Kee-Yoeup Paek
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Production of Biomass and Bioactive Compounds Using Bioreactor Technology

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Preface

Plants are the major source of secondary metabolites which are used as pharmaceuticals, flavours, fragrances, colouring agents, food additives and agrochemicals. In recent decades, plant cell, tissue and organ cultures have emerged as an alternative over whole plant cultivation for the production of valuable secondary metabolites. Cells, adventitious roots, hairy roots, shoots and embryos have been successfully cultured *in vitro* for the large scale production of secondary metabolites. Strain improvement, selection of high-producing cell lines, optimization of medium and culture environment have led to the enhanced production of bioactive and value added products. In recent years, a couple of bioreactor configurations have been developed and successfully adopted for the *in vitro* cultivation of plant cells and organs. Bioreactors such as mechanically agitated, airlift and photo-bioreactors have been designed and used for large scale cultivation of algal, higher fungal and plant cells. Bioprocess engineering parameters such as mixing, oxygen supply and shear stress have been investigated towards successful commercial scale cultivation. Various bioprocess operation modes including batch, fed-batch, two-stage cultivation, and bioseparation of intracellular metabolites have been suggested for enhanced and sustainable recovery of secondary metabolite products. More recently, overexpression of regulatory genes in up-regulating a series of enzyme activities in the metabolic pathways is also being achieved through genetic and metabolic engineering approaches.

This book provides recent progresses and limitations of production of biomass and bioactive compounds using bioreactor technology as mentioned above. It contains six parts: Part I describes bioreactor designing advantages and limitations of bioreactor cultures; Part II deals with the production of biomass and bioactive compounds from cell suspension cultures; Part III contains chapters on production of secondary metabolites from suspension cultures of plant organs – shoots, adventitious roots and embryos; Part IV deals with the strategies for enhanced production of secondary products, large-scale cultures and metabolic engineering of selected metabolites; Part V contains bio-safety assessments of plant cell and organ culture products; and the final Part VI contains physiological disorders in plants cultured in bioreactors.

We thank all the authors and reviewers who have contributed to this project. Our special thanks to Sophie Lim, Associate Editor, and Peter Butler, Editorial Director, for their helpful editorial work. We also appreciate the staff of Springer Publishers for giving us the opportunity to present this book to our readers around the world.

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Part I
Bioreactor Design, Advantages and Limitations of Bioreactor Cultures

Chapter 1
Design of Bioreactors for Plant Cell and Organ Cultures

Milen I. Georgiev

Abstract Demands for sustainable supply of plant biomass and/or value added-molecules (incl. native and heterologous therapeutic proteins, specialty proteins and industrial enzymes) have been the driving efforts to develop alternative ways for their bioproduction. Plant cell and organ cultures have been demonstrated an efficient, cost effective and eco-friendly alternative to classical technologies (i.e. by harvest from wild) and chemical (semi)synthesis. The progress has resulted in development of several commercial processes for large-scale production of plant biomass and high value molecules, besides numerous proof-of-concept studies at laboratory- and pilot-scale. This chapter summarizes the bioreactor configurations for plant cell and organ cultures, and attempts to outline the immense potential of plant *in vitro* culture-based bioprocesses for sustainable supply of biomass and value-added molecules for various purposes along with the major challenges that remain.

Keywords Bioreactors • Cell cultures • Organ cultures • Mechanically driven systems • Pneumatically driven systems

1.1 Introduction: From Simple Carboy Systems to Large-Scale Bioreactors

Per definition bioreactor is any device or vessel that is used to carry out one or more biochemical reactions in order to convert any starting material (*inter alia* substrate) into product(s) [1]. Bioreactor cultivation and subsequent up-scaling represent the final steps in the development of bio-based processes. In general, the basic function of a bioreactor is to provide optimal conditions for effective cell growth and metabolism

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by strict regulation of various environmental (chemical and physical) key factors [2, 3]. Though it is difficult to dedicate the very first attempt to cultivate plant cells in bioreactors, the pioneering work of Routien and Nickell [4] is a benchmark. In 1956, authors were granted the first patent for the cultivation of plant cells *in vitro* in simple 20-L carboy systems. Soon after that, the National Aeronautics and Space Administration (NASA) started a research program on plant cell culture for regenerative life support systems. Plants and the relevant *in vitro* cultures were grown under various conditions of microgravity (space shuttles, parabolic flights, biosatellites, the orbital stations Salyut and Mir) along with ground studies using rotating clinostat vessels (reviewed in Sajc et al. [5]). In the 1970s, further attempts to develop bioreactor configurations suitable for plant cells resulted in the development of a conical glass V shaped reactor (as called by the authors V-shape fermenter) for plant cell suspension cultures. This V shaped reactor has proved useful for both biomass and metabolite production [6]. Later, the concept of high shear sensitivity of plant cells was developed and only air-lift reactors were considered suitable [7, 8]. For instance, Kurz and Constabel [9] wrote "The most suitable reactor developed so far is the airlift reactor.... However this design is only applicable to cultures with a cell dry weight lower than 20 g L^{-1}". Accordingly, *Nicotiana tabacum* cell suspension culture was up-scaled in 360-L and 1,500-L bubble aeration-type bioreactors [10]. However, several industrial-scale processes developed in early 1980s utilizing stirred-tank reactors (STRs) subsequently challenged these perceptions [2, 8]. Nowadays, ca. 60 years after first dedicated attempts to grow plant cells in bioreactors has become nearly impossible to select the "best" bioreactor configuration for different plant *in vitro* cultivations.

