PLANT CELL BIOREACTORS

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Contents

Summary

For more than 50 years there have been intensive efforts to produce secondary metabolites, under sustainable and controlled conditions, for the pharmaceutical, food and cosmetic industries. This has resulted in the establishment of high productivity plant cell and tissue cultures, and the development of suitable *in vitro* production processes, in which undifferentiated plant cell suspension cultures predominate, despite possible somaclonal variations. In only a few cases are root cultures, which are more difficult to grow, used.

Meanwhile, commercially manufactured niche products include successful secondary metabolites such as Paclitaxel from Phyton (important for cancer therapies), mountain Ginsenosides from CBN Biotech (of interest for numerous diverse applications) or the PhytoCellTec compounds (PhytoCellTec Alp Rose, PhytoCellTEcArgan, PhytoCellTecMalusDomestica, PhytoCellTec Solar Vitis) from Mibelle Biochemistry (used in the manufacture of anti-aging products). The first pharmaceutical plant cell-derived proteins, such as Dow AgroSciences' Hemagglutinin-Neurominid protein from the Newcastle disease virus and Protalix'sTaliglucerase α, have already been approved.

To date, plant cell and tissue culture cultivations have been realized in bioreactors with capacities of up to 75m^3. The majority of the bioreactors that have been used are mechanically and pneumatically driven liquid-phase systems, or hydraulically driven

gas-phase systems, with cultivation containers that are either reusable or intended for single-use only.

Stirred bioreactors with bubble column modifications predominate among reusable systems, whereas wave-mixed bioreactors with bubble columns are the most often used single-use systems.

Based on typical plant cell and tissue culture characteristics, the most important demands on plant cell bioreactors, their operating mode and necessary instrumentation are delineated. Furthermore, a categorisation of suitable bioreactors, according to continuous phase and power input, is given. In the subsequent bioreactor description, a distinction is made between reusable systems for plant cell suspensions and root cultures, and single-use bioreactors for plant cell and tissue cultures. In conclusion, apparent trends for plant cell bioreactors are discussed. Neither shoots, embryogenic cultures or plantlets utilized for micropropagation and plant breeding purposes, or bioreactors used for their cultivation are presented in this chapter.

1. General Theoretical Background of Plant Cell and Tissue Cultures and Their Processes

The predominance of suspension cultures (among plant cell and tissue culture types used in *in vitro* production processes) can be explained by their less complicated morphology and easy scalability. A single plant cell can reach a size of between 100-500µm in length and 20-50µm in diameter. This means that a plant cell is between 10 and 100 times larger in size than a bacterial or animal cell. For this reason, plant cells double and grow more slowly. In fact, they normally have doubling times of several days and cultivation times of several weeks, resulting in high demands on a bioreactor's design and sterility.

Plant suspension cells, being spherical and elongated in shape, seldom grow as single cells. They tend to form huge aggregates caused by secretion of extracellular polysaccharides from the cell wall, especially in later growth stages. Good cell growth (meaning doubling times of five days or less) often results in a strong increase in the viscosity of the culture broth, which, as a result, displays non-Newtonian fluid flow behavior (e.g. Bingham plastic). This phenomenon has to be taken into account when choosing or designing the inoculation, transfer, sampling and harvesting devices used for a bioreactor. In order to prevent mass transfer limitations (in terms of nutrients and oxygen) in non-Newtonian culture broths within a bioreactor, sufficient power input must also be guaranteed. This in turn could lead to high shear forces, resulting from agitation and aeration, that act on the cells, potentially causing damage to them. Such damage (which can be both reversible and irreversible) is identifiable through morphological changes, a shrinking in cell viability, a reduction in product quantity and/or quality, and, in the worst case, immediate cell lysis.

