where downstream processing costs could be as high as 10 times the cultivation cost for value-added compounds. Hence, the application of integrated downstream processes suitable for the nature of the specific plant cell and tissue cultures are of great importance for the economic recovery and purification of secondary metabolites.

1.2 Mass Transfer Considerations

Mass transfer resistances play an important role in maximizing the separation efficiencies in downstream processes. Mass transfer occurs when compounds are transported from regions of high concentration to regions where the concentration is low or in other words from regions of high chemical potential to low. The driving force of mass transfer is molecular and bulk diffusions.

Molecular diffusion is the movement of compounds in a mixture under the influence of a concentration difference. Diffusion of compounds occurs in the direction in order to overcome the concentration gradient. On the other hand, bulk diffusion sometimes referred as convective mass transfer or turbulent diffusion is the transfer of compounds in the presence of bulk fluid motion. Molecular diffusion occurs whenever there is a concentration gradient. Additionally, if the bulk fluid is also moving, the overall rate of mass transfer will be higher due to the

contribution of convective motions. Analysis of mass transfer is most important in multi-phase systems where interfacial boundary layers provide significant mass-transfer resistances. Two mass-transfer situations which occur in downstream processing of plant cell and tissue cultures are liquid–solid mass transfer for the recovery of intracellular compounds from the biomass and liquid–liquid mass transfer between the organic solvents and the broth for the recovery of extracellular compounds.

1.2.1 Liquid–Solid Mass Transfer

Mass transfer between a liquid and a solid is important in plant cell and tissue culture processes due to the presence of clumps, pellets, flocs, and films of cells. The active compounds in the solid phase must be transported into the liquid phase as in the case of leaching and supercritical fluid extraction. Moreover, adsorption of compounds onto surfaces, such as in chromatography and adsorption requires transport from liquid phase to solid. Liquid–solid mass transfer is also important in crystallization as molecules move from the liquid to the face of the growing crystal.

Considering the transport phenomena, active compounds diffuse from biomass to the solvent phase or active compounds in liquid phase diffuse into or onto the solid as in the case of various adsorbents which are illustrated in Fig. 1.1. In each case, the fluid velocity is reduced and a boundary layer develops near the interface. The local concentration of the compounds at the surface decreases as a result of adsorption and a concentration gradient is established throughout the film. The concentration difference between the bulk liquid and the phase interface is the driving force for the mass transfer of the compounds from the liquid to the solid, allowing the adsorption to continue. For higher recovery of compounds from the liquid phase, the contact area for mass transfer is an important parameter. For instance, considering adsorption characteristics of digoxin recovery, the increase of the contact area for mass transfer significantly enhanced digoxin production (Hong et al. 1998).

In the case of active compounds diffusing from biomass to the solvent phase, first of all the target compounds in solid particles of biomass solvate, then the dissolved compounds diffuse to the outer surface of the solid particle and finally diffuse from the liquid film formed on the surface of the solid particle into the bulk solvent. Main factors affecting the extraction time are the external diffusion coefficient and the difference in chemical potential between the solid–liquid interface and the bulk liquid.

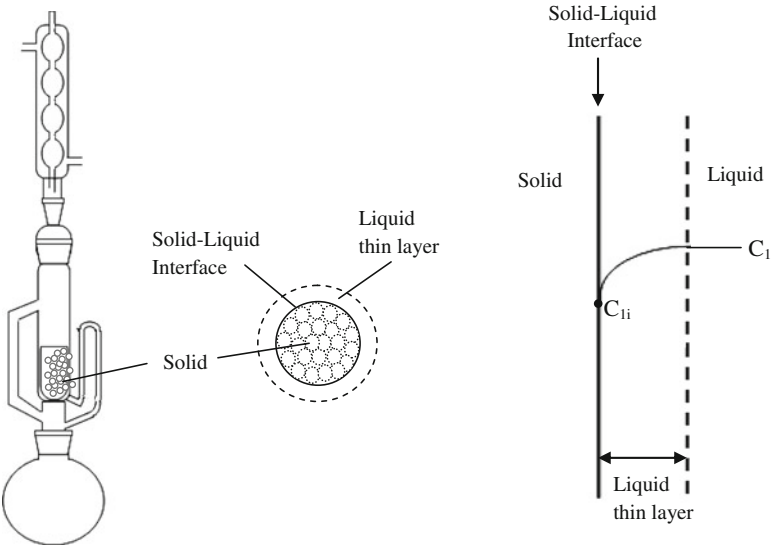

Fig. 1.1 Liquid–solid mass transfer; a Soxhlet apparatus and a schematic representation of active compound C_{1i} diffusing from biomass to the solvent forming the liquid phase

1.2.2 Liquid–Liquid Mass Transfer

Liquid–liquid mass transfer between the stream from the bioreactor and the solvent is encountered in the recovery of extracellular compounds. Organic solvents are used to isolate active compounds from the broths. Mass transfer of the active compounds from one phase to another involves transport from the bulk of one phase to the interface, and then from the interface to the bulk of the second phase. This phenomenon is expressed in the film theory as formation of a fluid film or mass-transfer boundary layer wherever there is contact between two phases.

