Scale-Up Production of Bioactive Compounds Using Bioreactors

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Abstract

The increased demand for plant secondary metabolites by the pharmaceutical, food, flavor, beverage, and cosmetic industries has necessitated the rapid and mass production of these metabolites using in vitro plant culture systems. Bioreactors provided a suitable alternative to conventional plant culture by facilitating large-scale propagation of plants and production of secondary metabolites. Bioreactors proved to be effective plant culture systems which are genetically stable, low cost, easy to operate, and fully automated. Bioreactors play a very important role in medicinal plant industry and have evolved over time. At present, a variety of bioreactor configurations are available each customized for specific plant cell/tissue so that a stable optimum yield of bioactives is obtained. The chapter discusses briefly about the use of bioreactors in scaling up the production of secondary metabolites, different categories and designs of bioreactors available, factors on which bioreactor function depends, and the different crops in which bioreactor scaling up is attempted.

Keywords

Bioreactor · Secondary metabolites · Plant cell cultures

3.1 Introduction

Plants have been the only source of medicine available to human beings from time immemorial until the modern medical research and drug synthesis emerged. With the realization of the innumerable side effects that modern medicines impose on the

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T. Belwal et al. (eds.), *Nutraceuticals Production from Plant Cell Factory*,
https://doi.org/10.1007/978-981-16-8858-4_3

health conditions, there has been a gradual readoption of the plant-based medicines. As per the reports of WHO, 80% of the global population relies on medicinal herbs for their healthcare needs. Many of the modern-day drugs contain plant extracts or active ingredients as their integral part. The very famous analgesic drug, aspirin, was derived from the plant species *Salix* and *Spiraea*, and anticancer drugs like paclitaxel is derived from *Taxus* species, while antimalarial drug quinine is obtained from *Cinchona* species. It is roughly estimated that one-fourth of the modern-day drugs contain active ingredients or bioactive compounds from plants. The active ingredients or bioactive compounds are the high-value compounds which are responsible for the pharmacological activities of the plant. Plant extracts provide a vast scope of applications by virtue of the chemical diversity present in them. Bioactives have numerous industrial applications in different sectors like pharmaceutical industry, cosmetic industry, food and beverage industry, etc. The global plant extract market for phytomedicines and herbal extracts segment is the largest as well as fastest-growing and is experiencing high demand from functional food and beverages which require ingredients for preventing chronic diseases. The plant extract market is estimated to be valued at USD 23.7 billion in 2019 and is projected to reach USD 59.4 billion by 2025 (http://www.marketsandmarkets.com, 2019).

Owing to all these factors, the demand for plant extracts has increased, but the supply is scanty; the reason is because nearly 72% of the species in high demand is continued to be sourced from the wild. The unscrupulous collection from the forest resources has threatened the survival of many important medicinal species. Apart from this, the destruction of forest resources due to increased anthropogenic activities and climate change scenario has also been responsible for the dwindling of populations of many economically important species. In such a scenario, it becomes essential to find the alternate sources for the supply of plant extracts or bioactives on a large scale for meeting the commercial demands. Traditionally, secondary metabolites were obtained through different extraction procedure using whole plants and tissues which again depend on the type of species, age of plant, plant part used, etc. Later on, with the advent of biotechnological methods like tissue culture techniques, large-scale in vitro plant cultures were used for the production of secondary metabolites. Findings from a study that anthraquinone synthesis occurred from undifferentiated cells of *Morinda citrifolia*, it was proved that both differentiated as well as undifferentiated cells can be used for production of secondary metabolites in plant cell cultures. Thus, the in vitro cultures for bioactive synthesis may be either callus cultures, cell suspension cultures, and/or organ cultures. Organ cultures such as root and shoot cultures were found to be better for the production of secondary metabolites as compared to undifferentiated cultures like callus and suspension cultures. In vitro culture of whole plant organs, viz., root and shoot cultures, was attempted in many medicinal plants for the production of secondary metabolites (Biondi et al. 2002). Analysis of secondary metabolites in organ cultures and intact plants showed that there was no difference with respect to quality and quantity of metabolites obtained. The added advantage of organ cultures is that they are less sensitive to shear stress and relatively more stable in the production of bioactives as compared to undifferentiated cells (Bais et al. 2002).