Due to the presence of a cellulose cell wall, plant cells are regarded as being more robust than their animal counterparts, which only possess a cell membrane. Their shear-sensitivity is classified as moderate and attributed to their high volume of intracellular vacuoles (up to 90 % of the entire cell volume). For instance, it has been demonstrated

that plant cells, if they are relatively large in size and contain many vacuoles, have a high sensitivity to shear stress. This phenomenon was observed during the late, exponential growth and early, stationary growth phases of cells.

Shear-sensitivity of plant cells is also influenced by other culture-specific factors, such as plant species, culture age and the rheological properties of the culture broth.

Root cultures are even more sensitive to shear stress than plant suspension cells. Changes in root length, thickness, branching, hairiness, and, callus formation at the root tips are indicators of root stress. There are two types of root cultures that have proven themselves to be effective in *in vitro* production processes: untransformed roots and hairy roots. Hairy roots generated through the transformation of *Agrobacterium rhizogenes* strains are more stable in geno- and phenotype than untransformed roots and plant cell suspension cultures. They grow more quickly than untransformed roots, and at a similar rate to suspension cultures. Hairy roots are able to deliver comparable amounts of secondary metabolites and proteins as plant cell suspension cultures. However, in hairy root cultivations, root tissue integrity should be maintained and non-uniform, overly dense biomass should be avoided. This can be ensured, *inter alia*, by root immobilization using vertical or horizontal meshes, cages or polyurethane foam carriers (also referred to as support matrix or scaffolding for root immobilization) in the bioreactor. This does, however, complicate the process of scaling these cultures up (see also Section 3.2).

Independent of plant cell and tissue culture type, product formation is either intra- (in vacuoles) or extracellular (secretion) and growth- or non-growth associated. The product formation mode influences the optimum bioreactor mode (batch, fed batch or continuous) and the equipment used. While feeding using culture medium or medium constituents (e.g. sucrose) is advantageous in growth associated product formations, growth and product formation are decoupled for non-growth associated processes. In the latter case, so-called two-phase processes are preferred, which generally rely on a growth and a production medium, both of which differ in their composition. In order to accomplish medium exchange and cell retention, internally or externally installed devices are required for plant cell suspension cultures. Because root tissues have an inherent barrier to liquid cell culture mediums, medium exchange in bioreactors using hairy root cultivations is clearly technically feasible. Up to now, the temperature shift that is performed at the beginning of the production phase for animal cell production processes has had no relevance for plant cell and tissue cultures.

Feature	**Range**
Temperature	23-29°C
pH[1]	5.0-6.0
Aeration rate	0.1-0.5vvm
Light[2]	0-10klux

[1] Addition of 0.1-0.5% CO_2 can stimulate cell growth and product expression, [2] Periodic (dark/light cycle:8h :16h) or continuous illumination

Table 1. Typical culture conditions for plant cell and tissue cultures

Appropriate ranges for temperature, pH, aeration rate, illumination intensity and duration (all depicted in Table 1) have to be managed in the bioreactor. Illumination (by externally positioned fluorescent lamps, internal illumination systems made with fluorescent tubes or glass fibers, or locating the bioreactor in illuminated rooms) is not always required. However growth and, in particular, product formation can be promoted by the periodical or continuous introduction of light in the case of heterotrophic, photomixotrophic, and photoautotrophic cultures.

2. The Plant Cell Bioreactor

2.1 Bioreactor Definition and General Instrumentation

The bioreactor plays a decisive role in process development and optimization. The simplest design is a non-instrumented cultivation container (e.g. plate, tube, flask) that is operated in an incubator or on a shaker. External equipment is responsible for the monitoring and control of essential cultivation parameters such as temperature and shaker speed. Such systems are small-scale, and are well known from screening experiments (cell line, culture medium).

Larger scale cultivations are carried out in instrumented vessels and bags, and the bioreactors possess their own measurement and control units. Typically, this means that temperature; agitation speed, aeration rate and dissolved oxygen (DO) are monitored and controlled online. Furthermore, plant cell bioreactors have online sensors for foam, pressure, filling level, pH and conductivity.