The situation at the interface between two liquid phases is shown in Fig. 1.2. Assume that the phases are two immiscible liquids and that C_1 is initially at higher concentration in the first liquid phase than in the other liquid phase. Compounds are present at bulk concentration C_1 in the broth, subsequently, this concentration falls to C_{1i} at the interface. In the solvent phase, the concentration falls from C_{2i} at the interface to C_2 in the bulk. Although the bulk liquids may be well mixed, diffusion of the active compounds is crucial in effecting mass transfer because the local fluid velocities approach zero at the interface. Therefore, diffusion is an important mechanism of mass transfer close to the interface between liquids.

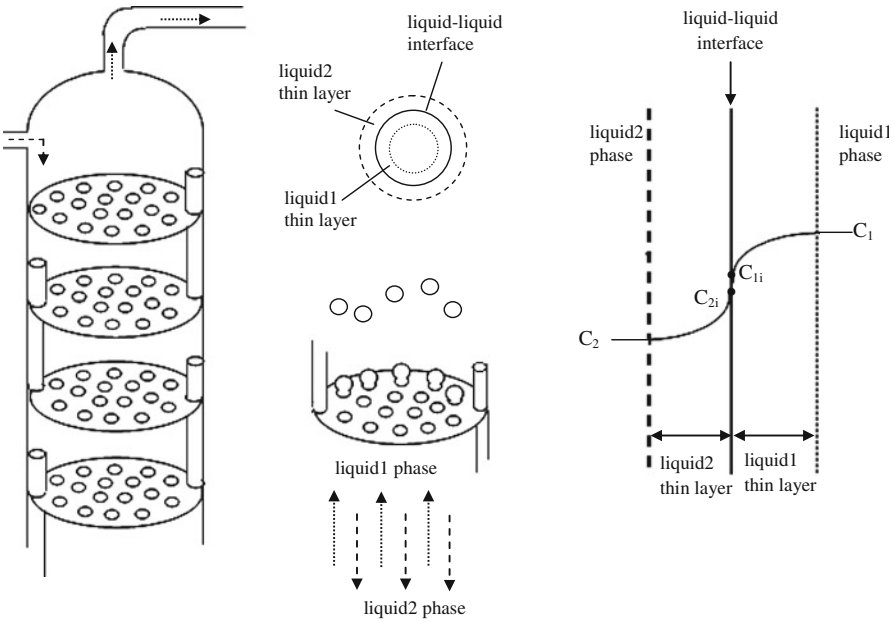

Fig. 1.2 Liquid–liquid mass transfer, schematic representations of an extraction column, and the transport phenomena between two liquid phases

1.3 Downstream Processes

Forming a mixture is a spontaneous, natural process that is accompanied by an increase in entropy or randomness. The inverse process, the separation of that mixture into its compounds is not a spontaneous process which requires an expenditure of energy (Seader and Henly 1998). The mixture to be separated may exist as a single homogeneous liquid or as two or more phases. Based on this, appropriate separation methods have to be applied with the ultimate aim of highest recovery at the lowest possible cost.

1.3.1 Solid–Liquid Separation

Removal of insolubles is generally the first step enabling the capture of the active compounds as a solute in a particulate-free phase. If the final product is a soluble compound, solid particles need to be separated from the liquid phase before it is further treated to purify the soluble compound. If the final product is biomass, then separation of cell aggregates is the major step in product recovery, leading to a significant volume reduction. Typical separation operations with respect to plant cell and tissue cultures are filtration and centrifugation which will be discussed in the scope of solid–liquid separation.

Filtration is used to separate solid particles from a fluid–solid mixture by forcing the fluid through a filter medium while the particles are retained. The solid layer deposited on the filter media is called the "filter cake." As the thickness of the filter cake increases, resistance occurs in further filtration. Therefore, filtration is performed using vacuum or positive pressure gradient, sometimes incrementally increased by time. Rate of filtration depends on the parameters such as the area of the filter cake, the viscosity of the mixture, the pressure difference, and the resistance to filtration.

As for various research studies, an attempt was to accomplish intermittent cell/medium separation of rosmarinic acid produced by *Anchusa officinalis* in the bioreactor by means of automated in situ culture filtration (Su et al. 1995). The cyanobacterium *Spirulina platensis* is an attractive source of chlorophyll, a green pigment used in food, pharmaceutical and cosmetic industries, and other high-value cell components. Filtration is applied as easy and cheap recovery approach from the cultivation medium (Soletto et al. 2005). Another study was conducted to show that auxin was required for the formation of active cyclin-dependent kinase associated complexes, promoting assembly of the complex in tobacco suspension cultures. The researchers found that kinase activity against tobacco retinoblastoma-related protein was maximal in fractions separated using get filtration (Harashima et al. 2007).

In centrifugation, separation occurs due to differences in density. In the case of the centrifugation of plant cell and tissue cultures, particles are generally small in size; the viscosities of the media can be relatively high, while the particle densities might also be similar to those of the suspending fluids. These obstacles have to be overcome in large-scale applications. In a study, a new protocol has been developed for somatic embryogenesis, plant regeneration, and transformation of a banana cultivar where liquid co-cultivation was carried out with centrifugation (Ghosh et al. 2009).