The selection and design of each bioreactor configuration and operational mode are unique, which however, underlying some basic principles, as low stress environment, adequate mixing, and oxygen and heat transfer [2]. In most cases, bioreactors available for microbial fermentation can be implemented for hosting plant cells with some slight modifications. In general, reactor design should ensure that nutrients are effectively provided to the cells. Cell growth and product formation kinetics should be assessed (by respective sampling) so that, the optimal environmental conditions can be defined and thus the most suitable operational mode to be determined. Transport phenomena, including mixing, shear forces, and oxygen transfer, should be continuously followed during the cultivation process in order to define the criteria for bioreactor design and up-scaling. Operating parameters, such as dissolved oxygen concentration (DO_2) and substrate concentration(s), temperature of cultivation, pH and agitation speed, among others, should be easy to monitor and set-up. In addition, the bioreactor configuration should be as simple and inexpensive as possible and it should be easily operated while ensuring long-term sterility [2].

1.2 High-Value Molecules Produced by Plants and Relevant *In Vitro* Culture

The vast chemical diversity of the plants has been exploited since time immemorial by humans to diminish and prevent pain, to produce pleasure, for use in religious ceremony and to cure various human disorders. The chemical entities responsible

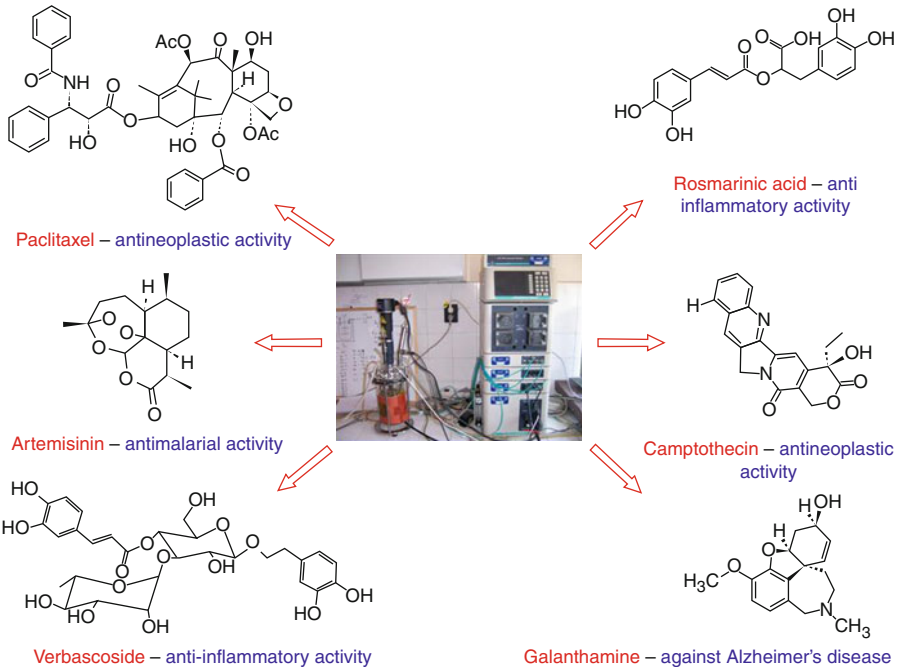


Fig. 1.1 Selected examples of high-value molecules produced by plant cell/organ culture, grown in bioreactors

for this biological activity are, in most cases, low-molecular weight compounds that are often accumulated at very low amounts in plants [11]. For instance, paclitaxel (Fig. 1.1) content in *Taxus* plants accounts on <0.02 % of the dry weight of the bark, where its levels are highest, therefore, commercial production of this complex diterpenoid by natural harvest is not economically feasible as *Taxus* plants grow very slow [12]. Recently, Wilson and Roberts [13] estimated that 340 tons of *Taxus* bark or 38,000 trees would be required to meet the 25 kg/year demand for the antineoplastic drug paclitaxel. In search of alternative solutions, two different routes for the total synthesis of paclitaxel were developed 20 years ago, however, the process involves 40+ reactions, utilizes harsh solvents, and has overall low product yields, which makes chemical synthesis of paclitaxel economically and environmentally unfavourable so far (reviewed in Wilson and Roberts [13]).

In addition, several plants accumulating metabolites of pharmaceutical interest are listed as endangered species (due to continuous overharvesting of natural populations), and therefore novel approaches have to be found in order to ensure the sustainable production of value-added molecules.

Today, over 25 % of modern medicines are derived either directly or indirectly from plants, especially in case of cancer therapy (60 %), infectious diseases (75 %), but also in metabolic syndrome and immuno-suppression therapy. Of course, these numbers also include microbial sources, but the significance of plants in the production of pharmaceuticals is undoubtful. To name a few, paclitaxel (Taxol®), galanthamine (Nivalin® and Reminyl®) and artemisinin (Fig. 1.1) are currently good

examples in this approach and amongst the blockbuster drugs worldwide [14]. The most important commercially relevant pharmaceuticals, derived from plants, are valued at over $ 25 billion per year in the USA alone [11]. In addition, World Health Organization (WHO) estimates, at least 80 % of the population in developing countries still relies exclusively on traditional medicine for their primary health care needs (Georgiev [14] and the literature cited therein). According to Food and Agriculture Organization of UN (FAO), world population is expected to grow by over a third, or 2.3 billion people, between 2009 and 2050 [15]. This purely means that in near future humans will face multiple challenges such as: (1) more food and fibre have to be produced to feed the growing population; (2) more feed stocks for a potentially huge bioenergy market and (3) more medicines (*inter alia* of natural origin) to cure human diseases.