Plant roots are also active sites of secondary metabolite synthesis and hence contribute as a potent explant for bioactive production in tissue culture. Hairy roots induced by *Agrobacterium rhizogenes* shows a high productivity of secondary metabolites and are yet another valuable source of phytochemicals used in pharmaceuticals.

Plant cell cultures are routinely used for the production of valuable bioactives by the pharmaceutical, flavoring, as well as fragrance industry. Around 20,000 chemical metabolites are produced in plants including both primary and secondary chemicals, and it is reported that every year about 1600 new plant chemicals are discovered (Sajc et al. 2000). Paek et al. (2005) described that in vitro cultures for secondary metabolite production were advantageous in terms of different factors like:

- Mass production of metabolites in short period of time.
- Ensures regular availability of bioactives independent of season and plant availability.
- Controlled and sterile environments with well-defined production system ensuring high yield with consistent quality.
- Prevention of abiotic and biotic stresses.

Considering the benefits of in vitro cultures, researchers implemented the automation of in vitro propagation using bioreactor system, thereby reducing the cost and labor for mass production of secondary metabolites. Bioreactor systems are particularly useful for species:

- Which is difficult to cultivate.
- Which has a long cultivation period.
- Produces commercially important metabolite which cannot be synthesized chemically.
- Which naturally produces only small quantity of metabolite of interest.

Bioreactor system is more advanced and efficient as compared to conventional tissue culture system as the bioreactor process is completely automated and allows for the manipulation of culture condition such as temperature, pH and dissolved oxygen, carbon dioxide, and nutrients at any point of time. The process of continuous aeration, mixing, and circulation of media also enhances the nutrient uptake by the cells and increases their proliferation (Ibrahim 2015).

3.2 Bioreactors

Bioreactors refer to large vessels with controlled and sterile environments containing liquid medium for culturing cells, tissues, or organs intensively with the purpose of mass multiplication or mass production of certain bioactives produced by these cells. Bioreactor systems mostly use liquid culture mediums for mass production of various plant cells and tissues. These systems were developed initially for mass

multiplication of microbes, but now it's applied to plants for rapid mass propagation of plant species which are difficult to propagate by conventional means and also for production of secondary metabolites. The objective of bioreactor system is to provide the optimum physical and chemical conditions for obtaining maximum yield and quality of explants and at the same time to keep minimum production cost by integrating automated facilities and simple low-cost devices (Preil 2005).

Three types of cultures are used in the bioreactor system as below:

- Cultures for mass propagation of planting material.
- Cultures for mass production of secondary metabolites and enzymes.
- Cultures used for biotransformation of exogenously added metabolites (which may be precursors in a metabolic pathway).

Bioreactor systems ensure optimal growth and biochemical functions of plant cell to synthesize bioactive compounds. The advantages of bioreactor system involve:

- It allows scaling up of bioactive production in suspension cultures under controlled conditions.
- It reduces manual handling of cultures, thus saving labor, time, and energy.
- Regulation and manipulation of culture conditions can be done at different stages of bioreactor.
- The system is easy for accessibility like inoculation or harvesting of cultures.
- Submerged culture conditions will enhance the nutrient absorption by the cells and thereby increases the secondary metabolite production. Large numbers of plantlets are easily produced and can be scaled up.

Bioreactor technology is already in place for mass propagation of many medicinal species like *Rauwolfia serpentina*, *Chlorophytum borivilianum*, *Bacopa monnieri*, *Swertia chirayita*, etc. However, with respect to bioactive production from medicinal plants, in spite of the enormous advantages, commercial scale production is achieved only in Japan in few species like *Lithospermum erythrorhizon* (for shikonin), *Panax ginseng* (for ginsenosides), and *Berberis aristata* (for berberine) (Bourgaud et al. 2001).