1.3.2 Cell Disruption Techniques

Cell disruption is used for the recovery of intracellular compounds after separating the cells and aggregates from the liquid broth. Cell walls can be ruptured by mechanical or nonmechanical techniques. The mechanical approaches are targeted more towards cell wall disruption while the nonmechanical techniques are mainly used for destabilizing the cell membrane.

The various mechanical disruption techniques involve using bead mills, rotor–stator mill, homogenizers, and ultrasonic vibrations. The bead mills are cylindrical tubes loaded with small beads. The beads collide with the biomaterial as the mill rotates, resulting in broken cell walls. The rotor–stator mill consists of a stationary block with a stator and a rotor. The cell suspension is fed into the gap between the rotating rotor and the fixed stator and expelled through the outlet due to centrifugal action. The generated turbulence and shear lead to disruption of cells. Homogenizer consists of a high pressure pump that injects the cell suspension

through a discharge valve causing an explosion of the cells. The cells can also be subjected to ultrasonic vibrations by introducing an ultrasonic vibration emitting tip into the cell suspension. The ultrasonic vibration could be emitted continuously or in the form of short pulses. The size of the emitting tips is determined based on the volume of sample being processed, whereas the duration of ultrasound is ascertained based on the cell type, the sample size, and the cell concentration.

Nonmechanical disruption techniques involve using detergents, organic solvents enzymes, and osmotic shock. Chemical lysis is carried out by adding detergents and solvents to solubilize cell walls. The detergents and solvents need to be subsequently removed from the product and this generally involves an additional purification step in the process. An enzyme can be used to disrupt plant cells such as cellulase. The main limitation of using an enzyme is its relatively high cost but, on the other hand, the process is less energy demanding and very specific. Another technique is osmotic shock resulting from a difference in solute concentration across a semi permeable membrane. A rapid influx of water into the cell takes place when a cell is suddenly transferred from an isotonic to hypotonic medium such as distilled water. Subsequently, the cell wall is expanded and ruptured.

Among these techniques, homogenization, sonication, steam explosion (Hartonen et al. 2007; Kurosumi et al. 2006), and treatment with supercritical fluids (Castor et al. 1998) were applied to plant cell and tissue cultures. The pretreatment efficiency depends on the type of tissue extracted and the metabolite localization, so ideally, any considered procedure should be tested prior to concentration and separation. The disrupted cells need to be separated from the other cellular compounds by removing the cell debris.

1.3.3 Concentration

Membrane processes are used to concentrate streams from bioreactors by permitting some compounds to pass through while retaining others. Hence, the feed stream leaves the membrane as a permeate containing small compounds and a retentate with large compounds. Membrane technologies have speedily gained importance in processes involving plant cell and tissue cultures. Various membrane processes are available with varying pore sizes and driving forces.

Microfiltration is a pressure driven process that separates micron range particles from liquid streams of plant cell and tissue cultures. Typical operating conditions are 0.3–3.3 bar pressures, 3–6 m/s flow rates with membrane pore sizes of 0.05–3 mm. Hwang et al. (2005) developed a simple and scalable, chromatography based process to purify a type of colony stimulating factor from recombinant rice cell culture. It consisted of cell removal by microfiltration, ammonium sulfate precipitation, and anion exchange chromatography. The purification process resulted in a final purity of about 95 % and an overall recovery yield of 48 % from the culture broth.

On the other hand, there are growing efforts to facilitate in situ medium exchange particularly to eliminate possible introduction of contaminants to the process using microfiltration. A study carried out in *Cyperus aromaticus* cell suspension culture to produce a bioinsecticide known as juvenile hormone III focused on the effect of different volumes and flow rates of replenishment toward cell biomass and the hormone. Medium replenishment of 75 % through the membrane system leads to higher cell biomass and metabolite production which corresponded to values as high as 64 and 112 %, respectively. The reduction in doubling time, extended exponential growth and increment increases in specific growth rates were also demonstrated. A 50 % medium replenishment with membrane filtration system was identified to be an economical and feasible application for the production of juvenile hormone III at large scale. The results obtained showed the potential of in situ membrane filtration system for the enhancement of cell biomass and metabolite yields in plant cell culture (Chan et al. 2010).

Ultrafiltration is another technique used for concentration and fractionation purposes in plant cell and tissue cultures. This is a low pressure process separating dissolved solutes of 5–100 μm in size and is used in numerous industries for concentrating large process streams. In one of the earlier studies, ultrafiltration was used to obtain a high molecular weight fraction of tobacco cell suspension cultures (Takahashi and Warren 1994). In the other study, ultrafiltration was applied to ascertain the responsible fraction for the plant activation of m-phenylenediamine in suspension cultures of tobacco cells (Gichner et al. 1995). Ultrafiltration was also used to deproteinize cytosol in which phosphoribosylpyrophosphate synthetase from *Hevea brasiliensis* Mull. Arg. latex was present (Gallois et al. 1997). Treatment of cell-suspension cultures of *Platanus acerifolia* with a crude elicitor preparation induced the synthesis of the hydroxycoumarin phytoalexins, scopoletin and umbelliferone, and their accumulation in the growth medium. Only the protein-containing fraction of the culture filtrate was involved in cell response. By ultrafiltration of this last fraction, a major eliciting glycoprotein able to induce 80 % coumarin synthesis was isolated (Alami et al. 1998).