Continuously increasing demands for plant biomass and therapeutic molecules, produced by ever greener processes, along with dramatic reduction in plant biodiversity, are the driving force to develop alternative ways to supply value-added molecules [8, 16]. Biotechnological production of secondary plant metabolites has been of interest for many decades. Nowadays, plant cell and organ cultures have become increasingly attractive and cost-effective alternatives to classical approaches (i.e. natural harvest and chemical synthesis) for the mass production of plant-derived metabolites ("green cell factories" concept), because of their several advantages. First, genetic modification in a contained system can readily be applied without the regulatory barriers associated with field grown crops. Second, a cell/organ culture system can be up-scaled in bioreactors with controllable production rates [16–18]. Furthermore, plant cell/organ culture is the only economically feasible way of producing some high-value molecules from rare and/or threatened plants. The progress in this field so far has resulted in the mass production of biomass and high-value molecules (*see below*) by different companies [2, 13, 19, 20].

1.3 Bioreactors for Dedifferentiated Plant Cell Culture

Stainless steel stirred tank reactors, bubble column reactors and air-lift reactors (Fig. 1.2a–c) directly derived from microbial bioprocesses are commonly used – with slight modifications – to grow plant cell suspension cultures up to 75 m^3 of culture volume [21]. Nowadays, stirred tank reactors are the most widely used reactor configurations for growing plant cells, because of their several advantages such as easy scale-up, good fluid mixing and oxygen transfer capacity, availability of numerous impellers types (reviewed in Georgiev et al. [2]) and compliance with current Good Manufacture Practices (cGMP) requirements [3]. Reasonably, most of the existing commercial processes with plant cell suspension cultures (discussed below) are based on STRs of m^3-scale. Towards minimization of production costs and validation efforts under cGMP regulations, several single-use bioreactor configurations for hosting plant cell culture have been developed recently, e.g. the wave-mixed reactors (Fig. 1.2d), slug bubble bioreactor (Fig. 1.2e), wave and

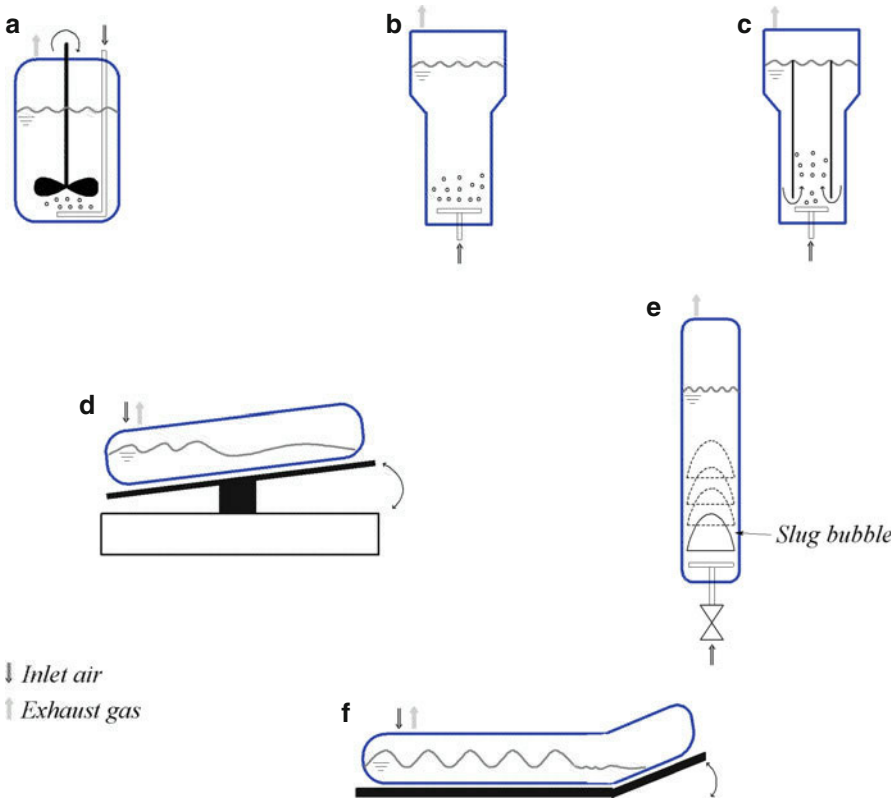


Fig. 1.2 Bioreactor configurations for plant cell culture. (**a**) Stirred tank reactor, (**b**) Bubble column reactor, (**c**) Air-lift reactor, (**d**) Wave bioreactor, (**e**) Slug bubble reactor, (**f**) Wave and undertow (Georgiev et al. [8] and the literature cited therein)

undertow bioreactor (Fig. 1.2f), CELL-Tainer, AppliFlex, Saltus Virbormix reactor, and OrbShake reactor [2, 8], among others. Single-use bioreactors are comprised of cultivation containers/bags, made of US Food and Drug Administration-approved plastics [21, 22]. Single-use bioreactors become increasingly accepted for biotechnological processes at small and medium size scale during the past decade. Numerous studies [23–25] have clearly shown their advantages, such as reduced contamination and cross-contamination rates, easy compliance with cGMP regulations, savings in time and costs, and reduced waste and thus clear environmental impact. Thus, single-use bioreactors have a wide range of applications for the production of therapeutic proteins [20, 26] and in the production of plant biomass and secondary metabolites for cosmetics purposes [2].

