Chapter 15

Plant Cell Cultures:
Bioreactors for Industrial Production

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Abstract

The recent biotechnology boom has triggered increased interest in plant cell cultures, since a number of firms and academic institutions investigated intensively to rise the production of very promising bioactive compounds.

In alternative to wild collection or plant cultivation, the production of useful and valuable secondary metabolites in large bioreactors is an attractive proposal; it should contribute significantly to future attempts to preserve global biodiversity and alleviate associated ecological problems. The advantages of such processes include the controlled production according to demand and a reduced man work requirement.

Plant cells have been grown in different shape bioreactors, however, there are a variety of problems to be solved before this technology can be adopted on a wide scale for the production of useful plant secondary metabolites. There are different factors affecting the culture growth and secondary metabolite production in bioreactors: the gaseous atmosphere, oxygen supply and CO_2 exchange, pH, minerals, carbohydrates, growth regulators, the liquid medium rheology and cell density. Moreover agitation systems and sterilization conditions may negatively influence the whole process.

Many types of bioreactors have been successfully used for cultivating transformed root cultures, depending on both different aeration system and nutrient supply. Several examples of medicinal and aromatic plant cultures were here summarized for the scale up cultivation in bioreactors.

Introduction

Plants produce several different secondary metabolites, called phytochemicals mostly of them used as pharmaceuticals. In recent years biopharmaceutical/nutraceutical industry renewed increased attention in production of health-promoting secondary metabolites using plant cell and tissue cultures. Different efforts to improve their productivity had limited success, especially due to the lack of suitable technologies for such scale-up applications.

The production of in-vitro secondary metabolites can be possible through plant cell cultures.[1] This technology represents a good model to overcome many problems linked to the conventional agriculture such as variations in the crop quality due to environmental factors: drought, flooding and other abiotic stresses and/or biotic stresses as diseases or pest attacks. Moreover crop adulteration, losses in storage and handling may decline the secondary metabolites production, which cannot be prevented by inability of some authorities.

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In order to optimize secondary metabolite production in in-vitro plant cultures the following strategies have been evaluated:

1. Establishment of cell suspension cultures of plant with a content of required phytochemicals,
2. Selection of highly productive clones,
3. Optimization of culture conditions.

Successful establishment of cell lines able to produce high yields of secondary compounds in cell suspension cultures has been reported first by Zenk.[2]

The accumulation of secondary products in plant cell cultures depends on the composition of the culture medium and on environmental conditions.[3,4]

For the production of secondary metabolites at commercial level some prerequisites have also to be considered, like: high demand, high product costs, uniform availability of raw material and technology cost inputs, otherwise the production will not be effective and commercially possible.

As consequence of these considerations, after the demonstration of desirable product presence, at reasonable levels, in plant cell cultures, we have to consider the problems of scale up from small (about 5 liters) to large vessels. Large volume automated culture vessels, called fermenters, have been successfully used to produce cultured plant cells and many bioreactor designs have been used for mass cell growth.[5]

The present chapter reviews various aspects of large scale plant cell and tissue cultures and the bioreactor systems, focusing the attention on some examples of medicinal and aromatic plant cultivation for secondary metabolite production.

Historical Background

For large scale cultures, the first success was obtained in 1960 by culturing cells of various plant species in a 134 liter bioreactor.[6]

In 1977, the culture of tobacco cells was obtained in a 20 L tank. In 1984, the first industrial production of shikonin (natural compound used as a dye and a medicinal compound) was performed in 750 L bioreactor by *Lithospermum erythrorhizon* cells in Japan. Later a German company started with the taxol production, an anticancer compound, using *Taxus* cell cultures with bioreactor capacities up to 75 L.[7]

However, this technology is still being developed and despite the advantages outlined above, there are a variety of problems to be overcome before it can be adopted on a wide scale for the production of useful plant secondary metabolites. The success of Mitsui Petrochemical Industry Co. Ltd. in Japan in producing shikonin on a commercial scale from *Lithospermum erythrorhizon* cultivations and that of Nitto Denko Co. Ltd. also in Japan in mass production of *Panax ginseng* or ginseng cells using 20 kL tanks and the other examples described above demonstrate that many of the problems can be overcome with perseverance. The economic feasibility of these processes is related to the value of the considered metabolite. It is possible to produce food additives, metabolites with pharmacological value, antioxidants, aromes, organic acids, aminoacids, vitamins and polisaccharides or secondary metabolites such as phenols, flavonoids, terpenoids, etc.[8]

There are known some significant differences between microbial and plant cell cultures that must be considered when attempting to apply plant cell cultures to the available technology. Table 1 shows a comparison of the characteristics of plant and microbial cultures during fermentation.

Large-scale cell culture production has been in part limited by the large size, rigid cell wall and extensive vacuole of plant cells, making them sensitive to shear stress.[9] In fact with normal blade impellers the cells may twist and can be broken triggering the cell death with the loss of entire cell culture. Thus, air-lift fermentors are recommended by some researchers. The low aeration requirement for plant cells is an advantage over microbial cultures in general. Furthermore the large size of the plant cells allows to extend their duplication time in comparison with the microbial ones, determining a prolonged period for a successful fermentation time. Other differences regard the product accumulation, since in plant cells they are stored in vacuoles, meanwhile microorganisms secrete the biosynthesised products in the medium. Thus, squeezing is necessary to obtain high metabolite yields from plant cell cultures, with the consequence of cells replenishment.

Table 1. Characteristics of microbial and plant cells during fermentation

Characteristics	Microorganisms	Plant Cells
Size	<2 μm	>10 μm
Shear stress	Insensitive	Sensitive
Water content	75%	>90%
Duplication time	<1 hour	Days
Aeration	1-2 vvm	0.3 vvm
Fermentation time	Days	Weeks
Product accumulation	Medium	Vacuole
Production phase	Uncoupled	Often growth-linked

Undifferentiated plant cells cultures often produce reduced quantities and different profiles of secondary metabolites in comparison with the intact plant. The poor production is attributed to a lack of differentiation in cultures. These features may change during the growth curve. On the other hand, there are cases of cultures that over-produce metabolites compared with the whole plant (Table 2).