1.3.4 Separation and Purification

Subsequent to the concentration of the valuable fraction, various methods such as extraction and chromatography are employed to recover soluble compounds.

1.3.4.1 Solvent Extraction

Solvent extraction is used to separate active compounds from the valuable fraction where the solute is contacted with a selected solvent exhibiting favorable solubility properties.

If the valuable fraction is concentrated liquid as in the case of extracellular compounds, liquid–liquid extraction is applied. In this operation, the solute, which is dissolved in the liquid broth, is transferred into a new solvent, which is either insoluble or partially soluble in the broth. The liquid broth is named as raffinate, and the solvent as extract solvent or simply the solvent. The limited solubilities of solvents constitute the basis of this operation. Thus two liquid layers are formed, each containing different fractions of all the components depending on the respective partial solubilities. The two layers separate from each other due to the density difference. The layer which contains a higher percentage of the extract solvent is called the extract phase and the layer containing a higher percentage of the raffinate solvent is called the raffinate phase. In many cases, there is more than one solvent that can be used for a specific extraction operation. The following properties of the solvents have to be taken into account in order to select the best one among the potential solvents. Selectivity of solvent (β) is defined as a ratio at equilibrium conditions. It indicates the separation power of the selected solvent for the solute dissolved in the raffinate solvent. If $\beta = 1$, this solvent has no selectivity for the solute and hence it cannot be used for the extraction. Higher the value of β, the easier it is to separate the solute from solvent.

$$\beta = \frac{y/y_c}{x/x_c}$$

Distribution coefficient (K) is defined as $K = y/x$ which should be as high as possible to accomplish the given extraction with minimum amount of solvent usage. The selected solvent should have no or low solubility in raffinate solvent to minimize the solvent losses. Separation of the solvent from the solute is achieved by rectification. Hence, the selected solvent should not form an azeotropic solution with the solute and if possible, the relative volatility of this solution should be high. Another parameter to be considered is the density of solvent. Density difference should exist between the phases for the separation of extract and raffinate phases. Hence, the density of the selected solvent should be different from the density of raffinate solvent. Furthermore, interfacial tension has to be taken into consideration. One of the phases is first dispersed in the other phase as small droplets by intensive mixing and then coalescence occurs by allowing the settling. Interfacial tension among the liquids plays an important role in dispersion and coalescence. Low interfacial tension means easy dispersion but difficult coalescence. Since short coalescence time is preferred, the selected solvent should exhibit high interfacial tension in the presence of the raffinate solvent. As the solvent is recovered by rectification and reused many times and during which it is heated and cooled repeatedly, it should be chemically stable to prevent decomposition. Finally, whenever possible the selected solvent should have low viscosity and vapor pressure, should be nontoxic, and should have low unit cost (Alpay and Demircioglu 2006).

If the valuable fraction is a dried or lyophilized biomass, then solid–liquid extraction which is known as leaching is applied. When the two phases are in

contact, the active compounds diffuse from the biomass into the liquid phase resulting in the separation of the compounds. However, the active compounds are generally intracellular, hence the rate of extraction may be slow due to the additional diffusional resistances of the cell walls. Drying the biomass leads to ruptured cell walls, thereby enhancing mass transfer.

Considering the transport phenomena, active compounds diffuse from the biomass into the solvent phase basically in three steps. Solvation of target compounds in solid particles of biomass, diffusion of dissolved compounds to the outer surface of the solid particle (internal diffusion), and finally diffusion from the liquid film around the solid particle into the bulk solvent (external diffusion). Main factors affecting the extraction time are external diffusion coefficients and differences in the chemical potentials in the liquid film.

The choice of solvent depends on the physical properties and oxidation sensitivity of the biomass produced by plant cell and tissue culture. Both nonpolar and polar solvents are used.

However, the disadvantages of solvent extraction can be listed as follows:

- Lower efficiency
- Solvent residues affecting the purity of the compounds
- High costs for high purity requirements
- Longer process durations
- Undesired side reactions affecting the quality of the product

Solvent extraction is widely used in industry due to its low investment costs, but additional separation steps have to be introduced in order to isolate the active compounds from the solvent.

In an earlier study, the recovery of capsaicin from a *Capsicum annuum* cell culture broth was reported. First, the solids were removed by filtration, and then liquid–liquid extraction was used to isolate the capsaicin. Chloroform was used to perform the extraction of the capsaicin in a reciprocating Karr column and the operating conditions determined in the pilot plant column were used to design a commercial-size reciprocating column (Tapia et al. 1993). Another study dealing with a new design was a two-liquid-phase bioreactor designed to extract indole alkaloids from *Catharanthus roseus* hairy roots with silicon oil with the aim of continuous selective extraction of secondary metabolites (Tikhomiroff et al. 2002). Such scale-up attempts and innovative designs can enhance large-scale cultivation of plant cell and tissue cultures providing cost-effective solutions. In more recent works, solvent extraction was applied to different fractions of cultures. For instance, lipids were extracted with chloroform–methanol and the remaining fraction containing vitamin D3 compounds was extracted with polar solvents from *Solanum glaucophyllum* grown in vitro in darkness (Curino et al. 2001). For the production of volatile flavor compounds from *Agastache rugosa* suspension culture, callus was obtained from the leaves of *A rugosa* and volatiles were isolated by liquid–liquid continuous extraction and identified by gas chromatography/mass spectrometry (Kim et al. 2001).