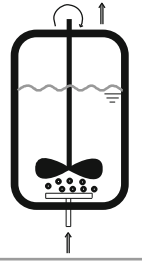
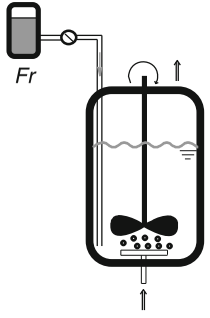
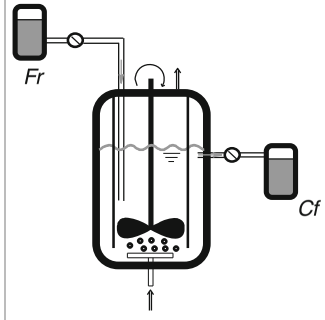
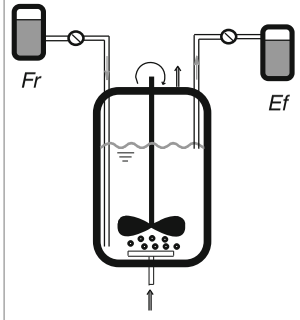
An effective bioreactor operational mode should provide adequate volumetric yield and overall high system productivity, which means more product(s) are formed per unit time per liter of bioreactor volume [2]. A major drawback of batch processes is that significant amount of time is taken up by the system and media sterilization,

filling and emptying, and cleaning the system. Thus, towards improving the cost-effectiveness of the plant cell culture-based bioprocesses, various operational modes have been developed, including multi-stage batch, fed-batch, single- or multi-stage continuous (chemostat), semi-continuous (draw-and-fill) and perfusion (continuous with cell retention) cultivation [2, 8]. A comparison of different cultivation modes and feeding regimes is summarized in Table 1.1. Among these, the most promising towards high productivity and thus most successfully applied ones for suspended plant cells are fed-batch and perfusion.

Towards the development of new bioreactor configurations, we designed a glass-column bioreactor, operated with pulsed aeration in the Laboratory of Applied Biotechnologies, Plovdiv, Bulgaria [27]. In bubble column reactors, the sole source of agitation is the pneumatic power input provided by isothermal expansion of the sparged gas from the bottom [1, 28]. The air balloon type of aeration (created *via* pulsed aeration) significantly reduces cell exposure to the local zones of high shear stress. Moreover, such type of aeration (also called slug bubble or Taylor-like) ensures both effective mass transfer of oxygen into the liquid medium and homogenization of the culture medium [3, 27]. The bioreactor was further used for cultivation of *Harpagophytum procumbens* (devil's claw) cell suspension and to study the production of pharmaceutically important verbascoside (a phenylethanoid glycoside, possessing desirable pharmacological activities for human health, such as antioxidant, antiinflammatory, antineoplastic, wound-healing and neuroprotective properties) [29]. As a result, both accumulated devil's claw biomass and the high-value verbascoside productivity in the column reactor with pulsed aeration were higher than the respective levels, reached in the shaken flasks and STRs. Consequently, the biomass [expressed as g biomass/(L day)] and verbascoside [expressed as mg verbascoside/(L day)] productivity were up to 30 % and threefold higher, respectively, in the pulse-sparged column bioreactor than other cultivation systems. The accelerated growth of the devil's claw cells and their high productivity imply that the pulse-aerated glass column bioreactor might be quite suitable system for hosting the plant cell suspension cultures. The construction of the bioreactor from glass also allows cultivation of phototrophic/photomixotrophic cultures [27]. A similar type of bioreactor, named the "slug bubble reactor", has been developed for the cultivation of *Nicotiana tabacum* cell suspension culture [30]. The slug bubble reactor is made of flexible gamma-sterilized biopharmaceutical grade polyethylene and can be operated in a single-use cultivation mode, as discussed above.

Nowadays, 30 years after the development of the first **industrial process** based on plant cells (shikonin production by *Lithospermum erythrorhizon* cell suspension culture), dozens of molecules are produced commercially using plant cell cultures. These include paclitaxel (Taxol®), berberine, ginseng biomass, *Echinacea* polysaccharides and several therapeutic and heterologous proteins, among others [2, 8, 13]. At present, in Ahrensburg (Germany), Phyton Biotech operates the world's largest cGMP plant cell culture facility with bioreactors specifically designed to meet the needs of plant cells in culture. The total production capacity of the taxanes train runs is up to 880,000 L/year. The Phyton Biotech is a global provider of chemotherapeutic agents including paclitaxel, docetaxel APIs (active pharmaceutical ingredients) and taxane

Table 1.1 Commonly used cultivation modes of feeding based on product yield and economical considerations

	Batch/ Repeated batch	Fed-batch	Perfusion	Continuous
				
Process manipulation	Low/medium	Medium	High	Medium
Cost (capital investment and labor)	Low/medium	Low	Medium	Low
Throughput	Low/medium	Low	High	Low
Product volumetric yield	Low	High	Medium	Low

Modified after Georgiev et al. [2]
Fr feeding reservoir, *Cf* cell-free spent medium, *Ef* effluent vessel

intermediates (www.phytonbiotech.com; accessed March 2014). The *Taxus* cell culture-based paclitaxel bioproduction was also commercialized by Samyang Genex Corporation (Taejon, South Korea) at m^3-scale [18]. In addition to relatively well established bioproduction of plant-derived molecules, in recent years, several biotech companies have been turning plants (and relevant cell suspension cultures) into drug factories in order to produce therapeutic proteins that could not be made otherwise or to make them cheaper [31]. Protalix BioTherapeutics (Israel) uses the next-generation recombinant protein expression system platform to produce a wide range of complex and biologically equivalent human proteins (e.g. ELELYSO, a plant cell-expressed form of the glucocerebrosidase enzyme for treatment of Gaucher's disease) in transgenic carrot and tobacco cell cultures in single-use plastic bag bioreactors (www.protalix.com; accessed March 2014). Another remarkable example includes the development of recombinant animal vaccine against Newcastle Disease virus, produced by transgenic tobacco cell cultures and marketed by Dow Agrosciences [20].