The culture of undifferentiated plant biomass can be obtained in semisolid agarized culture (callus) and in liquid culture (cell suspensions). The non-organised cultures might show genetic instability such as poliploidy that generally increase during the culture time.

Table 2. Secondary metabolites produced in high levels by plant cell cultures

		Yields (% Dry Wt)		
Compound	**Plant Species**	**Culture**	**Plant**	**Culture Type***
Shikonin	*Lithospermum erythrorhizon*	20	1.5	s
Ginsenoside	*Panax ginseng*	27	4.5	c
Anthraquinones	*Morinda citrifolia*	18	0.3	s
Ajmalicine	*Catharanthus roseus*	1.0	0.3	s
Rosmarinic acid	*Coleus blumei*	15	3	s
Ubiquinone-10	*Nicotiana tabacum*	0.036	0.003	s
Diosgenin	*Dioscorea deltoides*	2	2	s
Benzylisoquinoline Alkaloids	*Coptis japonica*	11	5-10	s
Berberine	*Thalictrum minus*	10	0.01	s
Berberine	*Coptis japonica*	10	2-4	s
Anthraquinones	*Galium verum*	5.4	1.2	s
Anthraquinones	*Galium aparine*	3.8	0.2	s
Nicotine	*Nicotiana tabacum*	3.4	2.0	c
Bisoclaurine	*Stephania cepharantha*	2.3	0.8	s
Tripdiolide	*Tripterygium wilfordii*	0.05	0.001	s

*s: suspension; c: callus.

It is possible to induce the undifferentiated tissue (callus) by hormonal treatments from fragments of in vitro or in vivo plant tissues (leaf, petiole). Normally auxines as Naphthaleneacetic acid (NAA) or 2,4 dichlorophenoyacetic acid (2,4D) were used at different concentrations and combined with light supply and temperature.[10] The juvenility of the starting plant material has a great importance in the callus induction: the best callus can be produced from young tissues.

Callus must be friable for cell culture development, i.e., it can be easily disaggregated in liquid medium. In the first week of culture the suspension is constituted by cells aggregates with different size. Then a filtration of this material is necessary to select small clusters and single cells for starting a synchronised controlled growth.[11]

Protocol optimisation for scaling up the biomass is a precompetitive method for the extraction of suitable amounts of secondary metabolites and for industrial production, after an economical evaluation of the system.

The Plant Bioreactors

Type of Bioreactors

As the bioreactor is a physical/thermal system for maintenance of cells at the best culture conditions for a fast growth, several models can be considered as bioreactors, starting from simple close vessels that can be externally agitated up to complex aseptic systems controlled and regulated by appropriate software.[12]

Tulecke and Nickel[13] firstly developed a successful 10 L system in a sample carboy for the cultivation of plant cells. For use at laboratory scale, bioreactor is a large culture vessel made up of glass (up to 10 L) but large scale reactors are made up of stainless steel. These are fitted with a microprocessor control unit for the control of pH, dissolved oxygen, gas flow rate, agitation speed, nutrient factors, temperature inside the vessel and cell density for optimal growth and/or production. For sterilization procedures small reactors can be autoclaved, while commercial scale reactors are sterilized in situ by passing steam at appropriate pressure.

Secondary metabolites produced in plant cells are either released into medium or accumulated in the cells. Thus the spent medium or the biomass is harvested after suitable incubation period for the extraction of bioactive compounds. Sometimes different media for growth and production are used to obtain maximum secondary metabolite production.

Cell growth and secondary metabolite production represent the main factors for selecting the suitable process mode. In fact if growth and production occur simultaneously, a process mode which supports growth of cells over an extended period should be chosen; otherwise if product synthesis follows a period of rapid growth, an appropriate process mode is selected for its ability to maintain cultures in a slow-growing rate and to retain its productivity. It is imperative to note that a bioreactor is used when basic studies related to optimization of product yield have been completed.

Different process modes are used, such as batch culture, fed batch culture, rapid feed batch culture, two-stage batch culture and continuous culture (Fig. 1).

Factors Affecting the Growth in Bioreactor

In order to manage the biomass growth in bioreactors, various culture conditions must be controlled, i.e. the oxygen supply and CO_2 exchange, pH, minerals, carbohydrates, growth regulators, the liquid medium agitation and cell density.[14,15] A complete review on this topic[16] reported several aspects relating to the gaseous atmosphere first in the culture vessels and then related to the bioreactor environment. In vessels the aerial part is composed mainly of nitrogen (78%), oxygen (21%) and carbon dioxide (0.036%). The culture vessel gas composition is influenced by the volume of the vessel and the extent of ventilation. Plants evolve CO_2 and consume O_2 during respiration, while during photosynthesis CO_2 is used and O_2 is produced. If photoautotrophic conditions persisted in the plant cell culture, CO_2 levels increased during the dark period, while they decreased during the light period. Ethylene, ethanol, acetaldehyde and other hydrocarbons are additional components of the gaseous atmosphere in vitro. Most

Figure 1. Scale up of *Salvia cinnabarina* hairy root culture in bioreactor (Applikon®, Germany).

of the effects of CO_2, O_2 and C_2H_4 on plant growth in vitro were reported for agar-gelled or cell suspension cultures.[17]

In bioreactors, the control of the gaseous phase depends on the gas flow and can be easily manipulated to provide the required levels of O_2, CO_2 and C_2H_4. The effect of aeration in *Linum album* in cell suspension culture in a 5 L stirred tank bioreactor equipped with low shear Setric impeller was reported to be important for particular compound production: 6-methoxypodophyllotoxin production was enhanced when cells were cultivated at 30% dissolved oxygen level.[18]

