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REVIEW

Technology-Based Production of Secondary Metabolites from Cell and Callus Culture

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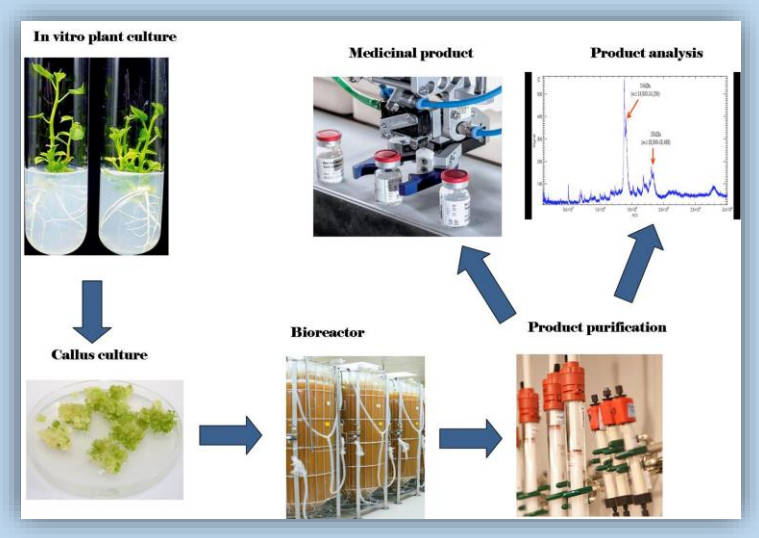
ABSTRACT

Recently, due to the wide spread of chemicals and their negative impact on human health and environment, the focus has increased on plant products, especially secondary metabolites in medical and in various industrial fields. Therefore, it seems necessary to focus and research on increasing the production of secondary metabolites using plant tissue culture techniques, especially callus culture, and improve its production on a large scale using bioreactors. Moreover, it is important to find the best methods to increase quality and quantity extraction of secondary metabolites. Callus culture and cell suspension culture in combination with developed extraction techniques represent powerful biomass production techniques to gain high quality secondary metabolites from plant materials. This article reviews the importance of callus and how to culture callus, in addition to the most important modern methods for producing secondary metabolites from callus and cell cultures.



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1. Introduction

Plants contain many medicinal compounds that are used in the pharmaceutical industry, so that about 25 percent of drugs approved by the FDA (Food and Drug Administration) and EMEA (European Medicines Agency) are derived from plants (Borkotoky et al. 2021; Thomford et al. 2018). Plant tissues culture (PTC), especially developing new techniques such as callus culture during the 1940s, 1950s, and 1960s has been increasingly popular as one of the methods to the development of agricultural crops, so that its impact on the economy is well known in recent years. In vitro PTC is the sterile cultivation of cells, tissues, organs, or whole plants in a controlled environment with controlled nutrients (Thorpe 2007). These controlled conditions such as the right average pH, the right temperature and the right gas-liquid environment provide a favorable environment for growth and reproduction of explants. Climatic and geographical conditions can't effect on PTC techniques, so these can be an incessant, sustainable, and economical techniques for the secondary metabolites production (Chandran 2020). Callus culture as a one method of PTC techniques generally consists of culturing young, friable, large, vacuolated and highly differentiated, but disorganized cells. Callus is a proliferating mass of disorganized and dedifferentiated cells which can be established and grows over a wounded or cut surface of plant under in vitro conditions on suitable nutrient media. A single differentiated cell can dedifferentiate and form a callus, and several complete callus cells can form the whole plant (Steward et al. 1958) (Nagata et al. 1971). On the base of macroscopic characteristics Calli are very diverse and can be classified into subgroups (e.g., friable or compact callus). Each group of callus has distinct gene expression profiles (Iwase et al. 2011). Callus culture can be applied for propagation, production of bioactive components and biological studies like genetic engineering and signaling pathway researches. One of the reasons for using the tissue culture technique, especially callus culture, is the lack of technology for the effective production of biologically active substances, the poor development of medicinal plant species in farms, and the extinction of many plant species in the natural environment. On the other hand, in vitro cells significantly differ from the cells in whole plant, which is largely related to the intensity of cell growth and the characteristics of the synthesis and repletion of biologically active components in them (Babich et al. 2020). In vitro callus cultures of higher plants are a new solution for natural product production that is the most widely used in practice.