1.4 Bioreactors for Differentiated Plant Organ Culture

In general, the differentiated plant organ culture consist of plantlets, shoot culture, adventitious (=normal roots) and transformed root culture (=hairy roots; harbouring T-DNA of *Agrobacterium rhizogenes* pRi plasmid). In the past two decades, plant organ culture have become increasingly considered as an attractive platform for bioproduction of plant-derived metabolites and therapeutic proteins, because of their several advantages, such as genetic and biochemical stability, and capacity for organogenesis-associated synthesis of metabolites, to name a few [2, 7, 19, 32, 33].

Diverse bioreactor designs (Fig. 1.3) have been used for cultivation of differentiated plant organ culture, including mechanically driven reactors (e.g. STRs, wave-mixed and rotating drum reactors), pneumatically driven systems (e.g. air-lift reactors and bubble column reactors), besides abundant bed reactors (e.g. mist reactors and trickle-bed reactors) and temporary immersion systems [2, 34]. The morphology of differentiated plant organ culture demands a special consideration for the adequate bioreactor configuration, which should (1) provide low-shear environment for tissue growth and (2) ensure reduced mass transfer limitations. The formation of strong nutrient and oxygen gradients in the tissue is a major issue in densely packed plant tissue beds, e.g. non-homogeneous growth [2, 21, 34, 35].

Thus, the use of ordinary STRs is, in general, not highly recommended because of the high stress-sensitivity of plant organ culture. However, slight changes in the STR internal hardware configuration – e.g. separation of the plant tissue from the impeller (by using a mesh for instance) or just a simple reduction of the agitation speed – have resulted in successful cultivation of transformed root cultures of *Beta vulgaris* [36] and *H. procumbens* [37] and *Atropa belladonna* [38] in bioreactors of different scale (Table 1.2). Pneumatically driven air-lift and bubble column reactors are probably the most frequently used configurations for hosting differentiated plant organ culture [2]. Among others, a modification of air-lift reactor, named balloon type bubble bioreactor

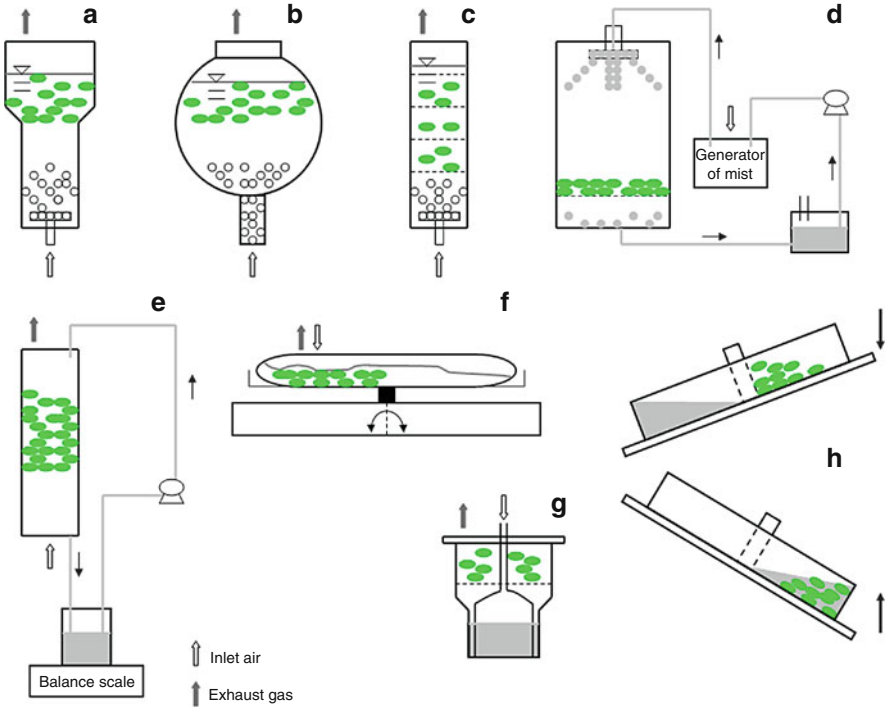


Fig. 1.3 Bioreactor configurations for plant organ culture. (**a**) Bubble-column bioreactor, (**b**) Balloon type bubble bioreactor, (**c**) Column photo-bioreactor with internal sections, (**d**) Mist bioreactor, (**e**) Trickle-bed bioreactor, (**f**) Wave bioreactor, (**g**) Temporary immersion system RITA®, (**h**) BioMINT reactor (Georgiev et al. [2] and the literature cited therein)

(Fig. 1.3b), appeared quite suitable for large-scale biomass and metabolite mass production by adventitious root culture of *Panax ginseng*, *Hypericum perforatum*, *Morinda citrifolia* and *Echinacea* (Baque et al. [19] and the literature cited therein). In a recent interesting study, Georgiev et al. [43] reported the successful cultivation of *Leucojum aestivum* shoot culture in modified glass-column bioreactor with internal sections (Fig. 1.3c) for production of galanthamine (naturally occurring alkaloid used in the treatment of mild-to-moderate Alzheimer's disease, marketed as Nivalin® and Reminyl®). The introduction of internal sections ensures submerged cultivation of the shoot culture and adequate mass and oxygen transfer, which resulted in high biomass accumulation (>20 g L^{-1}) and galanthamine production (1.7 mg L^{-1}).