Oxygen. The amount of O_2 in bioreactor depends on the presence of O_2 in the gas phase above and in the air bubbles inside the medium, as well as on the dissolved O_2 in the medium. Air is released through a sparger located at the base of the bioreactor. The available oxygen for plant cells in liquid cultures, determined by oxygen transfer coefficient (kLa values), is the part that dissolves in water. Its depletion as a function of the metabolic activity of the growing cell biomass can affect the culture yield. Plant cells have a lower metabolic rate than microbial cells and a slow doubling time and therefore require a lower O_2 supply. In general, high aeration rates appear to reduce the biomass growth.[19]

The requirements for O_2 may vary from one plant species to another and must be supplied continuously to provide adequate aeration, since it affects metabolic activity and energy supply as well as anaerobic conditions. The level of O_2 in liquid cultures in bioreactors can be regulated by agitation or stirring and through aeration, gas flow and air bubble size. The use of a porous irrigation tube as a sparger generated fine bubbles, high kLa values, low mechanical stress and provided a high growth rate.[20]

High aeration rates were found to inhibit cell growth in cell suspensions cultured in airlift bioreactors. This result was explained to be due to an effect of "stripping" of the volatiles produced by the plant cells, which are apparently necessary for cell growth.[21]

Increasing O_2 levels from 21% to 80% in bioreactor cultures of Boston fern clusters enhanced growth values from 0.61 to 0.92. The growth value (GV) was calculated as the difference between the fresh weight at the end of the growth period (FW_1) and the initial fresh weight (FW_0) divided by the initial fresh weight $GV = (FW_1 - FW_0)/FW_0$. Reducing O_2 levels to 10% (v/v) affected cell differentiation in bioreactor cultures of carrot embryogenic tissue.[30]

CO_2. The effects of CO_2 were reported for both agarized cultures and cell suspension cultures used for secondary metabolite production.[17,22,23]

The contribution of CO_2 supply during the proliferation and multiplication stage in media supplied with sucrose in bioreactors is not clear. It is implicit that if photoautotrophic conditions do not prevail, CO_2 enrichment beyond the 0.36% in the air supply is unnecessary. It is reported that high aeration rates rather than excessive oxygen inhibit growth and this reduction could be due to depletion of CO_2 or to the removal of various culture volatiles including CO_2.[24,25] The requirement for CO_2 was not related to photosynthesis but to some other metabolic pathways involved in amino acid biosynthesis.

Ethylene. Ethylene is produced by cell suspension; generally the level of ethylene in the headspace in liquid cultures in flasks differs from that in continuously aerated bioreactor cultures. High rates of aeration, which are often required at high biomass densities, can cause "stripping" of volatiles that are apparently important for some plants grown in culture. In clusters of *Brodiaea* cultured in liquid medium, ethylene had no effect on growth, although its level was reduced in the presence of silver thiosulfate (an inhibitor of ethylene action). The level of ethylene was reduced from 0.38 to 0.12 ml/L in highly aerated bioreactor cultures of this plant material without affecting biomass growth.[26]

As another example, growth of *Thalictrum* cell suspension cultures was suitable in airlift system. At high cell density for berberine production gas-stripping also played a significant role and it was discovered that CO_2 and ethylene were important for product formation. By supplying a mixture of CO_2 and ethylene into the airlift system, the specific berberine content was increased two fold.[25]

Mineral Nutrients. Media with various modifications in the inorganic and organic constituents of Murashige and Skoog medium (MS).[27] are used for most plant species in agar-gelled or liquid cultures in vitro. The availability of mineral nutrients depends on the type of culture, whether agar-gelled or liquid, the type and size of the plant biomass and the physical properties of the culture. Factors such as pH, temperature, light, aeration, concentration of minerals, the medium volume and the viscosity of the medium determine the rate of absorption of the various nutritional constituents.[28,29] Plant cells growing in liquid cultures are better exposed to the medium components and the uptake and consumption are faster. In bioreactors, in which either humidified air or condensers are used to prevent dehydration, the level of the nutrients in the medium is affected mainly by the absorption rate and by cell lysis.[30] A decline in pH (lower values than 4.5) with subsequent increase to pH 5.5 was often attributed to the initial utilization of ammonium (NH_4^+), followed by a later uptake of nitrate. In several species the depletion of NH_4^+ is the first limiting factor of biomass growth.[16]

Other limitations of growth are due to the availability of phosphate, nitrogen and carbohydrates and to a lesser extent to calcium, magnesium and other ions.[31]

Mixing of dissolved nutrients of the culture medium is generally not a problem in suspension cultures. But in presence of aggregates serious problems can be found, since cells adhered to the surface of the tank at the bottom of the bioreactors.

Carbohydrates. Cultured plants require a constant supply of carbohydrates as their source of energy. Sucrose and to a lesser extent glucose, fructose, or sorbitol are the most commonly used carbohydrates in vitro. In general, sucrose is removed rather rapidly from the medium and after 10-15 days it can be completely depleted or reduced to 5-10 g/L from an initial level of 30 g/L in both agar-gelled and liquid cultures. At the same time, glucose and fructose (derived from sucrose

hydrolysis) appear in the medium and can reach levels of 5-10 g/L *Catharanthus roseus* cell suspensions cultured in a column airlift bioreactor showed a lag phase of 5 days, during which there was a total hydrolysis of the sucrose to glucose and fructose.[21] In suspension cultures of alfalfa, sucrose also was hydrolyzed during the first 5 days. Most of the sugars uptake occurs after day 5 and glucose is taken up preferentially over fructose.[32]

pH. The initial pH in most plant cell cultures ranges between 5.5-5.9. Since most media are not buffered, changes occur during autoclaving and during the biomass growth. A rapid drop in pH to 4.0-4.5 took place within 24-48 h in cell suspension, organ and embryogenic cultures.[33-35] These changes were related to an initial ammonium uptake and acidification due to cell lysis. However, the pH increased after a few days and reached a stable level around pH 5.0-5.5, which was related to the uptake of nitrates. In spruce species cultured in liquid medium, the pH levels were shown to increase to 6.5-6.8 after 14 days in culture.[31]

Temperature. The control of the temperature in the liquid medium inside the bioreactor can be easily manipulated by using an heating element in the vessel or by circulating water in an enveloping jacket outside the vessel. There is, however, limited information on the effects of temperature in bioreactor cultures, which is usually kept constant at 25°C, with a short photoperiod.