3.3 Bioreactor Design

First bioreactor system for plant propagation was developed for in vitro mass propagation of *Begonia* × *hiemalis* (Takayama and Misawa 1981). Since then lot of bioreactor systems have been developed for different types of plant species. Standardizing a bioreactor system for plant propagation or secondary metabolite production is an interdisciplinary research which includes study of plant biotechnology, plant physiology, biochemistry, molecular biology, pharmacology, and engineering. Understanding all the complex processes involved is important to assess the tissue morphology, bioreactor conditions in terms of oxygen availability, heat

transfusion, agitation, mixing, kinetics of cell growth and metabolite production, control of culture environment, and the potential for process scale-up. The overall objective of bioreactor system is to develop an optimum protocol to achieve high productivity. The design and development of bioreactors depend on the purpose as well as the different engineering parameters like oxygen requirement, shear resistance, controlled physical and chemical environment, and amenability for scaling up. The factors affecting the metabolite production in bioreactors include: the gaseous atmosphere, oxygen supply and CO2 exchange, pH, minerals, carbohydrates, growth regulators, the liquid medium rheology, and cell density.

The basic requirements of bioreactor system are as follows:

- Provision for cell-to-cell contact.
- Provision for homogenous mixing with minimal shearing and sufficient aeration.
- Provision for effective heat transfer.
- Provision for adequate dispersion of air and gas.
- Should avoid segregation of substrates.
- Provision for measuring various growth and yield parameters.
- Provision for scaling up of bioactive production.
- Provision for sterile and stable environment.
- Provision for easy handling and maintenance.
- Should minimize all possibilities of contamination.

Bioreactors are classified into different categories based on certain criteria; one among them is based on agitation method. Based on agitation, bioreactors are fundamentally classified as mechanically agitated, pneumatically agitated, and non-agitated types. Mechanically agitated bioreactors include stirred tank bioreactor, rotating drum bioreactor, and spin filter bioreactor. Pneumatically agitated bioreactors comprise of simple aeration bioreactor, bubble column bioreactor, airlift bioreactor, and balloon-type bioreactor. Non-agitated bioreactors include gaseous phase bioreactors, oxygen permeable membrane aerator bioreactors, and overlay aeration bioreactors.

Mechanically agitated bioreactors use impellers or magnetic stirrers or vibrating perforated boards for aeration, mixing, and circulation; on the other hand, in pneumatically agitated bioreactors like airlift bioreactors, aeration and mixing are done by the inserted gas which is entering though the side or basal inlets via airlift pumps. The entering air will lift the plant biomass and provide sufficient oxygen (Ziv 2005). Mechanically agitated bioreactors have higher energy consumption, and tall bioreactors have sealing issues and are more prone to cell damage and shearing, whereas airlift bioreactors are advantageous in terms of its simple design, low shearing, high gas and nutrient transfer rates, low electricity consumption, and relatively high yields (Denchev et al. 1992). Since airlift bioreactors don't have any moving parts, the maintenance time and cost is also less. The disadvantages of this system include excessive foam formation and growth of cells at the top which can be avoided by using antifoams and wide top bioreactors or bubble bioreactors (Paek et al. 2001).

Recently, novel bioreactor systems based on the extent and period of immersion of explants or cells in culture media have emerged which have been categorized as continuous immersion and temporary immersion bioreactors. In continuous immersion system (CIS), the explant material is continuously in contact with the liquid medium. Stirred tank, airlift, and balloon bioreactors are examples of continuous immersion system. The limitations associated with this system are that continuous immersion causes hyperhydricity and malformations because of insufficient oxygen availability for the submerged tissues. The deficiency in oxygen availability induces oxidative stress producing reactive oxygen species causing injury to the plant tissue. To avoid this problem, oxygen should be provided either by agitation or by aeration or by exposing a part of the explant to air. This has led to the development of temporary immersion system (TIS) in which there is only temporary contact between the plants and the liquid medium, thus avoiding continuous immersion and providing adequate oxygen transfer to the cultures. The working principle of TIS system is to prevent complete immersion using different ways like using separate sides of culture vessels or keeping the explant above a platform. The medium comes in contact with the plant material for specific period of time and then the medium gets back to the storage tank. The whole process is controlled by electronic system. Bioreactors employing TIS system involve different types like:

- RITA®, Plantform™, and Rocker bioreactors: Explant material is kept separate from the liquid medium in the same culture vessel but in different zone or different compartments. Explant material is kept above the medium using different support materials like nets, glass beads, etc. The medium comes in contact with the material by the force of filtered air pumped at specific intervals in RITA® and Plantform™ systems and by mechanical movement of the vessel in the case of rocker bioreactors. Plantform™ systems are especially useful for plants that are more prone to hyperhydricity. The optimization of protocols in terms of immersion duration and frequency needs to be standardized for the species of interest.
- Two flask system: Explant material is kept in one vessel and medium is present in a separate vessel. Here also the explant material comes in contact with the medium by the driven force of filtered air pumped at specific intervals.