Furthermore, mist bioreactor (Fig. 1.3d), trickle-bed reactor (Fig. 1.3e), wave induced bioreactor (Fig. 1.3f) as well as several temporary immersion systems (Fig. 1.3g, h) have been validated as suitable for cultivation of plant organ culture (Table 1.2) (recently reviewed in Georgiev et al. [2]). It should be, however, noted that most of the temporary immersion systems are still of laboratory-scale, therefore, further more detailed experiments in large-scale volumes are pending to prove their efficacy.

Table 1.2 Selected examples of bioreactor configurations (operated in batch mode) used for cultivating plant organ cultures

Bioreactor type[a]	Plant organ culture[b]	Product	Final density (g DW/L)/productivity [g DW/(L day)][c]	Reference
Mechanically driven				
Stirred tank reactor (5 L)	*Beta vulgaris* HR	Betalains	12.9/0.68	Georgiev et al. [36]
Stirred tank reactor (3 L)	*Harpagophytum procumbens* HR	Iridoids	8.98/0.64	Homova et al. [37]
Stirred tank reactor with separate impeller (25 L)	*Atropa belladonna* HR	Tropane alkaloids	6.02/0.20	Lee et al. [38]
Wave reactor (0.5 L)	*Panax ginseng* HR	Ginsenoside	11.6/0.41	Palazon et al. [39]
Pneumatically driven				
Bubble column reactor (2 L)	*Harpagophytum procumbens* HR	Iridoids	6.6/0.31	Ludwig-Müller et al. [40]
Bubble column reactor (2 L)	*Beta vulgaris* HR	Betalains	12.7/0.79	Pavlov et al. [41]
Air-lift reactor (2 L)	*Nicotiana tabacum* HR	Murine interleukin-12	4.8/0.34	Liu et al. [42]
Column photo-bioreactor with internal sections (1 L)	*Leucojum aestivum* SC	Galanthamine	20.8/0.59	Georgiev et al. [43]
Balloon type bubble bioreactor (1,000 L)	*Echinacea purpurea* AR	Caftaric, chlorogenic and cichoric acid	5.1/0.15	Baque et al. [19]
Bed reactors				
Mist reactor (4 L)	*Nicotiana tabacum* HR	Murine interleukin-12	5.2/0.37	Liu et al. [42]
Mist reactor (1.5 L)	*Artemisia annua* HR	Artemisinin	14.4/0.38	Kim et al. [44]
Mist trickle reactor (5 L)	*Centaurium erythraea* SC	Secoiridoids	17.76/0.63	Piatczak et al. [45]
Trickle bed (14 L)	*Hyoscyamus muticus* HR	No product	36.2/1.45	Ramakrishnan and Curtis [46]

Modified after Georgiev et al. [32]

[a]Bioreactor working volumes are given in parenthesis

[b]*HR* hairy root culture, *SC* shoot culture, *AR* adventitious root culture

[c]*DW* dry tissue weight

Industrialization of plant organ culture bioprocesses is not yet fully developed, mostly due to the morphological features of differentiated *in vitro* cultures and the resultant challenges [2, 34]. One such challenge is the way to transfer plant tissue inocula from seed reactor to large-scale reactor, which apparently cannot be preformed pneumatically (as usually done in suspended culture-based processes). The recently developed commercial system at 10 m^3-scale for biomass and bioactive ginsenoside production from *Panax ginseng* adventitious roots by CBN Biotech Company, South Korea [19] could contribute towards solving "inocula transfer" issues. Monitoring of the plant tissue growth in bioreactors during the cultivation process (up to several weeks) is another worth mentioning challenging issue [2, 34]. A number of methods are, therefore, developed for (indirect) estimation of the tissue growth (in *off-line* or in *on-line* mode), such as measuring conductivity, osmolarity and redox potential of the culture medium (thoroughly summarized in Georgiev et al. [47]). Nevertheless, more reliable and accurate methods for biomonitoring are continuously sought.

1.5 Conclusions and Perspectives

For ca. 60 years of research, we have witnessed profound changes in development of plant *in vitro* culture-based bioprocesses for mass production of biomass, and plant derived-molecules and therapeutic proteins, which clearly outlined their immense potential for commercialization. Dozens of commercial processes were then developed and several others are on the pipeline. Nowadays, the design and configuration of bioreactors used adequately reflect the physiological requirements of plant cell and organ culture. It increasingly appears likely that single-use bioreactors, originally developed for highly sensitive mammalian culture, will become more often used (e.g. 3D bioreactor-based systems) for growing plant cells that do not exhibit Newtonian fluid behaviour. Wider commercialization of plant *in vitro* culture-based processes implies the development of more reliable methods for bioprocess monitoring (for plant organ culture bioprocesses in particular) and improvement of overall process performance. Recent and emerging "omics" platforms (and metabolomics in particular) are likely to accelerate this process.