Agitation Systems

Depending on the mode of agitation, bioreactors can be basically classified into following two types:

Mechanically Agitated Bioreactors. In these bioreactors medium is agitated with the help of a mechanically driven impeller and vary types of impellers have been used. One of these is flat-blade turbine impeller, in which high agitation breaks incoming air into small bubbles. Mechanically stirred bioreactors depend on impellers, including a helical ribbon impeller,[30] magnetic stirrers, or vibrating perforated plates.[19]

Pneumatically Agitated Bioreactors. These are classified in two types: bubble column and air-lift. These are tall and thin in comparison with agitation bioreactors.

In the bubble columns air is bubbled at the base of the column, thus medium is agitated. In air lift bioreactors, gas is sparged from the riser section to the top of the column and the medium flows downward in the down corner section. These two sections may be separated using a baffle, a concentric cylinder or an external loop. Circulation in the air-lift bioreactor promotes a better mixing and therefore offers advantages in uniformly suspending cells and clumps, although the oxygen transfer rate is low in the down-corner section. So the performance of an air-lift bioreactors is strongly dependent upon the geometry of the system. Mixing by gas sparging in bubble column or airlift bioreactors lacking impellers or blades is far less damaging for clusters than mechanical stirring, since they were shown to have a lower shearing stress.[26,34]

The main advantage of airlift bioreactors is their relatively simple construction, the lack of regions of high shear, reasonably high mass and heat transfer and relatively high yields at low input rates.[36]

Alternative Aeration: Silicon Tubing. A bubble free oxygen supply bioreactor with silicone tubing was found suitable for embryogenic cell suspensions and provided foam-free cultures.[37] A system of 8 independent units of bioreactor using silicon tubing as aeration system was established by Preil and Hvoslef-Heide, showing a good aeration percentage without damage cells.[38]

For hairy root culture, an acoustic mist bioreactor was found to increase root biomass significantly.[39]

Sterilization and Bioreactor Component

The sterilization routine is affected by the size of a bioreactor and its associated components. Hale et al[40] defined the following criteria for plant bioreactor design:

1. All components should be fully autoclavable and the sterility should be maintained for several weeks.
2. The growth chamber should be transparent for adequate light transmission and visibility.

3. Materials should selected to be more breakage resistant than glass.
4. Component should be easily assembled and disassembled for cleaning.
5. Reduce opening at minimum to avoid contamination.
6. The ventilation (oxygen) should be supplied to avoid damages of plant cells, tissues or organs.
7. Suitable mechanical agitation should be provided to protect the biomass from damage.
8. Glass or plastic bioreactor chambers must be strong to withstand the weight, the pressure and the turbulence.

Kinetics of the Cell Growth

The nutrient supply strictly conditioned the biomass growth into bioreactor.

The feeding system has implication on the growth pattern, therefore must be chosen considering the productivity objectives. A number of operating strategies can be applied in plant cell bioreactors:

Batch Culture (*Close System*): Inoculation of organisms in a fixed volume of liquid medium. Inside the vessel the growth conditions constantly change, with nutrient depletion and concomitantly a metabolites accumulation. The organisms perform a sigmoid growth curve reaching a plateau at the senescence. *Salvia officinalis* cells grown in batch culture exhibited this trend (Fig. 2).

Fed Batch Culture: Variation of the batch system. The culture medium is gradually added to the bioreactor with a consequent rise of plant biomass (linear relationship). An example of fed batch culture of *Cyclamen persicum* embryogenic cells is represented in Figure 3, in which dry weight biomass was measured during fermentations in fast or slow feeding.[41] A fast feeding fermentation was realised using 300 ml of 500 μm filtered suspension at minimum 80% of cell viability, put into the bioreactor with 200 ml of fresh medium; after 24 hours in constant conditions (batch phase) the fresh medium was added daily in increasing quantity of about 10% of the suspension total volume (feed-batch phase). The experiment stopped after 16 days when the volume reached 1.5 L.

A slow feeding fermentation in 500 ml suspension culture was performed with a growth cycle of 22 days; during that period the growth speed was lowered and 20-30 ml of fresh medium were added every 2 days and reaching 1.0 L of suspension at the end of the experiment.[41]

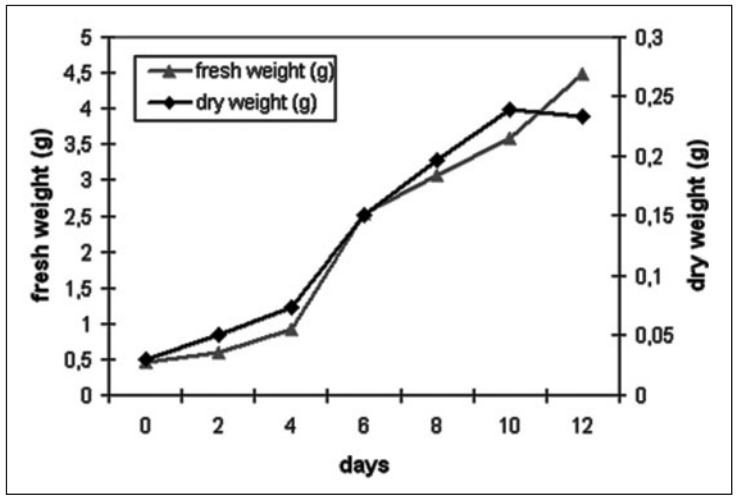

Figure 2. Growth curve of *Salvia officinalis* cells in batch culture. The typical sigmoid curve showed a logarithmic phase between the 4th and 10th day.