Taxol is another secondary metabolite widely used as a chemotherapeutic agent and applications of large-scale cultivation exist (Son et al. 2000). Suspension culture of *Taxus chinensis* cells was carried out in aqueous-organic two-phase systems for the production and in situ solvent extraction of taxol (paclitaxel). Three organic solvents, hexadecane, decanol, and dibutylphthalate, were tested at 5–20 % (v/v) in the culture liquid. All of these solvents stimulated taxol release and the yield per cell, though decanol and higher concentrations of the other two solvents depressed biomass growth significantly. Dibutyl phthalate at 10 % was the optimal solvent for improving taxol production and release with minimal cell growth inhibition. The time of solvent addition to the culture also affected taxol production, with the addition during the late-log growth phase being most favorable (Wang et al. 2001). In another study dealing with taxol, different organic solvents, including saturated aliphatic hydrocarbons, aliphatic alkanols, esters of dicarboxylic acids, monoterpenoids and unsaturated lipids, were investigated to test the effect of in situ paclitaxel removal from *Taxus chinensis* suspension cultures. The results indicated that dibutyl phthalate, as well as, oleic acid and terpineol were ideal solutions for in situ extraction of paclitaxel (Zhang and Xu 2001). Furthermore, a large-scale purification method which consisted of solvent extraction, synthetic adsorbent treatment, and two steps of precipitation for the isolation and purification was developed for producing paclitaxel from the biomass of *Taxus chinensis*. The biomass from plant cell cultures was mixed with methanol and stirred at room temperature for 30 min. Extraction was repeated at least four times. Each methanol extract was collected and concentrated at a temperature of 40 °C under reduced pressure to decrease the volume of the methanol extract down to 30 % of the original. As a second extraction step, the concentrated methanol extract was added to organic solvents (dichloromethane, chloroform, diethylether, and hexane) with varying polarities at a volume ratio of 25 % for liquid–liquid extraction and extraction was carried out at room temperature for 30 min. The extraction was repeated at least three times and the crude extracts thus obtained were pooled and dried at room temperature under reduced pressure (Pyo et al. 2004).

In our study, stem explants of young shoot of *Rosmarinus officinalis* were cultured in both woody plant medium and Murashige and Skoog media for callus initiation. The lyophilized calli were subjected to solvent extraction via sonicating at 35 kHz for 45 min with (50 °C) and without heat treatment at room temperature to obtain the methanolic extracts. Active constituents of eight calli extracts were analyzed by HPLC, and rosmarinic acid was determined to be the primary compound. Rosmarinic acid contents of the samples exposed to thermal treatment (50 °C) were higher than the untreated samples (Yesil-Celiktas et al. 2007) implying the importance of temperature-induced solubility. In another study, successful scale up of *Azadirachta indica* suspension culture for azadirachtin production was done in a stirred tank bioreactor with two different impellers. However, extraction of azadirachtin was not conducted on large scale, but rather at laboratory scale (Prakash and Srivastava 2007).

A variant plant of opium poppy (*Papaver somniferum* L.) containing high thebaine, which is a paramorphine, was obtained during a mutation breeding

experiment. The seeds of the variant plant were subjected to in vitro studies to investigate the prospects of thebaine production through tissue culture. Alkaloid profile showed higher thebaine in stems followed by leaf callus, stem callus, and cotyledons. Such a high content of thebaine from tissue raised plant material was argued to be a new vista for the extraction of thebaine at commercial level. However, the samples were extracted in 5 ml DMSO for more than 3 months which is not an appropriate and cost-effective protocol for possible scale-up studies (Chaterjee et al. 2010). Alkaloids were of interest in another study where a treatment involving an acid-based extraction from the cell culture of *Passiflora alata* was carried out (Machado et al. 2010). A cell suspension culture was developed from calli of grape rootstock for the bioproduction of resveratrol using methyl jasmonate as an elicitor. Liquid–liquid extraction of the culture medium and a solid–liquid extraction of the cells showed that other metabolites such as trans-epsilon-viniferin, trans-delta-viniferin, and a trans-3-methylviniferin as well as trans-piceatannol were also produced in a 2 L bioreactor cell culture of grapevine. Furthermore, a one step FPLC method was developed for the purification of resveratrol and epsilon-viniferin (Donnez et al. 2011). A recent study is a good example where extraction was combined with sonication in order to enhance the recovery of secondary metabolites. Cell suspension cultures of *Cephalotaxus fortunei* were manipulated to produce secondary metabolites of pharmaceutical interest and seven abietane diterpenoids were produced by the suspension-cultured *C. fortunei* cells by complementing an elicitation strategy with an in situ product removal strategy. After subculturing was completed, methyl jasmonate solution in ethanol was added to the culture medium together with autoclaved XAD-7HP resin. After 2 weeks of culture, the cells and the resin were harvested separately based on density. The suction-filtered resin was extracted with methanol three times at room temperature. Each extraction was performed by shaking on a rotary shaker at 100 rpm for 2 h followed by sonication for 20 min. The combined methanol extracts were filtered, and the supernatant was evaporated to dryness (Xu et al. 2011).