Plants have many secondary metabolites that produced in low rate, because they are not involved in plant growth or development (Kim et al. 2002). Secondary metabolites can be utilized in different industries such as agriculture, biomedical, biofuel, biocatalyst and environmental bioremediation. For example, reserpine, ajmalin, ajmalicin, serpentine and yohimbine are some of the well-known secondary metabolites that since ancient times have been used in many Ayurvedic and Onnaic medicinal preparations to treat diseases. Recently medicinal plants, as producers of secondary metabolites have become of particular interest. The production of secondary metabolites from tissue culture has been reported in several publications. For example callus culture and cell suspension culture of American ginseng were applied for the production of ginsenosides and polysaccharides (Qiang et al. 2020). Current review mainly illustrates and discusses the various in vitro biotechnological aspects such as callus culture and cell culture on secondary metabolite production, which provides significant ideas regarding the ongoing research activities and future prospects related to the improvement of secondary metabolite production and extraction from plants.

2. Wounding, Dedifferentiation and Callus Formation

The transition of cells from a certain state of differentiation to an undifferentiated or less differentiated state similar to that of stem cells and the re-acquisition of the ability to differentiate is called dedifferentiation (Grafi and Gideon 2004). Biotic and abiotic stimuli such as wound, cutting or auxin-specific hormones can lead to callus formation (Ikeuchi et al. 2013). Callus shape and transcription profile in callus induced by auxin and cytokinin and

callus induced by the wound is different (fig. 1) (Iwase et al. 2011). Studies in plants have shown that the expression and repression of certain genes by wounding stimulate callus that regulated by transcription factors. A group of AP2/ERF transcription factors called WOUND INDUCED DEDIFFERENTIATION1 (WIND1), WIND2, WIND3, and WIND4 play key roles in wound-induced callus formation (Minorsky and Peter V 2015). Cytokinin biosynthesis and signaling are upregulated by WIND1 24h after wounding that it's leading to activation of cell proliferation and tissue formation. There are many transcription factors that regulate callus formation but among them WIND1, 2, 3, 4 are very important (high callus formation and relative callus area compared by other transcription factors). They activate a multifaceted set of reprogramming regulators to promote cell proliferation and cell wall remodeling during regeneration (fig. 2 a,b) (Iwase et al. 2021). But neither endogenous auxin accumulation nor activation of the auxin response could be seen at the wound site (Iwase et al. 2011; Ikeuchi et al. 2016). For callus formation and organ regeneration, quiescent cells must re-enter to the cell cycle through reactivation of the basic cell cycle regulators CYCLIN (CYC) and CYCLIN-DEPENDENT KINASES (CDK). Recent studies revealed the mechanism of wound signal transmission to activate cell proliferation and callus formation.

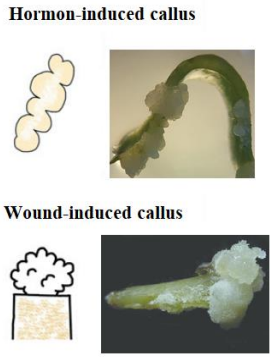

Figure 1. Comparison between callus stimulated by hormones and callus stimulated by wounding. With application of hormones callus induced from pericycle cells adjacent to the xylem poles but in wounding Calli generated from various cell types, such as vascular cells, cortical cells, and pith cells.