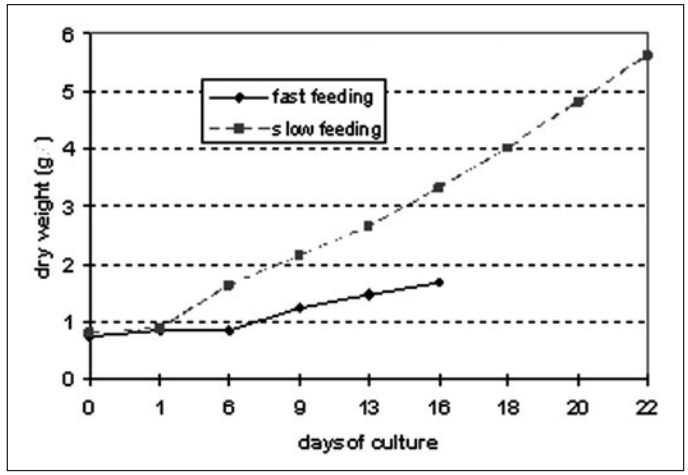

Figure 3. *Cyclamen persicum* embryogenic cell culture, scale up in bioreactor: dry weight biomass evaluation during fermentations in fast or slow feeding.[41]

Continuous Culture: During the exponential phase a volume of fresh medium is added and at the same time an equal volume of cell culture is discarded; a balanced growth can be obtained by using semi constant volumes of biomass quantity and nutrient and metabolite concentrations.

Bioreactors and Hairy Root Culture

Bioreactor cultivation represents the final step in the development of techniques for producing metabolites from plant in vitro systems.[42]

Many types of bioreactors have been successfully used for cultivating transformed root cultures such as conventional stirred tanks, stirred tanks with a separate impeller, bubble columns, mist reactors and balloon-type reactors.[43] Thus, it would be difficult, if not impossible, to select the "best" bioreactor design for cultivating transformed roots. However, for successful scale-up of hairy root-based processes, whatever type of bioreactor is used, several factors should be considered as morphology, unusual rheological properties and high stress sensitivity of hairy roots.[44]

However, the bioreactor cultivation of hairy roots has been considered in several reviews[43-45] and herein only recent advances (post-2002) will be considered.

The most traditional system to culture the transformed roots in liquid medium is the airlift bioreactors used for microorganisms or plant cells as reported for *Beta vulgaris* and *Artemisia annua.*[45,46]

A bubble bioreactor was found to efficiently support the scale up process for coculturing shoots and hairy roots from *Genista tinctoria* devoted to produce phytoestrogens.[47] To improve the homogenization of culture medium, an air lift mesh-draught reactor with wire helixes was designed for large scale culture of *Solanum chrysotrichum* hairy roots.[48] The principal advantage of this method is to reduce drammatically the volume of the culture medium and consequently to increase the concentration of the selected metabolites. This system was adopetd to ensure production of the antitumoral drug campthothecin from *Camptotheca* hairy roots.[49] Interestingly, in this study, coupled reactors of mean volume were used and the production capacity was enhanced by increasing the number of reactors. This represent a good alternative compared with the use of a single reactor of high volume and might reduce the risk occured in a bioreactor.

The development of disposable wave bioreactor systems represents a good advantage. The working principle of these systems is based on wave-induced agitation, which significantly reduces stress levels. Moreover, utilization of plastic disposable chambers minimizes the need

Figure 4. Temporary immersion system (RITA®, Vitpropic, Saint-Mathieu-de-Tréviers, France) for cultivating hairy root cultures.

for labor- and time-consuming cleaning and sterilization procedures and facilitates fulfillment of Good Manufacture Practice requirements.[50]

The ginsenoside production of *P. ginseng* hairy roots in 2 L wave bioreactors has been studied in detail.[51] The results showed that both biomass accumulation and ginsenoside production were significantly higher in 2 L wave systems than in shaken flasks. Large scale wave systems with capacities up to 600 L are now commercially available in Switzerland.

Temporary immersion systems (RITA®, Vitropic, Saint-Mathieu-de-Tréviers, France) (Fig. 4) have also been used for cultivating hairy root cultures from *B. vulgaris* and *Harpagophytum procumbens*.[52,53] Although the RITA® systems have been developed for plant in vitro propagation, their advantages (reduced hyperhydricity and lower consumable and labor costs) make them attractive for hairy root cultivation.[54] The scale of the RITA® systems is about 200 ml and the daily cumulative duration of the immersion stage (flooding) can vary from minutes to several hours. Pavlov and Bley[53] found that growth of *B. vulgaris* hairy roots is optimal with 15-min immersion/75-min standby cycles, while maximal amounts of betalain pigments are accumulated with 15-min flooding/60-min standby cycles.

Another significant problem occurring during the cultivation of hairy roots in bioreactor is associated with mass transfer limitations and root oxygen demands, greater in the meristems than the old tissues. The hairy roots typically grow in a "tuft-like" manner, which promotes the formation of oxygen and nutrient gradients in the tissue. Hairy roots can be grown in bioreactors at low tissue concentrations (<10-g dry weight/L) with virtually any configuration.[43] However, at high tissue concentrations (>10-g dry weight/L) in submerged bioreactors, several scale-up limitations may

arise. For high tissue density cultivation, Ramakrishnan and Curtis[55] developed a 14 L pilot-scale reactor that operates in a "trickle-bed" mode. The dry mass of *H. muticus* cultivated in this bioreactor reached 36 g/L within 25 days and the calculated growth index (based on the dry weight) and doubling time were 180 and 3.3 days, respectively.

In addition, an attempt to cultivate transformed root cultures on a large-scale (500 L) has been reported using a procedure involving inoculum preparation in a 10 L seed vessel for 2 weeks, followed by aseptic transfer of the root inoculum to the main 500 L reactor.[56] Here the roots were immobilized on barbs, which further facilitate their harvesting. Although the final biomass yield (about 4-g dry weight/L) could not be considered high, this procedure addresses many potentially problematic issues (mainly technological) concerned with transferring the tissue inoculum from the seed reactor to a greater one(s).