As mentioned earlier, developing methods for quali-quantitative analysis of metabolites produced by plant cell and tissue cultures are also very important for commercial applications. The hydrophilic metabolome of heterotrophic *Arabidopsis thaliana* cells grown in suspension was targeted and water-soluble metabolites were extracted using four protocols; perchloric acid, boiling ethanol, methanol, and methanol/chloroform. They were detected and quantified using 1H nuclear magnetic resonance (NMR) spectroscopy. The quantitative studies demonstrated that the four extraction protocols commonly used do lead to quite similar molecular compositions as analyzed by 1H NMR. The methanol/chloroform method proved effective and reliable to prepare series of physiologically significant extracts from plant cells for 1H NMR analysis. Reproducibility of the detected metabolome was assessed over long periods of time by analyzing a large number of separate extracts prepared from independent cultures. Quantitatively resolved 1H NMR of cell extracts proved to be robust and reliable for routine metabolite profiling of plant cell cultures (Gromova and Roby 2010).

1.3.4.2 Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) is an alternative technology for separation and purification of secondary metabolites from plant cell and tissue cultures. This technology has the potential to be an environmentally friendly "green" process. A fluid is said to be supercritical when its temperature and pressure values are slightly above the liquid–vapor critical point. Under these conditions, the fluid displays unique properties, such as high density, high diffusivity, and low viscosity. Furthermore, the extreme compressibility of supercritical fluids makes them very sensitive to minor temperature and pressure gradients causing supercritical fluids to be useful in many industrial applications. Supercritical fluid processes are applied to increase product performance to levels that cannot be achieved by traditional processes.

The critical temperatures, pressures, and densities of various fluids are provided in Table 1.1. Carbon dioxide is generally the most desirable solvent for supercritical fluid extraction due to its low critical temperature (31 °C) and pressure (73.8 bar). Additionally, being inert, nonflammable, and nontoxic and having a low surface tension and high diffusivity characters, CO_2 is attractive as a supercritical solvent for pharmaceutical and food industries. Since its diffusivity is one to two orders of magnitude greater than conventional fluids, a greater extraction rate is obtained compared to conventional solvent extraction.

CO_2 molecule is composed of two oxygen atoms covalently bonded to a single carbon atom displaying no permanent dipoles which makes CO_2 ideal for the separation of nonpolar compounds (Kaiser et al. 2001). On the other hand, the solubility of polar and high molecular weight compounds is very limited in CO_2. Hence, polar solvents with high dielectric constants are added at low concentrations in order to increase the solvating power of CO_2. These are named as modifiers, co-solvents, or entrainers (Taylor 1996; McHugh and Krukonis 1994; Dobbs et al. 1987). Therefore, adding of solvents such as methanol, ethanol, acetone, and water is required for the efficient extraction of compounds with polar properties existing in plant cell and tissue cultures.

A typical supercritical CO_2 extraction setup is comprised of a CO_2 tube, high pressure CO_2 pump, CO_2 flow-meter, temperature controllers, high pressure co-solvent pump, automated Back Pressure Regulator, cooler, and heater as illustrated in Fig. 1.3. The extractor volumes range between 10 and 1,000 ml for laboratory scale, 1.5–10 L for pilot scale and above 10 L as industrial scale. In Fig. 1.4, a 100 ml laboratory scale Thar and a 6.5 L pilot scale Natex supercritical fluid processing units can be seen. Liquefied CO_2 is introduced into the packed column through a piston pump with a cooling jacket. Both the pressure and temperature of the cartridge are automatically reached and maintained by a control unit according to the settings. After the desired pressure and temperature are reached, the dynamic extraction starts. The variables are temperature, pressure, CO_2 flow, and extraction duration and co-solvent ratio if polar compounds are of interest. Finally, extracts are collected from the separator outlet after releasing CO_2 from the system at laboratory scale applications, whereas CO_2 is recycled in

Table 1.1 The critical temperature, pressure, and density values of some solvents (Rivzi et al. 1986)

Solvent	Critical temperature (°C)	Critical pressure (atm)	Critical density (g/ml)
Methane	−83.2	45.40	0.162
Ethylene	9.3	49.64	0.218
Chlorofluoromethane	28.9	38.68	0.579
Carbon dioxide	31.06	72.83	0.468
Ethane	32.3	48.16	0.203
Propylene	91.9	45.59	0.233
Propane	96.7	41.84	0.217
Ammonia	132.5	111.52	0.235
Diethyl ether	194.6	35.92	0.265
n-pentane	196.5	33.26	0.237
Acetone	235.0	46.40	0.278
Methanol	239.5	79.84	0.272
Benzene	289.0	48.26	0.302
Toluene	318.6	40.56	0.292
Pyridine	346.9	55.56	0.312
Water	374.2	217.11	0.322
Xenon	16.6	57.63	1.113

