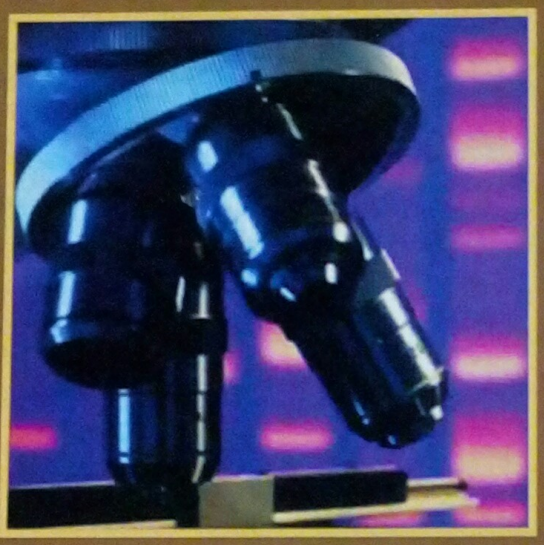
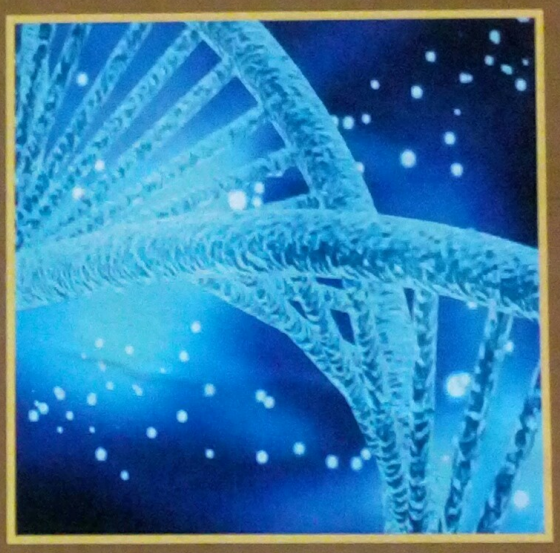
BIOTECHNOLOGY

Trends and Applications





Rachana Singh
Mala Trivedi

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Foreword

Biotechnology, the field is about the quality of life: repairing, replacing, enhancing and evolving. Biotechnology is the exploitation or harnessing of biological processes, organisms, cells or cellular components to develop new technologies and products that help to improve our lives and also of our planet.

Biotechnology provides breakthrough products and technologies to combat debilitating and rare diseases, reduce our environmental footprint, feed the hungry, use less and cleaner energy and have safer, cleaner and more efficient industrial manufacturing processes.

There is a wave of biotechnology, major applications are in agricultural, medical and industrial fields. In the last few years it has initiated a transformation of many parts of the chemical industry, agriculture, food, forestry, waste treatment, pharma and medicine, a transformation that has emerged from the laboratory into practical application with quite remarkable speed.

This book entitled "***Biotechnology: Trends and Applications***" consists of 15 chapters on important fields of biotechnology like environment, plant, animal biotechnology, stem cell research, genomics and proteomics microbes in biotechnology and some other. The title of the book has been chosen carefully. Applications and recent advances in biotechnology is the key part to know and promote the biotechnology. This book provides comprehensive information on the all the emerging and expanding area of biotechnology with their latest applications. The book is written to address everyone who is curious about the latest developments as well as fundamental basics. Experts actively working on the selected field of biotechnology from different part of world have contributed various chapter of the book.

The approach of the book is quite novel and the presentation is very articulate. It covers all the aspects of Biotechnology, supported with

authentic data and information. It is an excellent piece of treatise, useful to all students, researchers and teachers of this discipline.

The Editors of the book Rachana Singh and Mala Trivedi, must be complimented for their outstanding efforts in the bringing out this excellent reference book which will interest all those interested in different areas of biotechnology and their recent advances.

Professor (Dr.) Qumar Rahaman
Dean (Research & Science)
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About the Editor(s)

Rachana Singh is working as Assistant Professor at Amity Institute of Biotechnology, Amity University Uttar Pradesh, Lucknow. She did her Ph. D in Plant Virology from National Botanical Research Institute, Lucknow and University of Lucknow in 2006 and M.Sc in Microbiology from Dr. RML Avadh University, Faizabad. She has published 20 research papers 5 book chapters and many articles to her credit. She is member of Indian Science Congress, Indian Virological Society, Uttar Pradesh Academy of Science. She has two Indian patents to her credit. Working as fellow at NBRI, she has successfully transformed transgenic tomato resistant to tomato leaf curl virus. Currently she is working on the identification characterization of Soyabean viruses in India.

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First and foremost, we thank God for his blessings. Because of him we were able to complete this book. In the urge of contributing to the society AMITY family has given the heritage of knowledge to world unconditionally.

We would like to take this opportunity to extend our sincere gratitude and deepest thanks to ***Dr. Ashok Chauhan***, Founder President of AMITY University. We are grateful to him for providing such an excellent platform to excel academically as well as professionally.

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November, 2015

Editors
Rachana Singh
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Preface

Biotechnology is a group of technologies that involves manipulation of living organisms and their components to produce useful products that are very beneficial and can improve our lives.

It's a multidisciplinary branch which involves different biological and allied subjects. Being a vibrant branch, it has progressed very fast and still progressing with rigorous research and best knowledge. With the development in knowledge and advancement in technology, the field of biotechnology is further expanding in various areas related to industry, medical, plant, pharmaceutical, environment, microorganisms, agriculture, nanotechnology. The topic ***Biotechnology- Trends & Applications*** is taken up because of the rapid developments in this field in recent past that have revolutionized our concepts of applications of various branches of biotechnology in our day to day life. New approaches are demand of hour. To meet this demand, we brought together contributors of various specialized field of biotechnology who address the most recent trends and its application in the respective fields.

We started the book by applications of biotechnology in almost all the related fields including microbial technology, plant biotechnology and animal biotechnology, Immunology, bioprocess technology, virology, stem cell, plant biotechnology, plant transgenic production or cancer biology. The book also includes role of intellectual property right, its importance and how IPR is applicable in biotechnology.

The individual chapters of the book are organized according to the following format: chapter title and contributors, abstract, introduction to the chapter, chapter topics and text, and references cited for further reading. This format is designed in order to help the reader to grasp and understand the complexity of biotechnology in a better way.

The topics covered in this book will be of interest to all the persons that are part of different streams that are constituent of biotechnology stream viz. plant biologists, immunologist, plant virologists, molecular biologists,

pharmacologists, and pharmacologist, people in the field of bioinformatics, medical practitioners and in industrial fermentation, and research investigators in industry, IPR issues, working in research labs, and university.

Editors

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Plant Cell Cultures a Promising Biometabolite Reservoir

RADHIKA RAJENDRAN AND RAKHI CHATURVEDI*

ABSTRACT

Over billions of years, nature has developed a wide variety of plant kingdom producing diverse bioactive secondary metabolites useful to the mankind. These metabolites are used in pharmaceuticals, food, colors, flavors, fragrances and in agriculture. Although the demand for these plant products has increased manifold in the past few years, the supply is very limited. The production of these metabolites by in vitro *cell culture methods is an attractive alternative to traditional methods of metabolite production to meet the demands. The cell culture technique relies on the fact that many cells have the capacity to regenerate into whole plants, a phenomenon known as totipotency. The most important aspect of* in vitro *cell culture system is that it offers a defined production system, continuous supply of products with uniform quality and yield, irrespective of geographical, seasonal and environmental fluctuations. The current chapter focuses on the overview of plant cell culture technology, an alternative biotechnological approach and describe on the separation techniques for the target desired moiety and prominent future perspectives for commercial utilization of cell cultures.*