Monitoring the roots' growth during the processes in reactor systems is highly important. In fact during the cultivation of organ cultures (e.g., hairy roots) it is impossible to obtain homogeneous samples of the tissue, which complicates measurements of growth and other process parameters. Therefore, conductivity measurements have been widely used to obtain indirect growth estimates.[53,57] Changes in conductivity are due to the cellular uptake of ionic nutrients (nitrate anions usually). Huang and Chou[58] found that redox potential changes during the cultivation of hairy root cultures in a mist trickling reactor reflect the assimilation of ammonium, nitrate, sucrose and growth phases.

It is worth noting that, in some cases, such linear relationships between reductions in conductivity and biomass growth increase do not exist, indicating that the relationships should be individually determined for every culture.[59]

The osmolarity of the culture medium offers another possibility for indirect measurements of the root growth. Osmolarity measurements have several advantages over conductivity measurements, as osmolarity takes into account the total number of moles of all solutes present in the medium.[60] Use of such indicators could also be considered for monitoring hairy roots growth.

Scale-Up of Medicinal and Aromatic Plants for Secondary Metabolite Production

Zedoary (Curcuma zedoaria)

Zedoary (*Curcuma zedoaria* Roscoe), a member of the Zingiberaceae family, is a species, which grows wild in the eastern Himalayas and is cultivated in India, Sri Lanka, China, Japan, Thailand and Vietnam. Essential oils, curcumin and terpenoids are the main secondary metabolites of zedoary and show the principal pharmacological activities associated with the plant. The constituents of zedoary rhizome oil have been investigated extensively and zedoary has been recognized as a rich source of terpenoids.[61] Zedoary essential oils evidence antimicrobial and antimutagenic activities. Curcumin is well-known for its antitumor, antioxidant, antiamyloid and anti-inflammatory properties. Curcumin shows a free radical scavenger and antioxidant activity and inhibits lipid peroxidation and oxidative DNA damage.[62,63]

Cell suspension cultures for zedoary are generally considered the most suitable system for large-scale applications in the biotechnology industry; therefore zedoary was utilized for monitoring the possible use of these cells to produce of essential oil and curcumin.

A Vietnamese researcher team has established a model batch culture system in a small bioreactor, comparing the production of fresh biomass and metabolites in bioreactors with that obtained from usual in vitro protocols.[64] After the stabilization of the shaking liquid cell culture, the cell biomass were then collected and transferred into a bioreactor (Biotron, Inc. Korea) with a 5 L working volume and three impellers and then propagated at an agitation rate of 150 rpm and an aeration rate of 2.5 L/min for 14 days. Mixing and aeration were achieved using sterile gas from an air pump through a flow meter and an air filter. Determinations of the inoculum sizes, agitation rates and aeration rates suitable for cell biomass production were already known.[65] The

Table 3. Curcumin content and specific activities of POD, SOD and CAT in culture of zedoary cell (from Loch et al 2008). Different letters indicate significantly different means using Duncan's test (P < 0.05).

Culture Time (Days)	Content of Curcumin (% of Dry Cell Weight)	POD (U/mg Protein)	SOD (U/mg Protein)	CAT (U/mg Protein)
2	8.20^{ab}	0.01^{e}	13.12^{cd}	6.22^{de}
4	8.31^{ab}	0.02^{e}	13.57^{cd}	7.73^{d}
6	8.34^{ab}	0.13^{cd}	14.66^{c}	10.37^{c}
8	8.52^{ab}	0.15^{cd}	14.76^{c}	16.11^{bc}
10	8.55^{ab}	0.25^{c}	14.84^{c}	17.57^{b}
12	8.63^{ab}	0.41^{b}	14.96^{c}	17.96^{b}
14	9.69^{a}	0.63^{a}	16.60^{b}	19.59^{b}
16	8.63^{ab}	0.08^{d}	15.76^{bc}	12.84^{c}
18	8.31^{ab}	0.04^{de}	14.77^{c}	10.41^{c}
20	8.22^{ab}	0.01^{e}	12.17^{d}	7.77^{d}
Control	5.88^{b}	0.07^{d}	18.58^{a}	16.12^{bc}

online monitoring of the pH and temperature in the cultures (5.8 and 25°C, respectively) was measured by connecting the pH electrode and temperature sensor to the bioreactor. The biomass concentration reached a maximum value of 67.73 g fresh weight/L (approximately 6.77 g dry weight/L) after 14 days of culture.

The highest cell biomass was further used for the extraction of secondary metabolites (essential oil and curcumin) and for the determination of the activities of antioxidant enzymes peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT).[64] In general, the essential oil contents (percentage of dry cell weight) increased between days 2 and 14 and reached maximum values at the end of the log phase (14th day of culture) in both extractions via steam distillation (1.78%) and petroleum ether (0.69%). See Table 3 (from Loch et al 2008).

These results may prove useful in the development of a large-scale production protocol. Scale-up protocols will need to be designed and tested in the future.