Foam break down is performed almost exclusively by chemical silicon- and polypropylene-based agents. They reduce the surface tension of the culture broth, can inhibit (particularly at high concentrations) or conversely, even promote cell growth. Oxygen transfer in the bioreactor can also be impaired and downstream processing of the product complicated by the addition of an antifoam agent. Since plant cells tolerate a wide pH range, buffer substances contained in the chemically defined culture medium are sufficient to regulate the pH. With only a few exceptions, pH is not regulated by the addition of acid (CO_2) or base. However, pH monitoring is routinely performed in order to detect contamination and to indirectly observe initial ammonium assimilation (decrease of pH to a value of approximately 4) and later nitrate assimilation (increase of pH to a value of approximately 5). Another indirect method of determining plant cell growth is to measure the conductivity in the bioreactor. There is an inverse relationship between electrical conductivity and cell weight for different plant cell and tissue cultures that express intracellular products. A linear increase in dry weight correlates to a decrease in medium conductivity. Finally, despite the low respiration rate of plant cells, implementation of gas analytic systems for process monitoring is possible, but seldom used.

2.2 Plant Cell Bioreactor Categories

Numerous bioreactor types, used for the successful mass propagation of plant cell and tissue cultures, and the expression of their products, have been described in the literature. These are most commonly categorized according to the continuous phase that

exists within the bioreactor and its power input. Static systems (characterized by unenforced power input and only used in mL scale) play only a minor role. Dynamic bioreactors guaranteeing higher biomass or cell densities and higher product titre volumes become more important as bioreactor volume increases. By comparing their continuous phases, dynamic plant cell bioreactors can be subdivided into liquid-phase bioreactors, gas-phase bioreactors and hybrid bioreactors combining both types, as shown in Figure 1.