***Key words*:** Biotechnological approaches, Plant cell cultures, Analytical techniques, Secondary metabolites.

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1. INTRODUCTION

The evolution of plants produce diverse biochemicals that correspond to significant compounds such as, pharmaceutics, cosmetics, fine chemicals, or more recently nutraceutics. The use of these compounds, called secondary metabolites, in therapeutics is known since times immemorial. Based on the biosynthetic pathways, there are three groups of secondary metabolites present in higher plants, phenolics, terpenes and steroids, and alkaloids. Despite the availability of synthetic and semi-synthetic drugs, plant based natural products are much in demand since centuries and are the main source of lead compounds due to their unique and valuable properties[1]. However, the continuous extraction of metabolites from wild species causes depletion in the number of plant species. Moreover, varying cultivation period and environmental fluctuations result in uneven metabolite production from natural plant stock. In this context, plant, cell, tissue and organ culture technique has been proved very valuable since many plant cells are totipotent and have the capacity to regenerate into whole plants. Hence, the technique will be useful for large scale propagation of plants which could serve as reservoir for high value metabolites. Further, the *in vitro* cell cultures are an attractive alternative source to whole plants to generate the cell biomass for the production of high-value secondary metabolites and to reverse the effect of seasonal variations on metabolite production. This chapter provides in-depth information on applications of plant tissue culture towards the discovery of secondary metabolites and a few advanced strategies to identify and enhance the production of plant derived metabolites of high significance.

1.1. History of Plant Tissue Culture

The German botanist gottlieb haberlandt (1854–1945) is considered to be the father of plant tissue culture and was the first person to culture *in vitro* cells of plants. In his experiments, he cultured single isolated, fully differentiated cells from palisade tissues of leaves of *Lamium purpureum* and *Eichhornia crassipes*, the epidermis of *Ornithogalum* and epidermal hairs of *Pulmonaria mollissima* on Knop's salt solution for the development of entire plantlet. The cultured cells show an increase in size, change in shape, thickening of cell walls and appearance of starch in chloroplasts which initially lacked it. However, none of the cells divide. Later in 1904, Hannig successfully grew embryos of some crucifers to maturity on mineral salts and sugar solution. The failure on initiation of plant cell cultures laid the platform on further basic findings for the plant growth and development with elevating improvement and advantages towards large scale plant propagation, germplasm storage, crop improvement, development of disease and virus free *in vitro* culture system, production of high-value bioactive compounds. Some of the milestones in the development of plant tissue culture techniques are summarized in Table 1.

Table 1: Various landmark discoveries of plant tissue culture

Name	***Year***	***Discoveries***
G. Haberlandt	1902	Concept of *in vitro* cell cultures
E. Hannig	1904	Development of embryo cultures from cruci-ferous species
W. Kotte and W.J. Robbins	1922	*In vitro* culture of root- and stem-tips
F.W. Went	1926	Developed first plant growth hormone, IAA (Indolebutyric acetic acid)
P.R. White	1934	Vitamin B was supplemented in culture media as a growth supplement for tomato root-tips
R.J. Gauthret	1934	Developed the continuously growing cultured tissues from cambium cells
R.J. Gauthret, P.R. White and P. Noubecourt	1939	Endless proliferation of callus cultures
V.J. Overbeek, M.E. Conklin and A.F. Blakeslee	1941	Added coconut milk to the medium for cell division in *Datura* species
E. Ball	1946	Developed whole plants of *Lupinus* and *Tropaeolum* species by shoot-tip cultures
G. Morel and C. Martin	1952	Obtained virus free *Dahlia* plant *in vitro* for the first time from shoot-tips
W.H. Muir, A.C. Hildebrandt and A.J. Riker	1954	Isolated single cells from cell suspension cultures of tobacco and marigold
C.O. Miller, F. Skoog, F.S. Okumura, M.H. Von Saltza and F.M. Strong	1955	Kinetin as cell division hormone
F. Skoog and C.O. Miller	1957	Concept of hormonal control for organ culture
J. Reinert and F.C. Steward	1958 1959	Callus clumps and cell suspension cultures of *Daucus carota* regenerated embryos
E.C. Cocking	1960	Demonstrated the feasibility of enzymatic degradation of Plant Cell walls to obtain *via*ble protoplasts
L. Bergmann	1960	Plating of cell suspension cultures from *Nicotiana tabacum* and *Phaseolus vulgaris* to clone large number of isolated single cells
T. Murashige and F. Skoog	1962	Developed MS medium with higher concentrations of salts
K. Kanta, N.S. Rangaswamy and P. Maheshwari	1962	Developed "test tube fertilization" technique

(Contd...)

Table 1: *(Contd...)*

Name	*Year*	*Discoveries*
S. Guha and S.C. Maheshwari	1966	Demonstrated the generation of large numbers of androgenic cultures from pollen grains of *Datura innoxia*
J.B. Power, S.E. Cummins and E.C. Cocking	1970	Controlled fusion of isolated protoplast by chemical ($NaNO_3$) treatment
I. Takebe, G. Labib and G. Melchers	1971	Demonstrated totipotency in isolated protoplast
P.S. Carlson, H.H. Smith and R.D. Dearing	1972	Produced somatic hybrids between *Nicotiana glauca* and *Nicotiana langsdorfii*
M.D. Chilton, M.H. Drummond, D.J. Merlo, D. Sciaky, A.L. Montoya, M.P. Gordon and E.W. Nester	1977	Recognized gene transfer system in plant by bacteria
G. Melchers, M.D. Sacristan and A.A. Holder	1978	Produced the hybrid "pomato" by protoplasmic fusion of potato and tomato
P.J. Larkin and W.R. Scawcroft	1981	Proposed the term "somaclonal variation" and regenerated plants as "somaclones"
R. Horsch, R. Fraley, S. Rogers, P. Sanders, A. Lloyd and W. Hoffmann	1984	Developed transgenic plants of tobacco by *Agrobacterium* mediated transformation
J.C. Sanford, T.M. Klein, E.D. Wolf and N. Allen	1987	Designed the first biolistic gun for gene transfer

Adapted from[2].

1.2. An Overview of Primary and Secondary Metabolites

The primary metabolites have crucial role in controlling the basic life functions such as, cell division, growth, respiration, storage and reproduction. In plants, sugar produced by the process of photosynthesis plays a pivotal role as primary messengers. This carbon source modulates environment cue, co-ordinates the internal regulators to govern the growth and development[3,4,5]. Apart from the principle physiological functions, it also serves as a stress protector[6,7].

Sucrose and raffinose, serve as an osmoprotector whereas, galactinol acts against cellular damage occurred by oxidative stress[8,9]. The export of amino acids and utilization by the higher plants are highly successful by an interlinked complicated metabolism of nitrate and carbon sources. Further, the transcriptional changes accompanied by allosteric and post-translational regulatory enzymes performed by primary metabolism facilitates the amino

acids, nucleotide synthesis and induction of genes necessary for the protein, lipid and cell wall synthesis[10,11,12,13,14].

Contrastingly, central metabolism of primary metabolites acts as a precursor for the synthesis of secondary metabolites. The concept of secondary metabolite is defined as a chemically diversified metabolite opposed to primary ones exhibiting various biological functions rather involving in the basic functions of life[15,16]. In general, these secondary metabolites are often low in abundance, less than 1% of the total carbon level in the entire plant life system. However, it plays a chief role in adaptation of plant system to the environment by interacting with the ecosystem. The phytochemical metabolite attributes anti-microbial, anti-viral, anti-feedant properties and also acts as phytoalexins to fight against pathogens.

Furthermore, few compounds possess UV absorbing capacity to prevent leaf damages[17,18]. However, these economically viable competitive compounds change their chemical profile pattern due to the geographical and environmental constraints. These situations can be avoided by the development of incredible plant cell culture technology for the constant and continuous production of secondary metabolites[19]. The technology facilitates tremendous progress as witnessed in the improvement on paclitaxel production (> 100 fold), an anti-cancerous agent from *Taxus* species. The significance of cell culture technology towards product yield from various plant cultures was exemplified in Table 2.