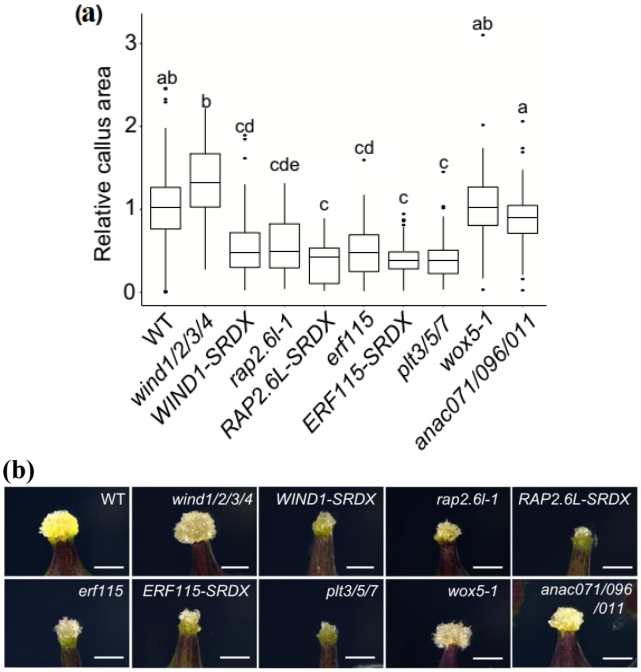

Figure2. Callus formation at wound sites induced by WIND transcription factors. **a.** Quantitative analysis of wound-induced callus formation in wild-type (WT), wind1/2/3/4 and other transcription factors. **b.** Images of wound-induced callus generated in WT, wind1/2/3/4 and the others (Iwase et al. 2021)

3. Callus culture and secondary metabolites production

If a certain metabolite be produced in a specific plant tissue, organ or in a specialized gland in the case of essential oils, it is important that differentiated tissues or organs be selected as an explant (Karuppusamy 2009; Rao 2002). Secondary metabolites with complex chemical composition that are produced in response to various forms of stress have different physiological roles in plants. Studies have shown that cultured tissue and suspended cells of differentiated tissues and calli can produce secondary metabolites and each cell can produce metabolites identical to those made by the mother plant under the suitable conditions (Isah et al. 2018). Callus and cell suspension culture are applied for the production of taxol, artemisinin, ginsenosides, raubasine, tropane alkaloids, tocopherol, ajmaline, serpentine, reserpine, flavonoids, scopolamine, paclitaxel, stilbene,

resveratrol, and anthocyanins (Efferth 2019; Patil et al. 2014; Sharma and Rasheeduz 2016; Baldi and V. K. Dixit 2008; Jeong et al. 2008; Ten Hoopen et el. 2002). Also callus cultures of medicinal plants can be used for the large-scale and stable production of bioactive phytochemicals as a secondary metabolite in pharmaceuticals and to treat a wide range of diseases such as cancer, cardiovascular diseases, neurodegenerative diseases, infectious diseases, etc. (Karwasara et al. 2010; Aziz et al. 2003).

4. Benefits of callus and cell culture for production of secondary metabolites

The ability of callus and suspension cultures to produce secondary metabolites provide opportunity to manipulating the biosynthetic pathways of plant cells to produce secondary metabolite derivatives with improved marketing features (Grzegorczyk and Wysokinska 2010). Additionally, by using biotransformation processes in cell and callus culture, specific substrates can be converted into desired end products. Also callus culture may help protect rare and endangered plant species, because sufficient amounts of phytochemicals can be extracted directly from calli without sacrificing the whole plant (Fischer et al. 1999). Callus cultures can be converted to single-cell suspension cultures growing in flasks on shakers or in biofermentors for the manufacture of the desired secondary metabolites (Ogita 2015; Wu et al. 2016). In extraction of secondary metabolites as medicinal compounds from plants callus is more reliable than extracting of them from the whole plant (Efferth 2019). Also, cell culture in compared with other production techniques has a more and faster potential application to the market (Xu et al. 2011). Because the use of callus and cell suspension culture enables controlled growth condition without the impacting of environmental factors, seasonal changes, microbial infections, pests, or geographic limitations, so this technique provides continuous production of metabolites with consistently excellent quality and homogeneity. Callus and suspension cultures have totipotency for the manufacture of secondary metabolites. They contain the whole genetic information of whole plants and in both plant cell cultures and whole plant; secondary metabolites are isolated and purified using the same chemical preparative procedures.

5. Strategies for high-yield production of secondary metabolites in plant cell culture

5.1. Select a high-producing strain

For high-yield production of secondary metabolites through callus and cell culture, it is necessary to choose a parent plant with high ability to produce of the desired metabolite. Plants with high content of secondary metabolites produce high-producing cell lines (Deus and Zenk 1982). So, the first step in establishing highly productive cell lines is the selection of suitable parent plant.

5.2. Environmental factors and secondary metabolites productivity

In addition to the selected parent type as a main factor for high-production of secondary metabolites, it may also be possible to increase productivity by altering environmental factors such as nutrient levels, light, and temperature (Parr 1989). The sources of nitrogen in medium culture play an important role in the accumulation of products in plant cells; therefore, depending on the plant species, a suitable nitrogen source should be selected (Tabata 1999).