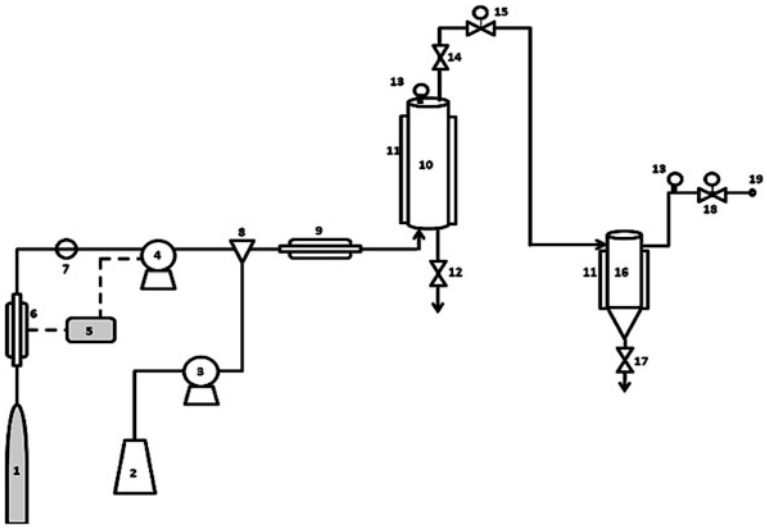

Fig. 1.3 The diagram of supercritical fluid process used (1—CO_2 supply in, 2—Co-solvent reservoir, 3—Co-solvent pump, 4—CO_2 pump, 5—Cooling bath, 6—Cooling heat exchanger, 7—Flow meter, 8—Mixer, 9—Heat exchanger, 10—Extraction vessel, 11—Heat jacket, 12—Bleed valve, 13—Gauge, 14—On–off valve, 15—Automated Back Pressure Regulator (BPR), 16—Fraction collector, 17—Drain valve, 18—Manual BPR, 19—Vent)

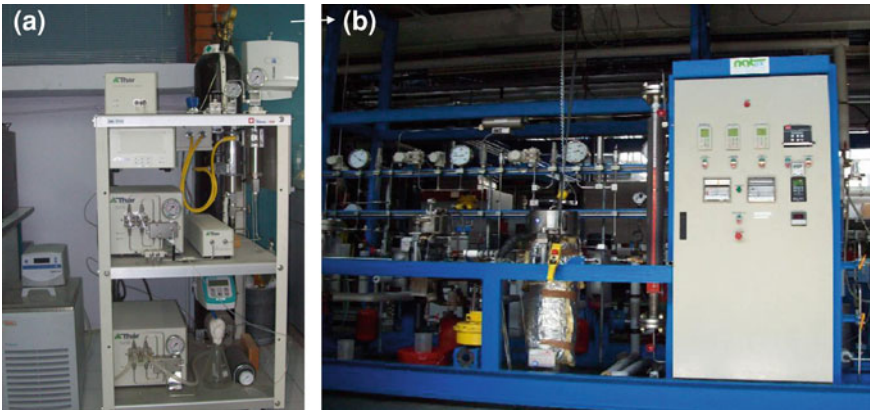

Fig. 1.4 A 100 ml laboratory scale Thar (**a**) and a 6.5 L pilot scale Natex (**b**) supercritical fluid processing units

most of the pilot scale and all industrial-scale applications. Therefore, the extracts obtained with SFE can be considered to be more sustainable and healthier, conforming with industrial requirements of healthy food and pharmaceutical products because they generally are solvent-free, even if co-solvents are applied in small amounts to enhance the extraction of high polarity active compounds.

While processing plant cell and tissue cultures, supercritical and subcritical fluids can also be used as a cell disruption technique releasing structural biomass constituents. These can further be subjected to a multiplicity of sub- and supercritical extraction steps with different solvation conditions used for each fraction (Darani and Farahani 2005), thereby conducting a two-step process in one single operation. Supercritical fluid extraction is a relatively new method and laboratory studies are still being carried out to investigate other potential applications. Only one study was identified for the supercritical fluid extraction of the biomass, where the supercritical CO_2 extraction of capsidiol from pepper fruit tissues activated with *Alternaria alternate* Keissler suspension culture as a biotic elicitor was investigated. The effects of separation parameters such as temperature, pressure, supercritical solvent flow rate, particle diameter and also initial capsidiol concentration on solubility, initial extraction rate, and extraction yield were studied. The optimal extraction conditions were obtained at a temperature of 40 °C, the pressure of 400 bar, the supercritical CO_2 flow rate of 2 cm^3/min, and the average particle diameter of 116 μm. The results showed that the ratio of the supercritical CO_2 extraction yield to the organic solvent extraction yield changed from 84 to 97 % depending on the initial capsidiol concentration (Salgin et al. 2005).

In terms of comparing the costs associated with SFE and traditional extraction plants, the capital cost of the SFE is generally higher than the traditional, while the operating costs are lower. However, it is necessary to take into account the capital costs of all the equipment required for a traditional process, such as evaporation systems for solvent recovery, costs associated with building requirements,

instrumentation, and electrical connections with appropriate safety requirements. The operating costs of the traditional processes, particularly the energy costs of water evaporation are considerably high. On the other hand, SFE is an attractive alternative for obtaining value-added compounds from biomass without any solvent residues, resulting in higher sales prices implying shorter return on investment. Consequently, SFE presents a potential for commercial applications with acceptable quality.