Plants cultured by TIS generally show increased vigor and better quality than those grown completely submerged in liquid medium or conventionally in semisolid medium.

Based on the nature of continuous phase used, bioreactors are classified as liquid-phase bioreactors, gas-phase bioreactors, and hybrid bioreactors.

Liquid-Phase Bioreactors In this bioreactor system, plant cells will be immersed in the liquid medium continuously, and oxygen will be given by passing air through the culture medium. Mechanically driven bioreactor, pneumatically driven bioreactor, and hydraulically driven bioreactor belong to this category. The limitation of liquid-phase bioreactors includes low solubility of gases, reduced availability of nutrients, growth inhibition, etc.

Gas-Phase Bioreactors Gas-phase bioreactors were introduced for very sensitive plant tissues like hairy roots which get damaged by other bioreactor system like stirred tank system. Gas-phase bioreactors include mist or spray technology in which the roots are exposed to nutrient mist/spray as in aeroponics. In these bioreactors, oxygen availability is not limited even at high culture density and also resistant to shearing. Studies also showed that the production of secondary metabolites is often greater in mist bioreactors than in liquid-phase reactors.

Hybrid Bioreactors These bioreactors are a combination of liquid-phase and gas-phase bioreactors. In this system, there occurs a shift from liquid to gaseous phase after the inoculation, distribution, attachment to immobilization points, and short growth phase of the cells. An example is the Wilson bioreactor for hairy root cultures.

Table 3.1 and 3.2 depicts the different types of bioreactors used in different medicinal species for secondary metabolite production and hairy root cultures.

3.4 Factors Affecting Bioactive Production in Bioreactors

3.4.1 Ventilation and Availability of Dissolved Oxygen

Regeneration capacity as well as viability of plant tissues in the culture mostly depends on the ventilation as well as availability of oxygen in the liquid medium. Bioreactor conditions have to be optimized with respect to agitation speed and aeration rate, gas mixing, etc. so that effective oxygen diffusion occurs from the gaseous to the liquid phase. The presence of sufficient oxygen in the liquid media is important for the growth as well as mass production of cells or tissues.

3.4.2 Mixing of Liquid Medium

In order to facilitate uniform distribution of nutrients to all the cells in the liquid media, there should be proper mixing of the medium. The mixing can be done either mechanically or pneumatically depending on the type of the bioreactor, but care should be taken to ensure that there is no cell or tissue damage happening during mixing and at the same time cells are getting enough nutrients.

3.4.3 Medium pH

The pH of the liquid media determines the mass production of cells, tissues, and thereby bioactive production in bioreactor systems. Studies have shown that liquid medium of pH 5 inhibits embryogenesis of cultures. pH of the medium also affects the availability of nutrients to the plant tissues.

Table 3.1 Different types of bioreactors used in different plant species for secondary metabolite production

Bioreactor type	Secondary metabolite	Plant species	Explant type	References
Shake flask system	Hypericin	*Hypericum perforatum* L.	Adventitious root	Cui et al. 2011
Airlift bioreactor system	Caftaric acid, chlorogenic acid, cichoric acid	*Echinacea purpurea* (L.) Moench.	Adventitious roots	Jeong et al. 2009
Airlift bioreactor system	Anthraquinones, phenolics, flavonoids	*Morinda citrifolia* L.	Leaf cells	Ahmed et al. 2008
Shake flask system	Anthocyanin dyes	*Melastoma malabathricum* L.	Callus cell cultures	Chan et al. 2010
Balloon-type bubble bioreactors	Ginsenosides	*Panax ginseng* Meyer	Adventitious roots	Smolenskaya et al. 2007
Shake flask system	Human serum albumin	*Oryza sativa* L.	Transgenic rice seeds	He et al. 2011
Shake flask system	Ginsenoside	*Panax japonicus* C.A. Mey. Var. repens	Callus cell cultures	Smolenskaya et al. 2007
Nutrient sprinkle bioreactor	Rosmarinic acid	*Dracocephalum forrestii* W. W. Smith	Shoot cultures	Weremczuk-Jeżyna et al. 2019
Stirred tank bioreactor	Resveratrol	*Vitis labrusca L*	Suspension culture	Chastang et al. 2018
Stirred tank bioreactor	Anthocyanin	*Vitis vinifera (L.) cv Gamay Fréaux var. Teinturier*	Suspension culture	Aumont et al. 2004
Nutrient sprinkle bioreactor	Caffeic acid	*Dracocephalum forrestii W.W. smith*	Shoot cultures	Weremczuk-Jeżyna et al. 2019
Nutrient sprinkle Bioreactor	Verbascoside	*Scutellaria alpina*	Shoots	Grzegorczyk-Karolak et al. 2017