5.3. Genetic and Metabolic engineering

Genetic engineering is the artificial modification of DNA or transfer of genetic material from one organism into foreign organisms. One of the goals of applying genetic engineering is the transfer of properties found in different plants or different cells and integration of specific and active regulatory mechanisms. Usually, the amounts of secondary metabolite produced in whole plants are small and restricted to single tissues of the plant or cellular organelles, that it affects their extraction process costs (A Benedito and Luzia 2014; Babich et al. 2020). So the production of metabolite should be enhanced in the plant. Metabolic engineering is a technique to enhance the production of high-value secondary metabolites in organism that improves cellular activities by manipulation of metabolic pathways of organism with the use of recombinant

DNA technology. Various engineering strategies such as metabolic engineering, inverse metabolic engineering, engineering of secretory pathways, and process optimization are applied to enhance the secondary metabolites production in plants (Wuest 2014). For example, terpenoids as a secondary metabolite in plants and some by bacteria or yeast are produced by Mevalonate (MVA) pathway and also 2C-methyl-D-erythritol-4-phosphate (MEP) pathway. Each of pathways comprises some enzymatic reactions (fig. 3). Overexpressing genes of each pathway is an effective strategy to increase the yield of pharmaceutical terpenoids (A Benedito and Luzia 2014).