Table 2: Plant cell derived secondary metabolites and its applications

Plant species	***Product***	***Applications***	***Ref. (s)***
Camptotheca acuminate	Camptothecin	Anti-tumor	[22]
Catharanthus roseus	Vincristine and Vinblastine	Anti-cancer	[23]
Coptis japonica	Berberine	Intestinal ailment	[24]
Lithospermum erythrorhizon	Shikonin	Treatment of burns and hemorrhoids	[25]
Macuna pruriens	L-DOPA	Treatment for Parkinson's disease	[26]
Morinda citrifolia	Anthraquinones	Anti-cancer and anti HIV	[27]
Panax ginseng	Ginsenoside	Health tonic	[28]
Rauwolfia serpentine	Ajmaline	Anti-arrhythmic	[29]
Vinca minor	Vincamine	Cerebral vasodilator	[30]

2. BIOTECHNOLOGICAL APPROACHES FOR THE DEVELOPMENT AND ESTABLISHMENT OF *IN VITRO* CULTURES

2.1. Micropropagation

In the conventional methods, the totipotency of somatic cells has always been exploited for vegetative propagation of plant species. Since in nature,

each and every part of the plant like, stem, leaf and root pieces, is able to differentiate to establish the new individuals. Harberlandt in 1902 confirmed the totipotency of plant cells and first plant tissue culture was established. Since then, the technique has developed a concept that a cell has the capacity and ability to develop into whole organisms. This foremost technique aids in large scale cloning of individuals, to select homogeneous, genetically stable, elite lines of plant species with improved metabolite production. The basic concept of micropropagation is the plasticity and totipotency. The plasticity favours plants to alter their metabolism, growth and development that best suits their environment and allows one type of tissue or organ to be developed into another type. As a result, whole plants can be regenerated with the same genetic potential as that of the parent plant and, thus, guarantees that the characteristics of the source plant are conserved[20,21]. The clonal propagation by nodal segment cultures (Fig. 1A–E.) and adventitious shoot proliferation by leaf-disc cultures (Fig. 1F–I.) are the two most popular methods of large scale micropropagation. Both these methods are useful to raise genetically stable parental stock under invitro culture which can serve as a resource for the constant production of bioactive phytochemicals throughout the year without the destruction of naturally available germplasm grows wildly in the field.

2.2. Callus Culture

"Callus is a mass of actively dividing undifferentiated cells obtained from explants grown *in vitro* on nutrient medium" (Fig. 2A-E). These callus cultures are biosynthetically totipotent which means that they retain the complete genetic information to produce the range of chemicals found in the parent plant. It is an attractive alternative to whole plant extraction for the production of high-value secondary metabolites[31,32,33,34,35]. The accumulation of secondary products in plant callus cultures depends on the composition of the culture medium, including the kind and concentration of plant growth regulators, mineral salts and carbon sources. The environmental conditions, such as temperature, light, and gas composition also affect the secondary metabolite production. However, the disadvantage of these callus cultures is that they induce somaclonal variations, usually during several subculture cycles. This may cause variation in the production of secondary metabolites from one subculture cycle to another. To overcome this problem, it is necessary to assess the different callus lines, when genetic stability is reached, for its growth rate as well as intracellular and extracellular metabolite concentrations. This allows an evaluation of the productivity of each cell line so that only the best ones will be taken for further studies.

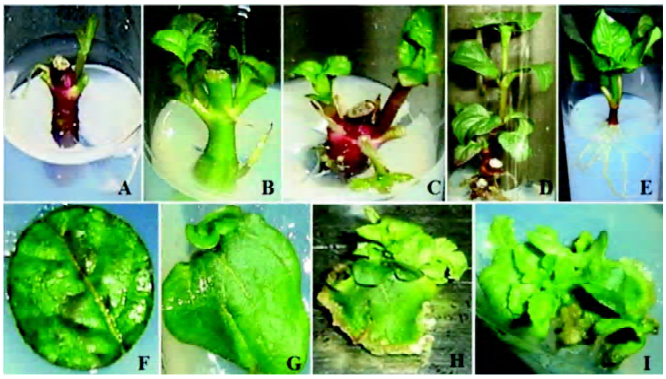

Fig. 1: Clonal propagation of *in vitro* cultures. (A) After one week, nodal segment culture (bar = 0.16 cm); (B) After two weeks showing formation of shoots from the axillary buds (bar = 0.16 cm); (C) After three week, showing slight elongation of shoots from nodal segment (bar = 0.16 cm); (D) After four weeks proliferation of single shoot from nodal segment explant (bar = 0.13 cm); (E) An entire plantlet (bar = 1.40 cm); (F) After one day, showing leaf-disc culture (bar = 0.15 cm); (G) After one week, showing an explant showing shoot bud initiation (bar = 0.05 cm); (F) After two weeks showing proliferation of shoot from leaf-disc culture (bar = 0.05 cm); (I) After four weeks showing development of multiple adventitious shoots from single explant (bar = 0.05 cm).

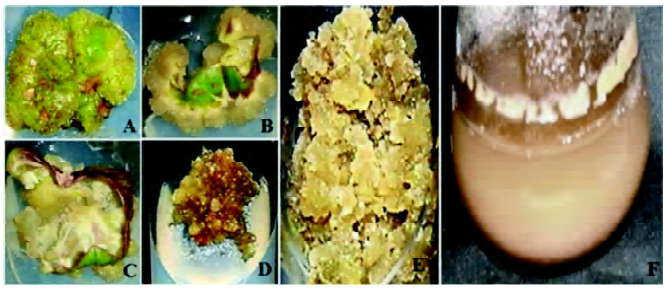

Fig. 2: Establishment of callus and cell suspension cultures. (A) After one week, showing swelling of an entire leaf-disc culture (bar = 0.06 cm); (B) After two weeks showing formation of calli at the cut end of leaf-disc explant (bar = 0.06 cm) (C) After three weeks showing formation of calli all over the surface of an explant (bar = 0.05 cm) (D) At the end of three weaks dedifferentiated cells were sub-cultured (bar = 0.05 cm); (E) After four week, development of biomass of callus cultures (bar = 1.26 cm) (F) After three weeks showing biomass of cells in suspension medium (bar = 0.08 cm)

2.3. Cell Suspension Cultures

A breakthrough in cell-culture methodology occurred with the successful establishment of cell lines, capable of producing high yields of secondary compounds in cell suspension cultures[36]. This finding opened the door for

the possible use of plant cultures for the production of secondary compounds of industrial interest. The cell suspension cultures offer a simple system to study growth and production kinetics which can help to evaluate and implement optimal conditions for the production of a number of high value medicinal compounds in good quantities[37]. The fine suspension of cells can be obtained by repeated subcultures of fast growing friable callus in liquid medium (Fig. 2F). In general, growth and division of cells in suspension is faster compared to the callus in semi-solid medium. It is noticed that the maximum biomass of cells in suspension was obtained in exponential phase, whereas, the metabolite accumulates occurs at the stationary phase[37,38]. Thus, liquid cell cultures sophisticated a good scope in future to isolate and increase the desired commercial products.

2.4. Hairy Root Cultures

The micropropagation, callus cultures and cell suspension cultures opened a new door for the development of genetically engineered hairy root cultures induced by the transfer of T-DNA from the plasmids of *A. rhizogenes*[39,40]. The explants when infected with pathogenic soil-borne bacteria *Agrobacterium rhizogenes,* induces 'hairy root' at the site of infection. The steps involved in hairy root induction are summarized in (Fig. 3). Being organized in nature, these hairy roots have the capacity to synthesize the metabolites stably for long at high concentration which is in contrast to the instable production of compounds by cell suspension cultures due to genetic instability[41,42,43,44]. They grow as fast as an unorganized callus or cell suspensions. Due to extensive lateral branching, the hairy root cultures produce large biomass as compared to the normal root system with added benefits of expression of all metabolic pathways as efficiently as any normal root cultures[45,46,47].