The data presented in Table 3 suggest that the accumulation of curcumin in zedoary cells can be associated with the increased levels of activities of POD, SOD and CAT showing the highest amounts at the 14th day of culturing.[64] The curcumin induction of such detoxifying enzymes proved the potential value of curcumin as a protective agent against oxidative stress, as reported in other detailed studies.[66]

*Coneflower (*Echinacea sp*)*

Echinacea sp is a traditional perennial herb plant native of North America and is widely distributed in the world for commercial purposes. Secondary metabolites obtained from roots and aerial parts of three species, *Echinacea purpurea* (called purple coneflower), *Echinacea angustifolia* and *Echniacea pallida,* are of particular importance.[67]

Roots from *E. angustifolia* were historically used in phytomedicine, but also *E. purpurea* extracts have antioxidative, antibacterial, antiviral, antifungal properties and are used for treating common cold, respiratory and urinary diseases.[68] The most important potential active compounds in *E. purpurea* are caffeic acid derivatives namely caftaric acid, chlorogenic acid, cynarin, echinacoside and chichoric acid. Of these, chichoric acid has immunostimulatory property and can promote phagocyte activity in vitro and in vivo. It also has anti-hyaluronidase activity, a protective effect

Table 4. Growth, productivity and bioactive substances in adventitious roots of* Echinacea purpurea *cultured in different conditions

	Biomass Yield		Caffeic Acid Derivatives (mg/g DW)			
Type of Culture	**Fresh Weight (Kg)**	**Dry Weight (Kg)**	**Caftaric Acid**	**Chlorogenic Acid**	**Chicoric Acid**	**Total**
500-L balloon-type bioreactors	26.3 ± 0.5	3.6 ± 0.1	2.8 ± 0.1	4.4 ± 0.4	20.2 ± 0.8	27.4 ± 0.5
1000-L drum-type bioreactors	40.5 ± 0.5	5.1 ± 0.1	3.9 ± 0.1	4.9 ± 0.1	22.5 ± 0.6	31.5 ± 0.6
Natural adventious roots	-	-	2.4 ± 0.2	-	6.2 ± 0.9	8.6 ± 1.1

on the free radical-induced degradation of collagen as well as antiviral activity[68] where it inhibits HIV-1 integrase and replication.[69]

Because commercial preparations are commonly made from root tissues, in vitro protocols for their proliferation could improve the commercial availability. Therefore many attention have been focused on the efficient biomass production for fast growth rates and stable metabolite productivity.[70,71]

Recently research efforts were focused on developing bioreactor methodologies for the efficient production of caffeic acid derivatives from adventitious *Echinacea* spp. root cultures.[67] Culture systems in airlift bioreactors (20 L, 500 L balloon-type, bubble bioreactors and 1000 L drumtype bubble bioreactor) were then developed for the production of chichoric acid, chlorogenic acid and caftaric acid. In the 20 L balloon type bubble bioreactors a maximum yield of 11 g dry biomass/L was achieved after 60 days.

Pilot scale balloon-type bubble bioreactors (500 L working capacity) and horizontal drum bioreactors (1000 L working capacity) were also used for the cultivation of adventitious roots. These bioreactors have sparger positioned at the bottom, used to generate air bubbles less then 0.5 mm in diameter. Aeration rate was controlled at 0.1 vvm.[67] 3.6 kg and 5.1 kg dry biomass were achieved in the 500 L and 1000 L bioreactors, respectively (Table 4).

Adventitious roots grown in pilot scale bioreactors were also efficient in accumulation of caffeic acid derivatives and the total caffeic acids contents were about 27 mg/g dry weight and 31 mg/g dry weight with adventitious roots grown in the 500 L balloon type bubble bioreactor and 1000 L drum bioreactor, respectively. The accumulation of 5 mg/g dry weight chlorogenic acid, 22 mg/g dry weight chichoric acid and 4 mg/g dry weight caftaric acids were achieved with adventitious roots grown in 1000 L bioreactors.

Sometimes during the scale up of plant cell and organ cultures, a decrease in productivity may occur.[72,73] However, the scale up of adventitious root cultures of *E. purpurea* showed no decrease in biomass production and caffeic acid productivity. Comparison of caffeic acid derivative contents in adventitious roots and field grown plants revealed that the contents of caftaric acid (1.6-fold), chichoric acid (3.6-fold) were higher in the adventitious roots than in the roots of field grown plants.

These results may be useful for biotechnological application of *E. purpurea* adventitious root cultures for the production of caffeic acid derivatives on a large scale.

*Basil (*Ocimum basilicum*)*

Ocimum basilicum L., a popular Lamiaceous plant known as sweet basil, is used as a kitchen herb in the production of "pesto", a typical Italian sauce known for its unmistakable aroma and as an ornamental in house gardens. The aromatic characters of each type of basil is determined by genotype and depends on their major chemical constituents in the essential oils. Basil aromatic leaves and essential oils are widely used as antioxidants, flavouring agents in foods, confectionary products, beverages as well as in perfumery.[74] Basil is known to contain the antioxidant phenolic compound, rosmarinic acid, one of the most common caffeic acid esters occurring in Lamiaceae family.

Hairy roots and cultured cells of sweet basil (*Ocimum basilicum* L.) are able to produce rosmarinic acid.[75,76]

The use of bioreactors for the growth of sweet basil cell suspensions and for the regeneration from nodal explants can both improve and scale up rosmarinic acid accumulation and plant micropropagation of this medicinal plant species.[5] Thus, proliferating callus tissue from nodal cultures were transferred for three weeks in an 5 L disposable presterilized plastic airlift bioreactor (Osmotek Lifereactors) (ml/reactor, 75 explants/reactor).[77,78]

During that incubation period, suspension cultures grew faster in the airlift bioreactor than in a 250 ml flask and the average biomass increased even more remarkable (1457%).[5]

In bioreactors, enhanced growth was highly positive correlated with rosmarinic acid accumulation ($r^2 = 0.99$), producing higher levels of metabolites than in 250 ml flasks suspension cultures. Further observations indicated that the culture fresh biomass growth was also positively correlated with radical oxygen species (ROS) production; ROS are by-products of normal cellular metabolism produced by mitochondria, chloroplasts and peroxisomes, so their increase in concentration might be associated with the rapid turn-over of primary metabolites.[5] In fact rosmarinic acid biosynthesis was previously associated with primary metabolic processes, leading to cellular growth.[76]

On the other hand, dry weight accumulation as well as soluble protein and carbohydrate concentrations were negatively associated with biomass growth. This culture growth was mainly due to cellular enlargement, a process associated with increased ROS formation.[79]

In conclusion both cell suspensions and nodal explants of *O. basilicum* represent appropriate sources of good rosmarinic acid biosynthesis under scale-up conditions.