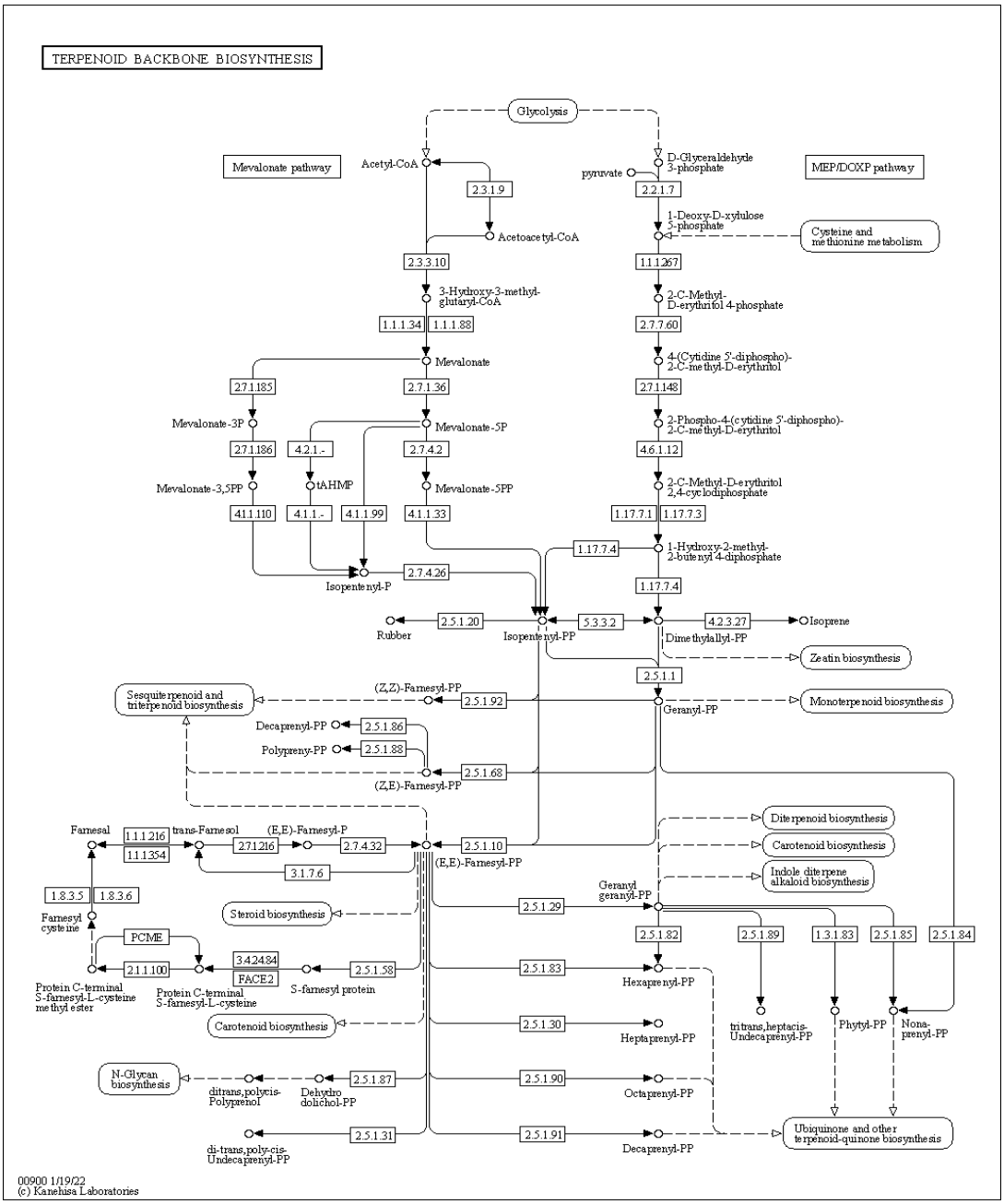

Figure 3. Biosynthetic pathways for terpenoid backbone production.

Metabolic engineering is applied in order to decrease costs of secondary metabolites production, increase yields, increase efficiency, increase consumer acceptance, and protection of environment by decrease of using traditional chemical methods.

5.4. Biotransformation

Biotransformation is another technique for the production of high-value metabolites from plant cells and organ cultures. It is the biochemical modification of chemical compounds using enzymes such as hydroxylase, glycosylase, oxidoreductase and hydrogenase. Enzymes with catalysis of reactions such as hydroxylation, glycosylation, glucosylation, oxidoreduction, hydrogenation, hydrolysis, methylation, acetylation, isomerization, and esterification have the potential to change inexpensive and abundant byproducts into rare and expensive goods. Plant cell cultures also can convert exogenous substrate into the interested products. Several different types of chemical compounds such as aromatics, steroids, alkaloids, coumarins, terpenoids, and lignin in plants can undergo biotransformations via plant enzyme. For example, in the Podophyllum species, podophyllotoxin, a precursor to a semi-synthetic anticancer drug, is derived from biotransformation of butanolide (Kutney 1993).

5.5. Cell Immobilization

In immobilization, cells entrap in porous polymers or microcapsules or bind to an inorganic or organic support matrix such as glass wool, Ca-alginate and cellulose beads (fig. 4). Immobilized whole cells as a competitive alternative provide an environment for increased cell densities and have been used in higher production of industrial and pharmaceutical metabolites specially antibiotics. The production of metabolites in immobilization of cells is several times more than in free cells. On the other hand, enzymes and enzyme-containing whole cells as biocatalysts are generally fragile and easily inactivated in that the immobilization techniques solve such challenges (Lou et al. 2021). The immobilization techniques decouple growth and metabolite production and reduce aggregate, growth, and foaming problems. Studies have shown that freely suspended and immobilized cells of *Capsicum frutescens* can convert protocatechuic aldehyde and caffeic acids to vanillin and capsaicin (Giri et al. 2001; Rao and G. A 2002). In addition, ginseng-cultured cells and roots can convert paeonol into its glycosides, which have radical scavenging properties. In plant cell immobilization techniques, produced secondary metabolite should be secreted to microenvironment. Release of single cells from cell aggregate may make process of secondary metabolite production more difficult. On the other hand, the medium may be unfavorable for secreted secondary metabolites and cause their degradation.

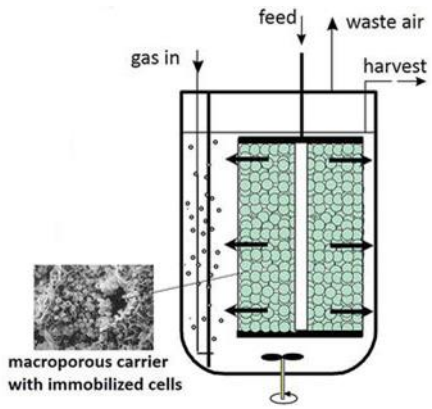

Figure 4. Fixed bed with radial flow integrated in conditioning vessel, plug flow (Graph courtesy of Pörtner and Rebecca, 2019).