3.4.4 Availability of Nutrients

Nutrients are the major chemical factors which determine the productivity of tissue culture systems in bioreactors. Nutrients have to be provided regularly at periodic intervals based on the amount of nutrient removed by the cultures in order to get sustained yields from bioreactors.

Table 3.2 Bioreactors used for hairy root cultures

Bioreactor type	Volume (l)	Plant species	Secondary metabolite	References
Stirred tank bioreactor	12	*Datura stramonium*	Tropane alkaloids	Hilton and Rhodes 1990
Stirred tank bioreactor	25	*Atropa belladonna*	Tropane alkaloids	Lee et al. 1999
Stirred tank bioreactor	2.0	*Panax ginseng*	Saponins	Inomata et al. 1993
Bubble column bioreactor	2.0	*Hyoscyamus muticus*	Tropane alkaloids	Cuello et al. 1991
Airlift bioreactor	1.0	*Catharanthus roseus*	Indole alkaloids	Toivonen et al. 1989
Nutrient sprinkle bioreactor		*Salvia officinalis* L	Rosmarinic acid	Grzegorczyk and Wysokinska 2011
Airlift bioreactor	2.0	*Duboisia leichhardtii*	Scopolamine	Muranaka et al. 1992
Airlift bioreactor	1	*Coleus blumei* L.	Rosmarinic acid	Bauer et al. 2015
Airlift bioreactor	1.5	*Nicotiana rustica*	Nicotine	Rhodes et al. 1986
Trickle bed bioreactor	2.0	*Hyoscyamus muticus*	Tropane alkaloids	Flores and Curtis 1992
Nutrient mist bioreactor	9	*Mucuna pruriens*	L-DOPA	Huang et al. 2004
Nutrient sprinkle bioreactor	5	*Leonurus sibiricus* L.	Caffeic acid	Sitarek et al. 2018

3.5 Case Studies of Medicinal Plants

3.5.1 *Catharanthus roseus*

This plant produces a very important compound—ajmalicine—in its leaves which has got antihypertensive property and also is used in the treatment of circulatory diseases. The low availability of the compound in the plant necessitated alternative production systems for the rapid and mass production of ajmalicine. The bacterium-induced hairy roots were considered as a better alternative for in vitro production of ajmalicine. Mass cultivation of hairy roots was carried out in different bioreactor systems like bubble column, rotating drum, modified bubble column, etc. Bioreactor conditions in terms of inoculum size and density, aeration rate, agitation speed, etc. were standardized. The yield of ajmalicine was only 4.6 ± 0.4 mg/l in rotating drum bioreactor, whereas it gave 34 ± 2.3 mg/l ajmalicine in shake flask bioreactor. The yield was still higher in a modified bioreactor where the hairy roots were anchored to a polyurethane foam (Thakore et al. 2017).