References

1. Chisti Y, Moo-Young M (2002) Bioreactors. In: Meyers RA (ed) Encyclopedia of physical science and technology, vol 2. Academic, San Diego, pp 247–271
2. Georgiev MI, Eibl R, Zhong JJ (2013) Hosting the plant cells *in vitro*: recent trends in bioreactors. Appl Microbiol Biotechnol 97:3787–3800
3. Georgiev MI, Weber J (2014) Bioreactors for plant cells: hardware configuration and internal environment optimization as tools for wider commercialization. Biotechnol Lett 36:1359–1367

4. Routien JB, Nickell LG (1956) Cultivation of plant tissue. US Patent 2,747,334
5. Sajc L, Grubisic D, Vunjak-Novakovic G (2000) Bioreactors for plant engineering: an outlook for further research. Biochem Eng J 4:89–99
6. Veliky IA, Martin SM (1970) A fermenter for plant cell suspension cultures. Can J Microbiol 16:223–226
7. Verpoorte R, Contin A, Memelink J (2002) Biotechnology for the production of plant secondary metabolites. Phytochem Rev 1:13–25
8. Georgiev MI, Weber J, Maciuk A (2009) Bioprocessing plant cell cultures for mass production of targeted compounds. Appl Microbiol Biotechnol 83:809–823
9. Kurz WGW, Constabel F (1979) Plant cell culture, a potential source of pharmaceuticals. Adv Appl Microbiol 25:209–240
10. Kato A, Kawazoe S, Lijima M, Shimizu Y (1976) Continuous culture of tobacco cells. J Ferment Technol 54:82–87
11. De Luca V, Salim V, Atsumi SM, Yu F (2012) Mining the biodiversity of plants: a revolution in the making. Science 336:1658–1661
12. Onrubia M, Cusido RM, Ramirez K, Hernandez-Vazquez L, Moyano E, Bonfill M, Palazon J (2013) Bioprocessing of plant *in vitro* systems for the mass production of pharmaceutically important metabolites: paclitaxel and its derivates. Curr Med Chem 20:880–891
13. Wilson SA, Roberts SC (2012) Recent advances towards development and commercialization of plant cell culture processes for the synthesis of biomolecules. Plant Biotechnol J 10:249–268
14. Georgiev MI (2013) Coming back to nature: plants as a vital source of pharmaceutically important metabolites – part II A. Curr Med Chem 20:851
15. Food and Agriculture Organization of UN (2009) Global agriculture towards 2050. www.fao.org/fileadmin/templates/wsfs/docs/Issues_papers/HLEF2050_Global_Agriculture.pdf. Retrieved March 2014
16. Davies KM, Deroles SC (2014) Prospects for the use of plant cell cultures in food biotechnology. Curr Opin Biotechnol 26:133–140
17. Lim E-K, Bowles D (2012) Plant production systems for bioactive small molecules. Curr Opin Biotechnol 23:271–277
18. Zhong JJ (2002) Plant cell culture for production of paclitaxel and other taxanes. J Biosci Bioeng 94:591–599
19. Baque MA, Moh SH, Lee EJ, Zhong JJ, Paek KY (2012) Production of biomass and useful compounds from adventitious roots of high-value added medicinal plants using bioreactor. Biotechnol Adv 30:1255–1267
20. Huang T-K, McDonald KA (2012) bioreactor systems for *in vitro* production of foreign proteins using plant cell cultures. Biotechnol Adv 30:398–409
21. Eibl R, Eibl D (2008) Design of bioreactors suitable for plant cell and tissue cultures. Phytochem Rev 7:593–598
22. Eibl R, Kaiser S, Lombriser R, Eibl D (2010) Disposable bioreactors: the current state of-art and recommended applications in biotechnology. Appl Microbiol Biotechnol 86:41–49
23. Eibl R, Brändli J, Eibl D (2012) Plant cell bioreactors. In: Doelle HW, Rokem S, Berovic M (eds) Biotechnology, encyclopedia of life support systems. EOLSS Publishers, Oxford (www.eolss.net)
24. Kwon J-Y, Yang Y-S, Cheon S-H, Nam H-J, Jin G-H, Kim D-I (2013) Bioreactor engineering using disposable technology for enhanced production of hCTLA4Ig in transgenic rice cell cultures. Biotechnol Bioeng 110:2412–2424
25. Raven N, Schillberg S, Kirchhoff J, Brändli J, Imseng N, Eibl R (2011) Growth of BY-2 suspension cells and plantibody production in single-use bioreactors. In: Eibl R, Eibl D (eds) Single-use technology in biopharmaceutical manufacture. Wiley, Hoboken, pp 251–261
26. Doran PM (2013) Therapeutically important proteins from *in vitro* plant tissue culture systems. Curr Med Chem 20(8):1047–1055
27. Georgiev MI, Ludwig-Mueller J, Weber J, Stancheva N, Bley T (2011) Bioactive metabolites production and stress-related hormones in devil's claw cell suspension cultures grown in bioreactors. Appl Microbiol Biotechnol 89:1683–1691