5.6. Bioreactors in plant cell culture

Bioreactors are manufactured fermentation systems and one of the alternative technologies to produce natural compounds such as secondary metabolites from organisms for using in the industrial and medical (Rodríguez-Monroy and Enrique 1999) (fig. 5). Several main factors in a bioreactor including: i) Agitation in order to mixing of cells and medium, ii) Aeration, iii) setting of factors such as Tm, pH, pressure, aeration, nutrient feeding and liquid leveled, iv) Sterilization and v) Withdrawal of cells and medium should be optimized. There are several types of bioreactors, which used extensively in industries such as Continuous stirred tank fermentor, Airlift fermentor, Fluidized-bed fermentor, Fluidized-bed fermentor, Photobiore-actor, Membrane bioreactor and Bubble column fermentor. In recent years for propagation of plants, a variety of conventional

bioreactor systems have been effectively developed that this systems was adapted for the unique requirements of micropropagation (Steingroewer et al. 2013). Also, for the production of biopharmaceuticals, new class of bioreactors is developed and modified that called Temporary Immersion Systems (TIS) (Georgiev 2015).

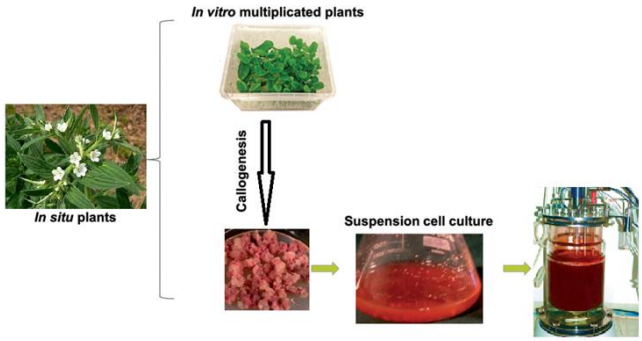

Figure 5. Bioproduction of secondary metabolites in bioreactor.

The accessibility of nutrients during their cultivation is one of the primary factors in bioreactors that should be considered because this factor influences both development and secondary metabolism in cells (Steingroewer et al. 2013). The factors that control the growth and multiplication of cultured cells/organs and biomass accumulation in plants differ from the stimuli that cooperate with biosynthesis of secondary metabolites. So, there is two phase or steps in plant cultivation process in bioreactors that these two-steps requires the control and monitoring of different parameters on each stage. In the first stage selection of high-yielding cells or organ clones, the optimization of the standard medium (salts, sugar, nitrogen, and phosphate sources), levels of plant growth regulators, and physical factors such as temperature, illumination, light quality, pH, agitation, aeration, and environmental gases have a crucial role and at first a plant cell line with the best genotype performance must be selected in order to produce a large amount of metabolite in bioreactor. But the second stage of the culturing process in bioreactors is impacted by the techniques of elicitation, replenishment of nutrient and precursor feeding, and product recovery that assist the accumulation of secondary metabolites in plant tissues. Control of plant in these two phases in bioreactors can help to produce high amounts of biomass of organic components (Murthy et al. 2014). For scale-up in bioreactors there are some strategies that include: design and operation systems, optimizing bioreactor design, improving culture strategies, and use of disposable bioreactors (Yancheva et al. 2019). Today, bioreactors are considered very valuable techniques for the production of pharmaceutical and health-cosmetic compounds in mass volume. Due to the optimization of the growth environment in bioreactors, the cells have the high ability to produce secondary metabolites, and as a result, the daily production volume in bioreactores increases, which has made bioreactors occupy a special place in the commercial production of these compounds. For example, derived shikinin (used against bacterial disease and inflammation) from roots of *Lithospermum erythrorhizon* in the traditional way and without using bioreactor be maximum of 2% of the active substance in its roots, but the root cells in the bioreactor create 23% shikonin in only 23 days (Renneberg et al. 2016). Table 1 showed examples for Rosmaric Acide (RA), reservitol and shikonin production by using cell culture in bioreactors.