3.5.2 *Panax ginseng*

Panax ginseng is valued for the saponins—ginsenosides—present in its roots which is responsible for the pharmacological activity. Cultivation of this plant being time consuming and laborious needed some alternate sources of ginsenosides. Large-scale biomass production of ginseng was achieved on industrial scale by Nitto Denko Corporation (Ibaraki, Osaka, Japan) in big dimension stirred tank bioreactors. Stirred tank and airlift bioreactors were commonly used for the suspension cultures. In stirred tank bioreactor system for ginseng, it was found that cell growth and ginsenoside production was affected by agitator design and the agitation rate (Furuya et al. 1984). Hairy root cultures were also tried by many workers for rapid saponin (Yoshikawa and Furuya 1987; Yu et al. 2000), but these hairy roots were observed to be lethal to mammalian cells. Therefore, adventitious root production was attempted for the production of secondary metabolite, and it was found successful (Yu et al. 2001), and ultimately balloon bubble bioreactor system was used for the scaling up of adventitious root production (Yu et al. 2000, 2001).

3.5.3 *Artemisia annua* L

Artemisinin obtained from the leaves of *Artemisia annua* is used for the treatment of cerebral malaria along with quinine and chloroquine. The availability of this bioactive is very limited in the plant accounting to only 0.01–0.5%; as a result only 6 kg of artemisinin can be obtained from 1000 kg dry plant leaves. This necessitated the in vitro production of artemisinin using plant cell and hairy root cultures. Different bioreactor systems have been tried in *Artemisia annua* for artemisinin production. It was found that artemisinin production was highest in mist bioreactors as compared to other systems (Patra and Srivastava 2014). The yield of artemisinin was 0.025 mg/l (Kim et al. 2003) and 0.088 mg/l (Xie et al. 2000), respectively, in bubble column type and shake flask bioreactors, whereas a higher artemisinin content of 0.031 mg/l was observed in nutrient mist bioreactors. It was also found that the genes responsible for artemisinin synthesis are overexpressed during scale-up of the cultures in bioreactor (Souret et al. 2003).

3.5.4 *Bacopa monnieri*

Bacopa monnieri yields the saponin bacoside which has got the pharmacological memory boosting property. In vitro production of bacoside has been reported by several workers from differentiated as well as undifferentiated tissues. Liquid medium for culturing *B. monnieri* is standardized by Ahuja et al. (2016). Shake flask bioreactor, Growtek®, and modified bench top air agitated bioreactors were used for shoot cultures of *Bacopa monnieri*. Air agitated bioreactor produced more biomass (169.67 g) as compared to Growtek® (23.17 g) and shake flask (15.70 g) bioreactors. Bacoside production was three times higher in the air agitated bioreactor

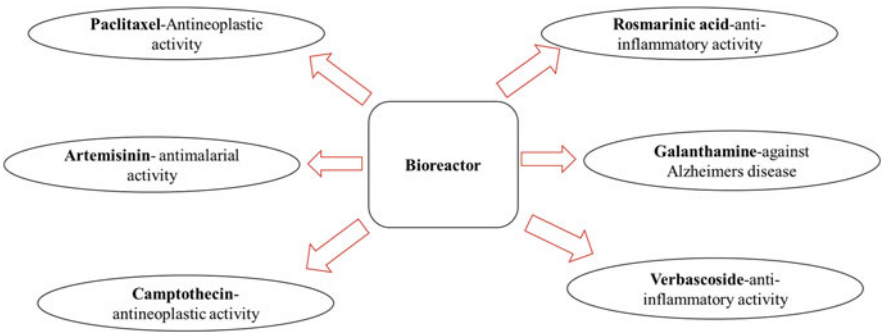

Fig. 3.1 Selected examples of high-value bioactives produced by plant cell/organ culture in bioreactors

than shake flask system. These findings suggested that bioreactor system is advantageous for *B. monnieri* shoot proliferation and bacoside production. Figure 3.1 shows high value compound which are commercially produced from few of the medicinal crops.

3.6 Conclusions and Recommendations

Plant bioreactors have turned out to be the most suitable system for the industrial production of plant secondary metabolites on a large scale. Most of the bioreactors utilize liquid culture media for the optimum growth of cultures and production of secondary metabolites. An efficient bioreactor system should provide a better control of the contact of the plant tissue with the culture medium and optimal nutrient and growth regulator supply, as well as aeration, medium circulation, the filtration of the medium, and the scaling up of the cultures. Better understanding of different metabolic pathways governing the biosynthesis of bioactive compounds is essential for developing a successful bioreactor system. Efforts should be made in the direction of shifting the bioreactor research from the laboratories to the commercial level so as to meet the trade demands of plant extracts in the international market worldwide.

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