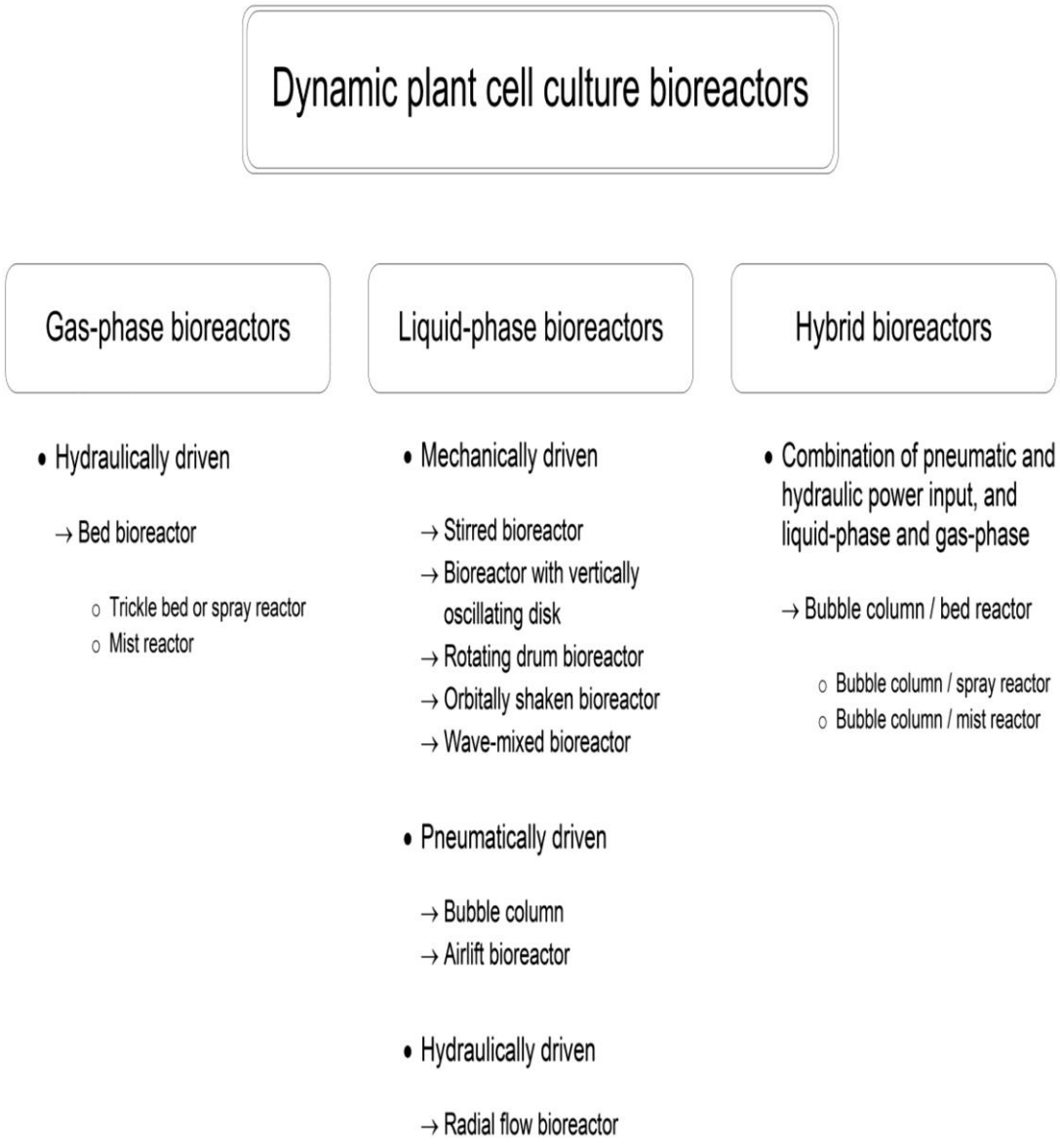

Figure 1. Categories of dynamic plant cell bioreactors according to continuous phase and power input

In liquid-phase plant cell bioreactors, cells are continuously immersed in the culture medium as air is introduced into the bioreactor. Mechanically, pneumatically and hydraulically driven plant cell bioreactors belong to this category of bioreactors. The largest group consists of those bioreactor systems which have internal or external

mechanically driven devices. These include plant cell bioreactors that are stirred, operated with a disk that vertically oscillates, rotated, orbitally shaken, or wave-mixed as a consequence of horizontal oscillation.

Although stirred bioreactors with a rotating impeller are most often used, their design must meet the highest hygiene standards. For example, safe dynamic sealing between the bioreactor's interior, its shaft and the environment has to be ensured. For this purpose, double mechanical seals or (alternatively) magnetic couplings deliver optimal performance. Tumbling und vibrating shafts employ static (bellows, metal compensators) and not dynamic seals. Pneumatically driven plant cell bioreactors use an even simpler design (no moving parts, shaft and seals). In these bioreactors, mass and heat transfer are affected by direct gassing of a tall vessel, whereas a compressed air or gas mixture is introduced at the bottom of the vessel through a sparger for aeration. Mixing and fluid circulation then result from rising bubbles in the culture broth. The most prominent examples are bubble columns and airlift bioreactors. The latter of which can be regarded as a modified bubble column bioreactor.

The main feature of airlift bioreactors is that the fluid circulates via a closed external or internal liquid circulation loop, which permits improved fluid flow and mixing in comparison to bubble columns. The circulation loop is created by the mechanical separation of an upflow and a downflow gas/liquid channel, which are then connected at the top and the bottom of the cultivation vessel. Possible disadvantages of bubble column and airlift bioreactors, despite both having more homogeneous energy dissipation than stirred bioreactors, are inadequate mixing, foaming, flotation and bubble coalescence for plant cell cultivations in which strong variations in biomass concentration and viscosity occur. Hence, bubble column and airlift bioreactors without additional design modifications are not suitable for highly viscous culture broths and high biomass concentrations.