Table 1. Production of Shikonin, Resveratrol and Stevioside from plants in bioreactors

Plant species	Metabolite	Culture	Bioreactor type and volume	Metabolite content mg/l	Reference
Lithospermum erythrorhizori	Shikonin	Root Cells culture	200-L Stirred bioreactor	25	Reinhard et al. 2016
Vitis labrusca L	Resveratol	Cell suspension culture	14-L Stirred bioreactor	72	Nivelle et al. 2017
Salvia officinalis L	Rosmarinic acid	Shoot culture	5-L nutrient sprinkle Bioreactor	59.04	Grzegorczyk et al. 2010
Stevia rebaudiana	Stevioside	Shoot culture	Temporary Immersion System (TIS) RITA® Bioreactor		Melviana et al. 2021

6. Extraction of secondary metabolites from callus

Organic and bioactive components in callus cultures should be extracted at a specific stage of growth cycle. Usually secondary metabolites are most abundant during the stationary phase of growth cycle. One of the methods in order to further scale-up and yield enhancement for extraction of secondary metabolites from callus is raising the callus in suspension. For this purpose, callus first is cultured in a shake-flask and then in a suitably designed bioreactor (Srivastava et al. 2020). Laboratory methods and quali-quantitative analysis required for extraction depend on the nature of metabolites (volatile or nonvolatile). For extraction of plant metabolites there are several traditional methods such as solvent based extraction, reflux, infusion, decoction, digestion, maceration, percolation hydro-distillation and enfleurage that among them solvent based extraction is more traditional methods for extraction of secondary metabolites (Wong-Paz et al. 2017). In this method selection of solvent is very important for high quality extraction. Methanol/water solutions effectively applied for extraction of semi-polar metabolites such as phenolic acids, flavonoids, alkaloids, and glycosylated sterols, while chloroform or n-hexane are more suitable for extracting carotenoids or aromatic (Bertoli et al. 2010). During the extraction process, it is important to consider some general factors like the chemical characteristics (e.g., thermolabile or pH sensitive) of the extracted secondary metabolites. During the extraction of plant metabolites, the interference between desired metabolite and co-extracted compounds should be minimized and also the metabolite should be extracted without any contamination. It should be noted that during the extraction, there is a possibility that the extracted metabolite be decomposed or a new compound be created as a result of the reaction with the extraction solvents, which must be prevented from occurring of these reactions (Jones and A. Douglas 2005). After extraction, the extract is analyzed for identification and quantification of the desired compound by HPLC, LC-MS, and other techniques. Each extraction procedure has advantages and disadvantages and the selection of a suitable method of extraction is very essential, which almost depends on the plant species and intended use of an extract.

6.1. Techniques used to enhance quantity of extracted metabolites

Investigations have shown that one of the limitations of traditional extraction methods in some cases is the low accumulation of secondary metabolites in cell cultures, which may be due to inhibition of feedback, enzymatic or non-enzymatic degradation of the product in the environment, or the volatility of the extracted compounds. To overcome that limitation, new extraction techniques are developed that are called as non- conventional extraction techniques. Some of the most developed techniques are ultrasound assisted extraction (UAE), enzyme-assisted extraction, microwave-assisted extraction (MAE), accelerated solvent extraction (ASE), pulsed electric field assisted extraction, supercritical fluid extraction and pressurized liquid extraction. Using of these techniques in secondary metabolite production,

decreased derivatives of metabolite and degradation of metabolite (Azmir et al. 2013).

Another method to increase the secondary metabolites content in plant is the using of enhancers. Enhancers are regulatory sequences that when bound by transcription factors increase the activity of a gene. So, by using genetic manipulation technique and insert of enhancer in vital gene for production of secondary metabolite can increase secondary metabolite synthesis (Bertoli et al. 2010).

7. Analytical methods for the quality control of extracted secondary metabolites

After the extraction, the quality and quantity of the extracted metabolite should be estimated. For this analysis, suitable techniques with the best selectivity, and sensitivity should be applied. Thin Layer Chromatography (TLC), High Performance-Thin Layer Chromatography (HP-TLC), Over Pressured Layer Chromatography (OPLC), High-Performance Liquid Chromatography (HPLC), Liquid Chromatography Mass Spectrometry (LC-MS), Ultra Performance Liquid Chromatography (UPLC), Gas Chromatography-Mass Spectrometry (GC-MS) and Direct Infusion (DI) are the prevalent analytical chromatographic techniques for the analysing of plant extracts (Bertoli et al. 2010). Recently developed analytical techniques provide more information about the phytochemical profiles of plant extracts. For example, ultra-performance liquid chromatography (UPLC) is a combination of a 1.7 µm reverse-phase packing material and a chromatographic system operate at 600-15000 psi pressures. This technique is very fast and high-resolution separation techniques with high-sensitivity mass instruments for the on-line identification of analytes. This has enabled better chromatographic peak resolution with widths in the order of 1-2 s for a 10 min separation. This technique in coupled to a Q-TOF mass spectrometer can be used for analysing complex mixtures (fig. 6).