28. Perez JAS, Porcel EMR, Lopez JLC, Sevilla JMF, Chisti Y (2006) Shear rate in stirred tank and bubble column bioreactors. Chem Eng J 124:1–5
29. Alipieva K, Korkina L, Erdogan Orhan I, Georgiev MI (2014) Verbascoside – a review of its occurrence, (bio)synthesis and pharmacological significance. Biotechnol Adv. doi:10.1016/j.biotechadv.2014.07.001
30. Terrier B, Courtois D, Henault N, Cuvier A, Bastin M, Aknin A, Dubreuil J, Petiard V (2007) Two new disposable bioreactors for plant cell culture: the wave and undertow bioreactor and the slug bubble bioreactor. Biotechnol Bioeng 96:914–923
31. Wolfson W (2013) Grown your own: Protalix BioTherapeutics produces drugs in carrot cells. Chem Biol 20:969–970
32. Georgiev MI, Ludwig-Mueller J, Bley T (2010) Hairy root culture: copying nature in new bioprocesses. In: Arora R (ed) Medicinal plant biotechnology. CAB International, Wallingford, pp 156–175
33. Murthy HN, Lee E-J, Paek K-Y (2014) Production of secondary metabolites from celland organ cultures: strategies and approaches for biomass improvement and metabolite accumulation. Plant Cell Tiss Organ Cult 118:1–16
34. Georgiev MI, Agostini E, Ludwig-Mueller J, Xu J (2012) Genetically transformed roots: from plant disease to biotechnological resource. Trends Biotechnol 30(10):528–537
35. Stiles AR, Liu C-Z (2013) Hairy root culture: bioreactor design and process intensification. Adv Biochem Eng Biotechnol 134:91–114
36. Georgiev MI, Pavlov AI, Bley Th (2006) Betalains by transformed *Beta vulgaris* roots in stirred tank bioreactor: batch and fed-batch processes. In: Sorvari S, Toldi O (eds) Proceedings of second international congress on bioreactor technology in cell, tissue cultures and biomedical applications. Karhukopio OY, Turku, pp 22–28
37. Homova V, Weber J, Schulze J, Alipieva K, Bley T, Georgiev M (2010) Devil's claw hairy root culture in flasks and in a 3-L bioreactor: bioactive metabolite accumulation and flow cytometry. Z Naturforsch 65c:472–478
38. Lee K-T, Suzuki T, Yamakawa T, Kodama T, Igarashi Y, Shimomura K (1999) Production of tropane alkaloids by transformed root cultures of *Atropa belladonna* in stirred tank bioreactors with a stainless steel net. Plant Cell Rep 18:567–571
39. Palazon J, Mallol A, Eibl R, Lettenbauer C, Cusido RM, Pinol MT (2003) Growth and ginsenoside production in hairy root cultures of *Panax ginseng* using a novel bioreactor. Planta Med 69:344–349
40. Ludwig-Müller J, Georgiev M, Bley T (2008) Metabolite and hormonal status of hairy root cultures of Devil's claw (*Harpagophytum procumbens*) in flasks and in a bubble column bioreactor. Process Biochem 43:15–23
41. Pavlov AI, Georgiev MI, Bley T (2007) Batch and fed-batch production of betalains by Red beet (*Beta vulgaris*) hairy roots in a bubble column reactor. Z Naturforsch 62c:439–446
42. Liu C, Towler MJ, Medrano G, Cramer CL, Weathers PJ (2009) Production of mouse interleukin-12 is greater in tobacco hairy roots grown in a mist reactor than in an airlift reactor. Biotechnol Bioeng 102:1074–1086
43. Georgiev V, Ivanov I, Berkov S, Ilieva M, Georgiev M, Gocheva T, Pavlov A (2012) Galanthamine production by *Leucojum aestivum* L. shoot culture in a modified bubble column bioreactor with internal sections. Eng Life Sci 12:534–543
44. Kim YJ, Weathers PJ, Wyslouzil BE (2002) Growth of *Artemisia annua* hairy roots in liquid- and gas-phase reactors. Biotechnol Bioeng 80:454–464
45. Piatczak E, Chmiel A, Wysokinska H (2005) Mist trickling bioreactor for *Centaurium erythraea* Rafn growth of shoots and production of secoiridoids. Biotechnol Lett 27:721–724
46. Ramakrishnan D, Curtis WR (2004) Trickle-bed root culture bioreactor design and scale-up: growth, fluid-dynamics and oxygen mass transfer. Biotechnol Bioeng 88:248–260
47. Georgiev MI, Pavlov A, Bley T (2007) Hairy root type plant *in vitro* systems as sources of bioactive substances. Appl Microbiol Biotechnol 74(6):1175–1185

Chapter 2
Disposable Bioreactors for Cultivation of Plant Cell Cultures

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Abstract The trend for using disposable bioreactors in modern biotechnological processes has also been adopted for plant cell cultivations. In fact, plant cell cultures are now being grown in disposable bioreactors with volumes up to 400 L. This trend has been witnessed for both the development and commercial manufacture of therapeutic proteins, secondary metabolite-based pharmaceuticals and cosmetic compounds. Prominent examples of commercial products are Protalix's ELELYSO and Mibelle Biochemistry's Phyto Cell Tech-derived bioactive compounds.

This chapter discusses the current state of disposable bioreactor technology for plant cell cultures. After a brief introduction to the general fundamentals of disposable bioreactors (relevant technical terms, advantages and limitations of disposable bioreactors) a current overview of disposable plant cell bioreactors and their instrumentation will be provided. We will describe the working principles and engineering characteristics of disposable bioreactor types that are scalable and successfully being used for the cultivation of plant cell suspension and hairy root cultures. In addition, we will provide selected application examples focusing on the cultivation of geraniol producing tobacco cells. The chapter will end with perspective on future developments of disposable bioreactor technology for plant cell cultures.

Keywords Bubble column • Disposable bioreactor technology • Hairy root culture • Instrumentation • Mist bioreactor • Orbitally shaken • Oscillating • Plant cell suspension culture • Recombinant protein • Secondary metabolite • Stirred • Tobacco

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