In gas-phase bioreactors solely used for growing root cultures, the roots that are immobilized on a matrix, are continuously exposed to humidified air or other gas mixtures. The cells are periodically (seldom continuously) subjected to the nutrients from the culture medium, which is pumped from a medium storage vessel and delivered in the form of droplets which are generated by spray nozzles, compressed gas atomizers or ultrasonic transducers.In order to accomplish the optimum spray/mist cycle, it is usual for the pump to be coupled to a timer. A distinction can be made between spray or trickle-bed and mist bioreactors, all characterized by lower shear stress than the previously described liquid-phase bioreactors.

Hybrid plant cell bioreactors are alternately operated with liquid and gas as the continuous phase. They were developed for the purpose of sterile transfer and in order to homogeneously distribute large amounts of root material at benchtop and pilot scales. Distribution of inocula is performed by fluid spreading in the bioreactor. Usually, after inoculation the bioreactor is switched from liquid to gas-phase mode, an approach that can contribute to a shortening of the cell growth phase.

The plant cell bioreactors presented in Figure 1 are available with non-sterile, autoclavable cultivation containers made from either glass, stainless steel or (more

rarely) plastic (referred to as reusable or standard bioreactors, or disposable cultivation containers). Disposable (single-use) cultivation containers are rigid or flexible and are always made of FDA-compliant plastic materials (polypropylene, polysulfone, polyethylene, polytetrafluorethylene, ethylene vinyl acetate). It is also worth mentioning that with increasing volume, flexible cultivation containers, called bags, require a collecting tray or support container, which fix, stabilize and shape the bag. Disposable cultivation containers are sterile and ready to use. After the final product harvest, they are decontaminated and disposed of. By using single-use plant cell bioreactors, time- and cost-intensive cleaning and sterilization procedures become unnecessary.

Furthermore, time for development and production, as well as overall time-to-market can be reduced for plant cell and tissue-derived products. The risk of contamination is lower, so processes in single-use bioreactors become safer. However, potential cost savings are only achievable for high-value products, due to the current high prices for single-use cultivation bags equipped with standard or single-use sensors. Interestingly, there have been no reports of leachables and extractables secreting from plastic bags and inhibiting cell growth for plant cell and tissue cultures. Despite this, reusable plant cell bioreactors currently predominate in research and development and commercial manufacturing.

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Biographical Sketches

Regine Eibl was born on May 9th, 1964 in Meiningen (Germany). She earned an engineering degree in biotechnology from the Technical University in Köthen (Germany) in 1987. In 1990 she finished her training as a translator for biological sciences (German-English) at the Martin Luther University in Halle (Germany) and received a Ph.D. in biotechnology from the Technical University in Köthen. In 1991 Regine Eibl moved to Switzerland.

Since 1993 she has been working at the University of Applied Sciences in Wädenswil (Switzerland) as a *lecturer* (bachelor and master level), as well as being part of a *working group* ("cell cultivation techniques") and a *project leader*. Regine Eibl has an extensive background in both plant and animal cell cultivation techniques and is a well-known expert in bioreactor and process development, particularly in conjunction with single-use technologies. She has a specialist interest in the following product areas: biomass, secondary metabolites, and proteins for pharmacy and cosmetics. She was *guest scientist* at the pharmacy departments of the University of Helsinki (Finland) and the University of Barcelona (Spain) and can draw on a strong professional network. In 2002, Regine Eibl successfully finished her postgraduate studies in scientific communication at the former University of Winterthur (Switzerland). For 5 years she has been very active in giving further training courses in cell cultivation techniques for industrial clients in Switzerland and abroad.