Generally single analytical techniques will not provide sufficient detection of the plant metabolomic profile. Analysis of extracted compound by the classical analytical methods generally is time and solvent-consuming and almost the results aren't reproducible. Therefore, chromatographic based systems combined with new extraction techniques such as ASE, MAE are rapid and effective analytical methods in order to analysis the quality and safety of plant extracted metabolites (Bertoli et al. 2010).

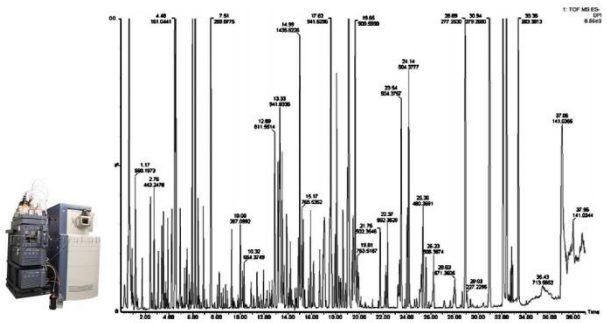

Figure 6. UHPLC-quadrupole-TOF-MS base peak ion chromatogram of combined methanol extracts from soybean and M. truncatula (Lei et al. 2011).

8. Commercially important products produced through cell culture and callus

The presence of secondary metabolites in plants has caused plants to assign a special place in the industry and medicine (fig. 7). In previous years, the extraction of metabolites at the commercial level was done only through cultivation at the field level. For example, neonicotinoid insecticides extracted from whole plant of tobacco and commercialized in 1991 (Gosta et al. 2008). But the low amount of metabolites in the plant and the effect of various environmental factors on their production content have caused various laboratory techniques, including callus and cell culture, and even the use of elicitors as stimuli to increase metabolite production. Till now a lot of bioactive secondary metabolites extracted from callus and cell suspension cultures and commercialized as a medicine or other consumptions (fig. 7, table 2).

Table 2. Bioactive metabolites extracted from callus and cell suspension cultures as a medicine

Plant species	Bioactive metabolite	Method	Applying	Reference
Taxus cuspidata	Paclitaxel	Cell culture	a chemotherapy medication used to treat a number of types of cancer	(Yamamoto et al. 2022)
Lithospermum erythrorhizori	Shikonin	Cell culture in bioreactor	used against bacterial disease and inflammation and Health-cosmetic	(Fujita et al. 1981)
Solanum elaeagnifolium	Solasodine	callus culture	functions of resisting cancer and inflammation, and relieving asthma	(Jha et al. 1988)
Cephaelis ipecacuanha	Cephaeline	callus culture	Anticancer and emetic agent	(Jha et al. 1991)
Taxus mairei	Taxol	Cell suspension	anticancer drug as a Paclitaxel (PTX) medicine	(Wu et al. 2001)
Coptis japonica	Berberine	cell suspension	protective capacities in digestive diseases	(Sato and Yasuyuki 1984)
Catharanthus roseus	vincristine and vinblastine	callus culture	Anticancer	(Iskandar 2016)
Taxus cuspidate	Toxoids	cell suspension	Antitumor	(Ketchum et al. 2003)

Figure 7. Some commercialized plant metabolites.

9. Conclusion and future prospects

The cell is the smallest and at the same time the main place for production of secondary metabolites in an organism. A collection of similar cells called callus can be considered as a green factory for the production of secondary metabolites. Generally, the content of most secondary metabolites in plants is very low and is affected by environmental and genetic variations. Optimizing the growth environment of cells or callus increases their ability to produce secondary metabolites. Therefore, selecting the appropriate cell line and optimizing the growth environment of cells in vitro and bioreactors is essential for metabolite production. Also, in order to increase the secondary metabolite production, it is necessary to have more knowledge in the field of the biosynthetic pathways of metabolites and the regulatory systems of the biosynthesis pathways. It is possible to increase the production of these

compounds or even their derivatives through the use of techniques such as metabolite engineering and manipulation of metabolic pathways. In order to commercially produce of secondary metabolites, in addition to increasing the cell's ability to produce these compounds, it is essential to use a suitable technique with high resolution and sensitivity for metabolite extraction and analysis.

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