1.3.4.3 Adsorptive Separation

Adsorption involves adsorbing the active compounds on the surface of solid particles. In adsorption operations, the substance being concentrated on the surface is called the adsorbate, whereas the material to which the adsorbate binds is the adsorbent. The ideal adsorbent material has a high surface area per unit volume which can be achieved if the solid contains a network of fine internal pores providing a large internal surface area. Silica gel, oxidic resins, activated carbons, and polymers with different surface functionalizations are commonly used for adsorption of biological molecules. As the polarity of the resins strongly influences the adsorption behavior, the preselection of applicable sorbents focuses on covering a wide range of polarities. Additionally, the resins are characterized by different specific surface areas, pore diameters, and porosities (Winkelnkemper et al. 2011). A typical adsorption operation consists of the following stages: adsorption step which loads solute onto the adsorbent, a washing step to remove residual unadsorbed material, desorption or elution of adsorbate with a suitable solvent, washing to remove residual eluant, and regeneration of the adsorption resin to its original condition.

Cell and tissue culture research focused on adsorption process in 1990s. The main drive was to test suitability and efficacy of various adsorbents for the recovery of value-added compounds from cultured plant cells and tissues. However, the findings from the studies have also highlighted the potential of adsorbents to overcome growth inhibition. The use of a polycarboxyl ester resin, for the in situ separation of berberine, isoquinoline alkaloid, produced from plant cell culture of *Thalictrum rugosum* was studied (Choi 1992). The suitability of adsorbent polymeric resins was investigated for the accumulation of sanguinarine from *Papaver somniferum*cell cultures. In addition to sanguinarine, the resins were found to adsorb growth regulators and vitamins from the culture medium. Growth inhibition was overcome by delaying resin addition for approximately 4 days after cell inoculation in fresh medium. Resin addition to actively growing unelicited cultures led to increases in sanguinarine production and release of 30 to 40 and 60 %, respectively. The addition of resins to elicited cultures led to increases in alkaloid production of up to 50–85 % with similar increases in alkaloid release as observed for unelicited cells. Overall yields of sanguinarine increased from 21 mg/g biomass dry weight for elicited cultures to more than 39 mg/g when elicitation was combined with resin addition (Williams et al. 1992). Another study focused on the

integrated recovery of solavetivone from fungus elicited hairy root cultures of *Hyoscyamus muticus* using volatile organic solvents and solid-phase adsorbents in an external loop extraction configuration. Hexane and pentane were shown to be toxic when added directly to the culture; however, growth of roots was not inhibited when cultures were exposed to media saturated with these hydrocarbons. Solid-phase neutral adsorbents displayed higher capacity and better solavetivone partitioning capability than the hydrocarbons; however, their selectivity for the sesquiterpene solavetivone was found to be poor in comparison to hexane (Corry et al. 1993). Various hairy root clones were derived by transforming two kinds of *Catharanthus roseus* plants with *Agrobacterium rhizogenes*. Hairy root growth and indole alkaloid production were then investigated to select a high yielding hairy root clone. One of the Amberlite resins tested greatly enhanced the release of catharanthine and ajmalicine from hairy root cultures, with an increase in total production. The researchers introduced a permeabilizing agent (dimethyl sulfoxide) and a fungal elicitor to provide physical and biochemical stress, respectively, together with in situ adsorption with the ultimate aim of enhancing alkaloid production and secretion. The results indicated that in situ adsorption sequentially applied with permeabilization and fungal elicitation had a synergistic effect on the production and secretion of indole alkaloids (Sim et al. 1994). Another compound of interest was digoxin, a cardiac glycoside. Selection of proper resins was attempted in order to use in situ adsorption by biotransformation from digitoxin in plant cell suspension cultures. Among various kinds of resins tested, one of the Amberlite adsorbent was found to be the best for digoxin production in considering adsorption characteristics as well as the effect on cell growth. However, the findings of this study were not in parallel to the rest of the other mentioned studies in this section because of the fact that the presence of resins was suggested to be as short as possible to increase the productivity. Another comment was to prevent the cells from direct contact with resin particles with a suggestion of designing immobilized systems (Hong et al. 1998).

Recent studies are related to continuous removal of value-added compounds, introducing innovative process designs, and testing novel biomaterials as adsorbents. Continuous removal of anthraquinones by Amberlite polymeric adsorbents through in situ adsorption in *Morinda elliptica* cell suspension cultures was studied for product recovery and improvement of the overall titer. Ethanol was the best eluting solvent for effective recovery of anthraquinones from all adsorbents. Pretreatment of the adsorbent with sodium acetate not only enhanced intracellular anthraquinones, but also their release and subsequent recovery from the adsorbent. High amount of adsorbent and longer contact period for the cultures entering stationary growth phase was reported to stimulate anthraquinones release and recovery, but at the expense of cell growth (Chiang and Abdullah 2007). A recent study was conducted to develop an optimal tandem simulated moving bed process containing two four-zone simulated moving bed units in series for separation of paclitaxel, 13-dehydroxybaccatin III and 10-deacetylpaclitaxel. The bed was loaded with a Curosil-PFP resin with an average particle size of 5 μm which was used as the adsorbent. Based on the estimated adsorption isotherm and