Prof. Eibl is the platform leader for "Single-use technology" of the Swiss Biotechnet and a member of the DECHEMA (Society for Chemical Engineering and Biotechnology), the ISPE (International Society for Pharmacoepidemiology) and the ESACT (European Society for Animal Cell Technology). She is the editor of three books, and author of numerous research papers and book chapters. Prof. Regine Eibl's most recent publications include the following:

Eibl R., Eibl D., Pörtner R., Catapano G., Czermak P. (2009). Cell culture and tissue reaction engineering. Berlin Heidelberg: Springer.

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Johanna Brändli was born on January 17th, 1970 in Switzerland. After finishing school in Wattwil in 1988 with a Type B Maturity, she studied medicine and pharmacy in Zurich and biotechnology at the University of Applied Sciences in Wädenswil (Switzerland). In 1998 Johanna Brändli received her diploma (Dipl. Ing. FH Biotechnology).

She has over 11 years of experience in plant and animal cell cultivation techniques. Prior to joining Cytos Biotechnology AG (Schlieren, Switzerland) in October 2000, she worked as a*scientific assistant* in cell culture technology at the University of Applied Sciences Wädenswil. At Cytos Biotechnology she held the position of *scientist*, where she worked for 6.5 years in upstream processing and optimized eukaryotic

as well as prokaryotic production cell lines, and their related processes. She was also responsible for developing new methods for quality control. In February 2007, she returned to the University of Applied Sciences in Wädenswil. She is currently serving as a *senior scientist* in the cell cultivation technique group of the Institute of Biotechnology.

In addition to her responsibility for quality assurance in the cell cultivation technique group, Ms. Brändli teaches practical aspects of plant cell cultivation techniques and investigates plant cell-based production processes aimed at products for the cosmetics and pharmaceutical industries. She has also been involved in writing papers and book chapters. Ms. Brändli's publications are listed below.

Ivanova L., Brändli J., Saudan Ph., Bachmann M. F. (2005). Hybrid Sindbis/Epstein-Barr virus episomal expression vector for inducible production of proteins.BioTechniques **39**, 209-212.

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Dieter Eibl was born on October 15th, 1957 in Bad Frankenhausen (Germany). He has held an engineering degree in "Foodstuff techniques" since 1981 and a Ph.D. in biotechnology from the Technical University in Köthen (Germany) since 1987.

His diverse work experience includes internships and temporary work placements in companies and universities in Russia, Poland, Slovakia and Germany. From 1981 to 1987, he was *senior assistant* at the Institute for Beverage Technology and Biotechnology of the Technical University Köthen and lectured in bioprocess techniques. Following a four month guest assignment at the University of Applied Sciences in Wädenswil (Switzerland – formerly HTL Wädenswil) in 1991, he started lecturing in chemical and biochemical engineering and developed the biotechnology course and department in Wädenswil. He is the current *head* of *"biochemical engineering and cell cultivation techniques"* and *working group leader for"*biochemical engineering" in the Institute of Biotechnology of the University of Applied Sciences in Wädenswil. Dieter Eibl brings more than 30 years of professional expertise in bioreactor and process development, scaling-up, facility design, project and team management, and fundraising. He collaborates with numerous well-known companies in Switzerland and abroad (e.g. GE Healthcare, Hoffmann-La Roche, Nestlé, Sartorius Stedim Biotech).

Prof. Eibl is member of the Society for Chemical Engineering and Biotechnology (DECHEMA, where he is also the leader of the temporary working group for "Single-use technology in biopharmaceutical manufacturing", the International Society for Pharmacoepidemiology (ISPE), the International Association for Pharmaceutical Technology (APV), the European Society for Animal Cell Technology (ESACT) and the Swiss Society of Food Science and Technology (SGLWT). He has edited three books, is author of more than 30 research papers and book chapters, and has been a speaker at various conferences and training sessions on single-use bioreactors and production facilities. A selection of his recent main publications is given below.

Eibl R., Eibl D. (2009). Application of disposable bag-bioreactors in tissue engineering and for production of therapeutic agents, Advances in Biochemical Engineering and Biotechnology **112**, 183-207.

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