In *Datura* species, the tropane alkaloid production which is concomitant with the actively growing hairy root lines of genus was highly stable unlike when produced from cell suspension cultures[48]. The technique serves as an alternative strategy to plant cell cultures to study root derived chemicals and to establish a link between growth and production of metabolites.

3. APPROACHES TOWARDS HIGHER PRODUCTION OF METABOLITES

3.1. Optimization of Media Constituents

The established *in vitro* culture system show improved performance by monitoring metabolite production response as influenced by single factor, keeping others at an unspecified constant level, is termed as "one-variable-at-a-time". However, this methodology is extremely non-profitable due to

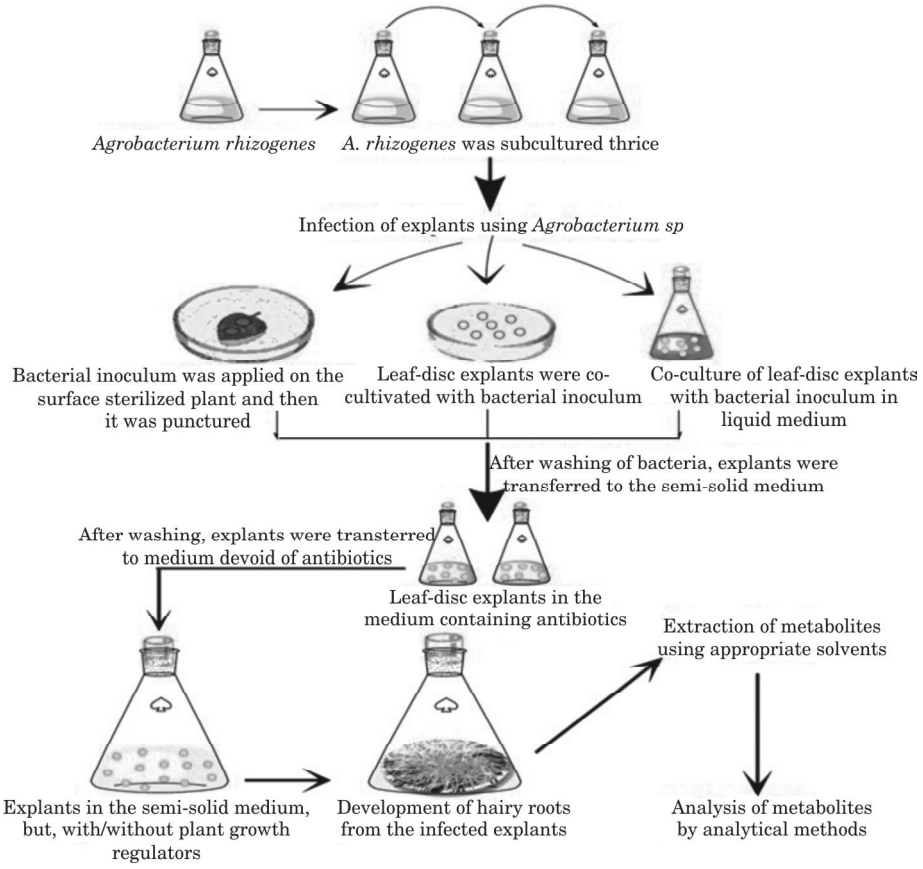

Fig. 3: Development of hairy root cultures from *Agrobacterium rhizogenes* for the analysis of various biactive metabolites

increase in number of experiments, time, cost and loss of details of interactive effects of chosen variables on response[49]. To prevail over these drawbacks, the analytical procedures have been optimized by multivariate statistic techniques like, 'Response Surface Methodology (RSM)'. The RSM is the combination of mathematical and statistical techniques based on the fit of a polynomial equation to the experimental data which must describe the behaviour of a data set when a response or a set of responses of interest are influenced by several variables[50]. The ultimate objective is to simultaneously optimize the variables to improve the best possible response on growth and metabolite production.

The RSM was implemented in plant tissue culture to optimize the media constituents such as, sucrose, nitrogen and phosphorous to improve the production of β-carotene 13.61 µg/g DW over control 9.63 µg/g DW and

from cell suspension cultures of *Daucus carota*[51]. Using RSM methods, shoot regeneration was increased from shoot-tips of *Basilicum polystachyon* using two different optimal plant growth regulators, 2.36 µM naphthaleneacetic acid (NAA) and 8.19 µM benzyl-6-aminopurine (BAP)[52]. Similarly, optimized concentrations of media constituents yielded in accumulation of 3.3 µg/L of azadirachtin in hairy root cultures[53] and 4.97 µg/g DW of azadirachtin in dedifferentiated cultures of zygotic embryos of neem which was much higher than the predicted value[54].

3.2. Application of Stress Factors

The stress factors can be categorized as elicitors and precursors. Elicitors are biological (fungal extracts), chemical (heavy metals, pesticides and detergents) or physical (cold shock, UV irradiation, High hydrostatic pressure (HHP), pulsed electric field (PEF) factors which induce enzymatic activity against stress[31]. Precursors on the other hand are intermediate products in the biosynthetic pathway of secondary metabolite. The plant cell cultures treated either by precursors, elicitors or through physical factors are schematically shown in the (Fig. 4). These secondary metabolites are complex chemical substances with signaling functions that are mostly produced due to environmental stresses and protect the plant from environmental changes[55]. As most of the target secondary metabolites are intracellular and is difficult to recover, treatment of plant cells with elicitors and precursors triggers the production of these secondary metabolites. Phenylalanine when added as precursor substance in cell suspension cultures, it induces the production of rosmarinic acid[56,57], taxol[38] and capsaicin[58,59]. The enhancement of capsaicin was also attempted by an addition of near precursor isocarpic acid. The amino acid, leucine, is a lead precursor for the improvement of volatile monoterpenes. Similarly, decarboxylation of basic amino acids such as, valine, leucine, isoleucine, phenylalanine and tyrosine acted as precursors for the production of amine moieties of *N*-alkylamides (NAAs) and geraniol in nerol and citronellol[60].

Treatment of plant cells with biotic and abiotic elicitors is one of the most useful strategies to enhance the production of secondary metabolite in plant cell cultures[61,62]. The methyl jasmonate, an important elicitor, enhances the biomass growth and production of ginsenosides and, taxol from cell suspension cultures of *Panax ginseng* and *Taxus chinensis,* respectively. It also channelized increased production of eleuthroside and chlorogenic acid from embryogenic cell lines[63,64,65]. Another most effective elicitor, Jasmonic acid, stimulated significant increase in the production of oleanolic acid[66]. Elicitor response varies with different parameters. Therefore, elicitors play a crucial role in enhancing the secondary metabolites production in intact plant cells and the effect of which vary with the type, concentration and duration of treatment[67].