Lavender (Lavandula vera)

Lavandula species, belonging to Lamiaceae plants, are mainly grown for their essential oils, which are used in perfumery, cosmetics, food processing and aromatherapy products.[80] Lamiaceae plants are well known producers of phenolic compounds and cell suspensions of *Lavandula vera* MM specie represented a promising producer of rosmarinic acid.[81]

Many investigations were focused on transferring the suspension cell process in a laboratory bioreactor and on the optimization of cultivation conditions. Experiments were performed in a 3 L propeller-stirred bioreactor (BioFlo 110, New Brunswick) using 1,8 L cultivation nutrient medium, inoculated with 7-day-old shake-flask suspension.[52] During cultivation of plant cell suspensions were selected different nutrient media and the dissolved oxygen and agitation speed were separately optimized.

Significant production of rosmarinic acid was achieved up to 3489.4 mg/L in the 3 L bioreactor cultivation although yields of biomass were relatively invariable in the different culture used (Table 5). The obtained data confirmed the proposed algorithm for the optimization of rosmarinic acid biosynthesis by *L. vera*, which is of technological significance and revealed possibilities for the next scale-up of the process.[52] (Table 5).

Ginseng (Panax ginseng)

The use of modified nutrient medium, as well as elicitors and air lift bioreactors are common methodology to improve production of the active ginsenosides (saponins) from Ginseng (*Panax ginseng* C.A. Meyer), a worldwide important medicinal plants, whose active components have

Table 5. Biomass, RA yield and productivity detected in Lavandula in different bioreactor conditions (adapted from ref. 52)

Parameter	Shake-Flask Culture in Standard LS Nutrient Medium	Shake-Flask Culture in Modified LS Medium	3-L Bioreactor Culture in Modified LS Medium
RA Yield, mg/L	68.0	1786.7	3489.4
Biomass, g Dry Weight/g sucrose	0.54	0.72	0.59
RA productivity, mg/L day	8.5	148.9	268.4

attributed cardio-protective, immunomodulatory, antifatigue, hepato-protective physiological and pharmacological effects.[82]

It is well known that synthesis of secondary metabolites and enzymes in plants is usually associated with plant defense responses to different stress conditions.[83,84]

The effects of methyl jasmonate and salicylic acid on changes of the activities of major antioxidant enzymes and ginsenoside accumulation were investigated in ginseng roots (*Panax ginseng* L.) in 4 L air lift bioreactors.[85]

Selected adventitious roots were collected at different time of growth after methyl jasmonate and salicylic acid elicitation in airlift bubble type bioreactors. These elicitors considerably increased the saponin accumulation without changing biomass until seven days, however, biomass decreased in salicylic acid-treated roots compared to methyl jasmonate. Moreover both treatments induced an oxidative stress in *P. ginseng* roots, increasing superoxide anion (O_2^-) formation and conseguently lipid peroxidation.[86] The results suggested that methyl jasmonate and salicylic acid act as signalling molecules inducing saponin accumulation and O_2^- may function as a signal for the induction of defence genes and could enhance the ginsenoside production.[87]

The finding that methyl jasmonate and salicylic acid enhance both the antioxidant defence systems and the secondary metabolite formation without affecting biomass accumulation of *P. ginseng* roots increases the usefulness of this culture system for production of pharmacologically ginsenosides.[85]

Sage (Salvia miltiorrhiza)

Salvia miltiorrhiza Bunge (Lamiaceae) roots or Danshen in Chinese, is a well-known Chinese herb, which is widely used in modern and traditional medicine for the treatment of menstrual disorders and blood circulation diseases and for the prevention of inflammation. A major class of bioactive ingredients of Danshen is ascribed to the lipophilic diterpene pigments generally known as tanshinones.[88] The hairy root culture of *S. miltiorrhiza* has been established as an alternative more efficient production of tanshinones then the whole plant growth in farms.[87,89]

Elicitation is one of the most effective means for improving secondary metabolite production in plant tissue and cell cultures including hairy root cultures;[84] the effect of elicitation is a general productivity which depends on the biomass growth rate and biomass concentration in the culture.[90]

The most common elicitors used are fungal carbohydrates or polysaccharides, jasmonic acid or methyl jasmonate, chitosan and heavy metal ions. In addition to these agents, hyperosmotic stress had an effective stimulation for the production of various secondary metabolites in plant cell cultures.[90] Although single elicitors have been mostly used, the combined dose of two different elicitors has been shown more effective due to a synergistic effect.[91,92]

Wu and Shi[90] observed an increase (three to four fold) in the total tanshinone content of *S. miltiorrhiza* roots by osmotic stress and yeast elicitor treatment, separately, but more significantly by their combination (about eight fold).

The application of three different means to *S. miltiorrhiza* hairy root cultures, such as a semi-continuous culture process, a yeast elicitor addition and in situ adsorption of tanshinones (with a hydrophobic polymeric resin, X-5) applied at the late exponential growth phase, increased the root biomass to 30.5 g dry weight/L (versus 8-10 g dry weight/L in batch mode). Moreover the volumetric tanshinone yield reached 87.5 mg/L (about 15-fold increase), with 76.5% adsorbed to the resin.[93]

These results demonstrated that the integration of multiple elicitation, in situ adsorption and semi-continuous operation can synergistically enhance tanshinone production in *S. miltiorrhiza* hairy root cultures.

Conclusion

Bioreactors offer a great hope for the large scale synthesis of bioactive compounds in medicinal and aromatic plants. Since the biosynthetic efficiency of population varies a high yielding variety is recommended as a starting material. There are also several difficulties associated with large scale bioreactor technology, mainly focused on product yield cost, optimising growth rate and product release, expression of desirable traits and culture stability. Genetic transformation may provide increased and efficient system for in vitro production of secondary metabolites. Recent progress in the scaling up of hairy root cultures is making this system an attractive tool for industrial processes.

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