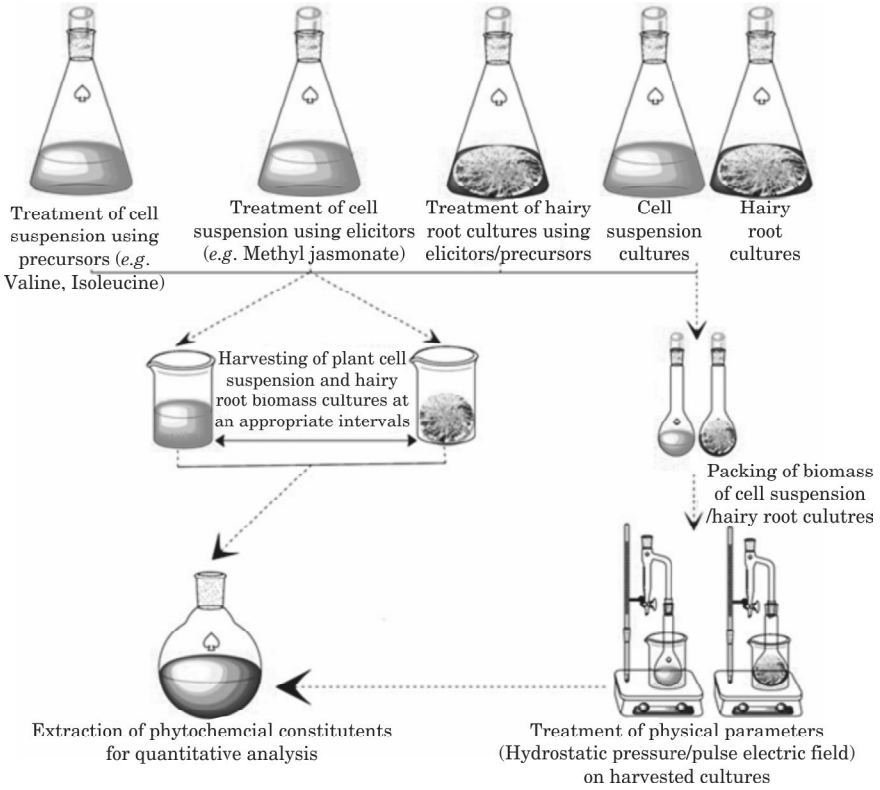

Fig. 4: Effect of precursors/elicitors/physical parameters on *in vitro* cultures for enhanced production of secondary metabolites

High hydrostatic pressure (HHP) and Pulsed electric field (PEF) are grouped into physical stress factors that induce secondary metabolite biosynthesis. HHP causes reversible and or irreversible changes in 3-D protein structure of the plasma membrane and the intracellular system[68]. On the other hand, application of Pulse Electric Field (PEF) influences the membrane integrity at different voltages between 1.6 KV – 2.0 KV and also affects protein channels within the cell membrane. Thus, it is positively resulted in the intracellular secondary metabolite recovery from the plant cell culture system[55]. Other stress factors like UV-irradiation and osmotic shock also aids in the stimulation of natural biosynthetic pathway for the enhancement of metabolites of plant cell cultures[69].

3.3. Immobilization of Plant Cell Cultures

The main bottleneck in commercialization of cell culture based process for secondary metabolite production is the high production cost due to slow growth of plant cells, low product yield, genetic instability of the selected

lines and low shear resistance of cells and intracellular accumulation of products. The immobilizations of plant cells are advantageous over cell suspension cultures to reduce the shear hydrodynamic stress and for higher volumetric productivities[70]. The cells are grown on inexpensive matrices for large-scale culture of immobilized cells[71,72]. The entrapment of cells in gel or behind semi-permeable membranes is the most popular method for immobilization of plant cells. In this technique, cells are confined within a reactor system, preventing their entry into the mobile phase which carries the substrate and the products. The steps involved in the immobilization of plant cells are summarized in (Fig. 5). Cells are reutilized for a comparatively larger period provided that the metabolites do not accumulate within the cells but are secreted into the culture medium[73]. Immobilization is only relevant where the production process involves two stages - first is to optimize the conditions for biomass production in suspension culture and second is to optimize the conditions for product formation by immobilized cells showing

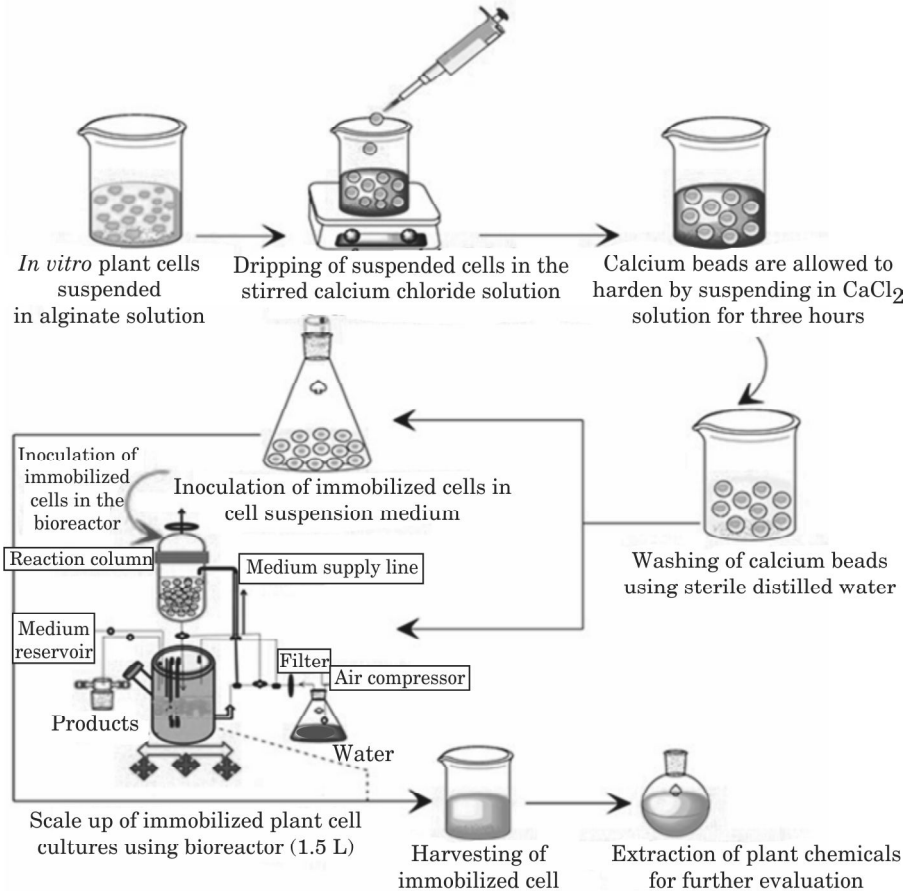

Fig. 5: Immobilization plant cell cultures

little or no growth[2]. The gel entrapment method for immobilization of cells was attempted on *Catharanthus roseus*, *Digitalis purpurea* and *Morinda citrifolia*[74]. The immobilized cultures of *Plumbago rosea* produced two to three folds higher plumbagin than control using un-cross linked alginate and calcium chloride[75].

3.4. Biotransformation of Plant Cell Cultures

Biotransformations are bio-chemical reactions exhibited during conversion of exogenously supplied complex substrates into product of interest by living plant cells, permeabilized cells or entrapped enzymes derived from cell cultures[76]. The cell cultures have the ability to biotransform the substrates of synthetic origin to yield already known aromatic, alkaloids, coumarin, lignin, steroid and terpenoids or to produce rare, novel, expensive products, and, subsequently, to establish a biosynthetic pathway unknown in many different plant species[77]. It may be a single step (mediated by single enzyme) or a multistep (mediated by two or more enzymes) process. However, the yield decreases with increase in the number of steps between precursor and product. Nevertheless, undifferentiated mass of plant cells require longer doubling time with its limited range of enzymes specifically necessary for the production of wide varieties of fine chemicals as compared to microbial cells[78]. Nonetheless, isolation of chiral metabolite from *Nitraria schokeri* by non-enzymatic reactions clearly reveals that the plant cell system do not always necessarily produce metabolites through enzymatic reactions[79]. The chemical isoeugenol was biotransformed in immobilized cell cultures of *Capsicum frutescens* to vanillin flavour and capsaicin[80]. Few other biotransformation reactions using plant cell cultures are listed in the Table 3.

Table 3: Biotransformation reactions in *in vitro* plant cell cultures

Plant species	***Product***	***Applications***	***Ref.(s)***
Artemisia annua	Oxidoreduction	Artemisinin	[81]
Catharanthus roseus	Hydroxylation	5-hydroxyneodihy droxycarveol	[82]
Datura innoxia	Nitroreduction	2,4,6-aminodinitro toluene (ADNT)	[83]
Dioscorea deltoidea	Reduction of C–C double bond	Carvone	[84]
Mentha piperita	Epoxidation	(-)-7-hydroxyisopi peritonone	[85]
Nicotiana plumbaginifolia	Glucosylation	6-O-butyryl-D-glucose	[86]
Rauwolfia serpentina	Reduction	Raumacline	[87]
Salix matsudana	Glucosylation	Salicin	[88]
Spirodela oligorrhiza	Hydrolysis	(R)-alcohols	[89]

3.5. Large Scale-Up of Plant Cell Cultures

The scale-up of plant cell cultures in bioreactor systems is an active field and possible final step for the production of valuable compounds at a reasonable amount for potential commercialization. Inspite of its significance, large-scale culture of plant cells often show sensitivity to shear stress, slow growth rate, and low oxygen demand. As the volume of cell suspension increases, mixing inside the bio-reactor vessel become difficult, resulting in non-uniform concentration of the nutrients and limited oxygen transfer to living cells. Eventhough unique problems exist during large scale cultivation of plant cells, the rheological characteristic of plant cells varies with different species resulting in the selection of innovative reactor designs to develop a sophisticate plant cell system[90]. In this context, air-lift or bubble reactors, used for plant cell cultivation, may reduce the lysis of cells during agitation compared to the traditional propeller system[91]. The main focus on designing the reactors was based on the relationship between biomass growth of plant cell cultures and the metabolite production which lead to the development of different types of reactors namely, single step and two-step process reactors. The single step reactor type comprise of extraction of metabolite which were associated with the growth of the cells. Whereas two-step reactors are required where the product is non-growth associated where first the biomass is increased in growth medium and then transferred to a production medium at the end of the growth phase[92,90].

The bio-reactor studies has a unique place in the plant cell and tissue culture history for the production of paclitaxel (taxol) from *Taxus chinensis* species using stirred tank reactor with modified impellers for improved mixing and low shear stress followed by ginseng production from fine cells of *Panax notoginseng*[93,94]. The cell lines of *P. ginseng* were cultivated in bioreactors using different impellers types such as, flat-blade, angled-blade disc-turbine, and anchor impeller to improve the production of ginsenoside. However, application of same reactor did not support the growth and production of metabolites in *Coptis japonica* cell lines[28]. This illustrates that each and every part of the reactors promotes the longevity of cell line and, hence, improved production of secondary metabolites for commercialization. A few limitations such as, product inhibition due to controlled addition of limiting nutrient in the batch cultivation lead to the design of fed-batch cultivation to resolve the problems faced by batch cultivation. Fed-batch cultivation has been able to improve the productivity of ginseng by *P. ginseng*, taxane by *T. chinensis* and berberine from *C. japonica*[95,96]. Similarly, few other developments emerged in the progress cells in bioreactors[97]. The steps to scale-up the plant cells in bioreactors are given in (Fig. 6). Few historical records using bioreactors for large scale cultivation of plant cells and production of secondary metabolites are listed in the Table 4.

Table 4: Production of secondary metabolites from plant cell cultures using bioreactors

Plant species	***Reactor type and capacity***	***Product (mg/L)***	***Ref.(s)***
Anchusa officinalis	Stirred tank bioreactor, 2.5 L	Rosmarinic acid, 3700	[98]
Catharanthus roseus	Air-lift reactor, 20 L	Catharanthine, 22	[99]
Holarrhena antidysenterica	Stirred tank bioreactor, 6 L	Conessine, 106	[71]
Lithospermum erythrorhizon	Stirred tank bioreactor, 200 and 700 L	Shikonin, 4000	[100]
Nicotiana tabacum	Fed-batch	Cinnamoyl putrescine, 400	[101]
Podophyllum hexandrum	Stirred tank bioreactor, 3 L	Podophyllotoxin, 13.8	[90]
Taxus cuspidata	Wilson type reactor	Taxol, 22	[102]

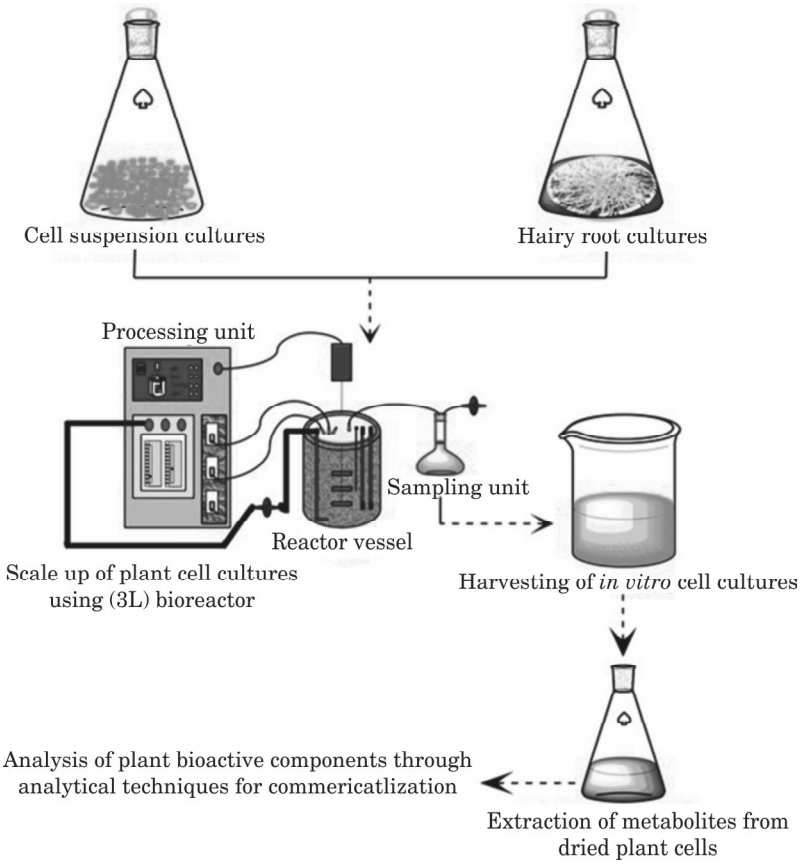

Fig. 6: Scale up of plant cell cultures using bioreactors for higher production of secondary metabolities

4. ANALYTICAL APPROACHES FOR THE SEPARATION OF PLANT DERIVED CHEMICALS

The analytical platforms are the main technique to identify the bioactive molecules with diverse atomic arrangement, polarity, solubility and volatility. Due to the superior sensitivity of chromatography techniques, spectroscopical methods draw the attention in characterizing the structure and quantity of phytochemical compounds. These analytical approaches are a powerful tool to explore the discovery of new compounds, saving time in re-isolation and re-identification of already known compounds even at the micrograms scale[103].

4.1. Thin Layer Chromatography

Thin layer chromatography (TLC) is one of the oldest, versatile and reliable analytical methods for the identification of secondary by products and to perform purity tests. The technique is readily available with improved precision and accuracy and has occupied an important place for the identification of most of the secondary metabolites present in the plant extracts. TLC consists of a solid stationary phase and a liquid mobile phase. Glass plates coated with stationary phase are used for the separation of compounds. Silica gel is the most commonly used coating material, although alumina may also be used[104]. The mobile phase consists of a solvent system with varying polarity. Using this chromatographic technique, large number of samples, like phospholipids, steroids and other metabolites from complex mixtures can be analyzed simultaneously and quickly[105]. The compounds isolated from TLC can be detected by exposing the plates under UV light, or spraying it with Dragendorff and anisaldehyde reagents[105,106].

Preparative TLC is another alternative method and is used for separation and recovery of specific phytochemicals in large amount by applying larger samples in long bands onto plates with increased layer thickness[107]. However, low reproducibility, sensitivity, resolution and several other factors such as, vapour, environment inside the developing chamber and the unstable colorations when using chromogenic reagents for detection hinder the wide spread use of TLC. The short comings of regulated environment and quality control precisely have developed a High Performance Thin Layer Chromatography (HPTLC). HPTLC is a densitometry method for the qualitative and quantitative analysis of bioactive phytochemical compounds that allows evaluation, electronic and forgery-proof picture documentation of all instrumental parameters in visible, long and shortwave UV ranges of chromatograms with naturally detectable zones. This led to the comprehensive, quality control, stable identification and assessment of secondary products by reducing the detection limits and resulting in an

improved sensitivity[107]. The HPTLC allows faster separation of conessine in extracts and polyherbal formulations by eliminating the need for two-dimensional TLC. Ecdysone from whole plant material of *Sida rhombifolia* was also analyzed and derivatized for the development of dietary supplements[108]. Nonetheless, TLC and HPTLC have lesser feasibility to analyze isomer moieties. Other complex and volatile molecule requires alternative techniques for derivatization such as GC-MS, HPLC, and LC-MS.

4.2. Gas Chromatography-Mass Spectrometry (GC-MS)

Gas chromatography is an analytical technique equipped with mass spectrometry (flame ionization, FID) that separates the volatile compounds from a crude mixture of extracts when passed through a column packed for separation of compounds by gas adsorption or gas-liquid partition chromatography. This also facilitates a feasibility to identify known compounds within a few minutes using mass spectral libraries and mass spectral deconvolution softwares. Gas chromatography (GC) comprise of a solid stationary phase packed with solid like, activated carbon, alumina, or silica gel and mobile gaseous phase. Hydrogen is preferred as a carrier gas as it has lower elution temperature that allows shorter time of analysis and reduced the risk of thermal degradation of compounds[109]. Gas-phase chromatography can be applied to any appreciable vapor pressure material to identify diversified compounds such as, acids, alcohols, amines, esters, and several hydrocarbon molecules[110]. Additionally, the hydrolyzed phospholipids, methyl transesterification producing fatty acid methyl esters and other fatty acid metabolites have also been analyzed by GC-MS[111,112].

Inspite of several tested advantages of GC-MS, its applications on herbal products is unsuitable for the analysis of thermo-unstable compounds, volatile compounds and essential oils for which derivatization is not always possible[113,114]. However, the various compounds of essential oil from *Cucurbita* and *Turnera diffusa* were investigated through low-pressure GC-MS using mega-bore analytical columns. The modern improvement in GC-MS has led to an analysis of essential oils in a reduced analysis time with decreased detection limits although concurrent loss of efficiency occurs in separation of organic compounds from complex sample mixtures. In contrast, fast GC-MS analyses using micro bore capillary column for the essential oils has increased the efficiency of resolving power and intensities of chromatogram of a compound by increasing velocity of solute and inlet pressure. Thus, GC-MS analytical technique has gained widespread acceptance in several applications such as, process control in recovery of plant chemicals, quality control in food industry, monitoring sample composition in oil industry, environmental and biomedical sciences[115,116,117].

4.3. Liquid Chromatography-Mass Spectrometry (LC-MS)

In contrast to GC-MS, Liquid Chromatography-Mass Spectrometry (LC-MS) elutes thermally unstable, non-volatile compounds and has great advantage over GC-MS as the columns are operated at or near room temperature. This method has been successfully implemented in analysis of herbicides, steroids and insecticidal compounds by the development of controlled surface porosity columns supported by the use of high speed and pressure to generate fast end separations of biologically active materials. LC-MS also facilitates the structural identification of large, polar and semi-polar molecules using MS detectors with soft ionization sources to provide valuable information. The organic compound, benzodiazepine metabolite is a drug and can be detected through LC-MS from urine samples of the victim to extract forensic information for the investigation of specific crimes although the same was not confirmed by Gas Chromatography[118,119]. Later, LC-MS method was also validated for simultaneous determination of flavonoids, such as, puerarin, daidzin, baicalin, liquiritin, alkaloids such as, berberine, palmatine, jateorhizine and triterpene glycosides such as, glycyrrhizic acid from rat plasma samples that laid the foundation for identifying more than one component in a complex[120,121]. Similarly, sensitive and accurate matrix effect was experimented on leaves and cell cultures of *Arabidopsis thaliana* to evaluate the metabolomic profiles. Further, other plant secondary metabolites such as, alkaloids, saponins, phenolic acids, phenylpropanoids, glucosinolates, polyamines and flavonoids are also examined in keeping reproducible operating procedures[122].

4.4. High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography is a popular analytical technique characterized by high sensitivity and resolution coupled with continuous flow detectors capable of handling small flow rates and to detect very small amounts. It favours rapid analysis of samples at relatively high inlet pressure and controlled flow of the mobile phase. The principle is that a small volume of liquid sample injected into a tube (column) packed with tiny particles called stationary phase and the sample is moved down through the column with a liquid (mobile phase) at high pressure. The separated components are detected at the exit of the column by a detector that measures their amount and the output is called 'liquid chromatogram'. The liquid chromatogram is based on the difference in the rates of migration of sample through the column arising from different partition of the sample between the stationary and mobile phase[123]. The most widely used separation technique is the reverse-phase chromatography due to broad application range and is able to handle compounds of a diverse polarity and molecular mass[124,125,126]. The molecules with hydrophobic characters such as, proteins,

peptides and nucleic acids, were mostly separated using reverse-phase column with high quality recovery[127]. HPLC directly overtakes TLC by avoiding few components of sample extracts from auto-oxidation and also enables the system to elute the isolated compounds for further analysis[128,129]. Separation of phytopharmaceutical compounds, such as, terpenes trilactones, biflavones, flavonol glycosides and other phenolic compounds, were detected using HPLC adapted with ultra-violet (UV), fluorescence and refractive Index (RI) detectors. Pentacyclic triterpenoids were identified from unorganized callus cultures proliferated from leaf-discs of *Lantana camara* using RP-HPLC. The samples from undifferentiated redifferentiated and cell suspension cultures of *Spilanthes acmella* were analyzed to separate and identify *N*-alkylamides (NAAs) through HPLC-UV technique to evaluate their biological activities[37,130,131]. Scopoletin, a coumarin, was analyzed from nodal segment cultures of *Spilanthes acmella* through the system equipped with HPLC-fluorescence detector[132].

With the development of genomics, proteomics and metabolomics, modified conventional HPLC with high resolution and sensitivity such as, Ultra High Performance Liquid Chromatography (UHPLC), Ultra Performance Liquid Chromatography (UPLC) and High Temperature Liquid Chromatography (HTLC), are developed. Due to small peak widths afforded by the UPLC, a high acquisition rate can be easily obtained. HTLC can also be used to perform rapid analysis with conventional column lengths since mobile phase viscosity and backpressures are decreased[133]. UHPLC is well equipped to withstand pressure up to 1000 bar. The usage of solid-phase column of 2 µM particle size can dramatically increase the separation power with superior sensitivity at high resolution and speed. Furthermore, the data acquisition can be performed with modified detector facility in a shorter analysis time with less consumption of solvents and small flow volume as compared to conventional HPLC method[133,134]. The anthelminthic monepantel and its sulfone metabolite were identified from goat's milk and ovine muscle by generating shorter and easier methodology using UHPLC-MS/MS[135]. In the same way, the ultra-fast scanning speed efficiently yielded eighteen purified preservatives to maintain the growth and freshness of corresponding materials from vegetables[136]. In addition, tropane alkaloids and other prohibited analytes in sports such as, stimulants, diuretics, narcotics, and anti-estrogens were also analyzed through automatic tool for peak picking based on retention time and mass accuracy[137]. The secondary metabolites with high pharmacological properties such as, catalpol, harpagid, harpagoside, and traces of loganin and catalposide are identified from in vitro plant cultures of *Rehmannia glutinosa* with highest accumulation of aucubin, verbascoside, and isoverbascoside using UHPLC[138].

4.5. Mass Spectrometry

Mass spectrometry (MS) is the most versatile method in proteomics and metabolomics. It determines the mass of molecules that carries electrical charge and the resulting ions are identified by their mass-to-charge ratio (m/z). It provides the valuable structural information on new and known compounds with a blend of rapid, sensitive and selective qualitative and quantitaive analyses. The significance of MS drives the attention in coupling with Gas chromatography, Liquid chromatography, High performance Liquid Chromatography and other analytical techniques. The two different hard and soft ionization techniques programme the pattern of mass spectrum based on the degree of fragmentations obtained by imparting internal energy onto the sample with numerous ion fragmentations. In addition, ionization of some molecules shows the same molecular mass due to molecular ion stability[139].

The development of macromolecule ionization methods, including Electron Spray Ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), assist to study the proteins by MS. Chowdhury SK. Katta V, Chait BT. Electrospray ionization mass spectrometric peptide mapping: A rapid, sensitive technique for protein structure analysis. Biochem Biophys Res Commun. 1990 Mar 16; 167(2):686-92. ESI is a soft ionization technique vastly used for the production of gas phase ions (without fragmentation) of thermally labile large supramolecules. The high end ESI-Mass Spectrometers transfer analyte moieties into gaseous phase for mass analysis to yield protonated $[M+H]^+$ deprotonated $[M-H]^-$ or adduct $[M+Na]^+$ formation of the parent ion molecules[140,141].

However coupling of ESI with HPLC is more attractive due to its efficiency and faster analysis. Generally, the precursor ions are produced that leads to successive fragmentation by collision induced dissociation (CID) or by spontaneous dissociation. In general, ESI generates stable ions by CID rather than any earlier ionization mode[142]. The construction of MS libraries for plant metabolites catalyzes the search of new natural products and dereplication of known compounds for lead compound discoveries[143].

4.6. Nuclear Magnetic Resonance (NMR) Spectroscopy

In commensurate with growing and advanced technology, Nuclear Magnetic Resonance (NMR) Spectroscopy has a cornerstone in the field of chemical research since 1946. NMR was discovered by famous Nobel laureates, Edward Mills Purcell and Felix Bloch in physics, 1952. This instrument provides appropriate and irreplaceable structural determination of purified natural products with minimal use of the sample needed for probe at 1.5–2.5 µl. The potential value of new spectroscopic technique reveals that the nucleus

of ^{1}H dominates the NMR landscape even today and particularly ^{13}C and ^{15}N were preferably used to recognize the carbon skeleton of organic moieties[144]. The two dimensional NMR (2D) experimental studies allows for the structural characterization of unfractionated and partially fractionated plant extracts over one dimensional (1D) NMR. However, 1D NMR is extremely useful in analyzing similar groups of samples. The identification of large number of components is highly difficult due to over lapping of signals.

The 2D NMR subdivided as homonuclear correlation spectroscopy (COSY), nuclear over hauser effect spectroscopy (NOESY), heteronuclear single quantum correlation (HSQC) and heteronuclear multiple-bound correlation (HMBC) NMR spectra for the understanding of environmental molecular orientations. Further, distortionless enhancement by polarization transfer (DEPT) NMR experiment is necessary for the automated dereplication of natural products by superimposing with spectral database for the analysis of 1D and 2D NMR spectra[145,146]. Similarly, the biologically synthesized unsaturated pattern of purified isobutylamide compounds from different plant species such as, *Achillea*, *Spilanthes*, *Echinacea* and *Heliopses* species are analysed using ^{1}H and ^{13}C NMR for the structural confirmation[106,147]. The significant analysis of secondary metabolites from plant cell cultures can also be achieved by the recent advancement and hyphenation technique LC–DAD–HRMS–SPE–NMR equipped with microvolume, cryogenic, flow probes and other combinations[148]. The overall schematic representation of extraction and identification of biologically active phytochemical compounds by high through-put screening methods, derived from plant cell cultures, are shown in the (Fig. 7).

5. SUMMARY

During the last few decades, the advancement in technologies revolutionized the availability of natural products all over the world and also leads to the discovery of economically valuable compounds from nature. The plant tissue and organ culture technology is a suitable alternative biotechnological approach to whole plant cultivation for the analysis of desired secondary metabolites. Perhaps, improved underpinning of secondary metabolite pathways has a greater impact on the usage of plant cell cultures for the commercial production of economically important compounds. Explorations of media combinations, bio-transformation, plant cell immobilization, hairy root culture, precursor feeding and elicitation of plant cells are some of the strategies towards improvement in the quality and quantity of compound production. Moreover, the vast array of higher sensitivity and resolution, miniaturization, high-throughput screening chromatographic methods such as, TLC, GC, LC, HPLC and other spectroscopical studies advanced the scope of analysis of metabolites in the field of functional food, cosmetics,

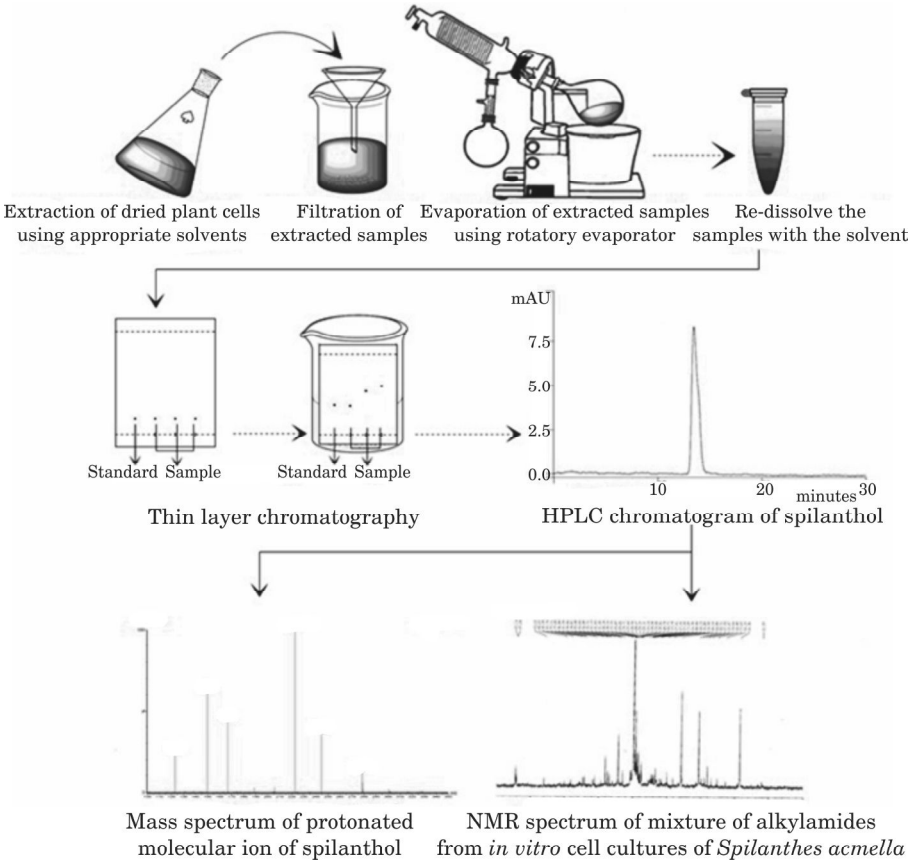

Fig. 7: Extraction and identification of phytochemical components through high-throughput screening methods

plant resistance and phytomedicine. Thus, the direct infusion of rapid and effective analytical methods can define the quality and safety of plant cell tissue and organ culture products.

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