BIOREACTORS AND CULTIVATION SYSTEMS FOR CELL AND TISSUE CULTURE

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Summary

Cell and tissue culture is the complex process by which cells, mostly of mammalian or plant origin, are grown under controlled conditions. The importance of cell and tissue culture in large scale is reflected in the many valuable products for human health. Products from mammalian cells such as monoclonal antibodies, cytokines, recombinant glycoproteins, and, increasingly, vaccines dominate the biopharmaceutical industry. Regenerative medicine using human primary and stem cells opens new therapeutic concepts. Additionally, plant cell and tissue cultures for the production of biologically active substances (low molecular secondary metabolites and recombinant proteins) are of growing importance. Numerous bioreactors and cultivation systems for cell culture,

either for production of biopharmaceuticals or for tissue engineering, have been developed. Due to the special characteristics of these cells specific solutions are required. The following contribution gives an introduction to the characteristics of cell culture technology and introduces bioreactor systems and cultivation strategies applied.

1. Introduction

Efficient cultivation of mammalian, tissue or plant cells requires comprehensive knowledge of biological as well as biochemical fundamentals (e.g. characteristics of cell growth and metabolism, cell line establishment, culture medium optimization) and related engineering principles (e.g. bioreactor design, process scale-up and optimization).

This chapter gives an introduction to the special demands on design and operation of the before mentioned cells due to their specific characteristics. It shall help to understand the importance of cell culture technology as well as differences compared to microbial fermentation technology.

The first sections are related to mammalian cells. Due to the importance of these techniques they are explained more in detail. Further sections address special cell culture applications having a high potential for further development, including the insect cell-based recombinant protein production and the plant cell-based bioprocessing. Finally, cultivation of primary tissue and stem cells is discussed.

2. Mammalian Cells

2.1. Products from Mammalian Cells

Mammalian cell culture or *animal cell culture* is devoted to the application of cells isolated originally from mammalian tissues and further cultivated and reproduced in an artificial medium under controlled conditions (*in vitro*) (Figure 1). An overview on applications is given in Figure 2.

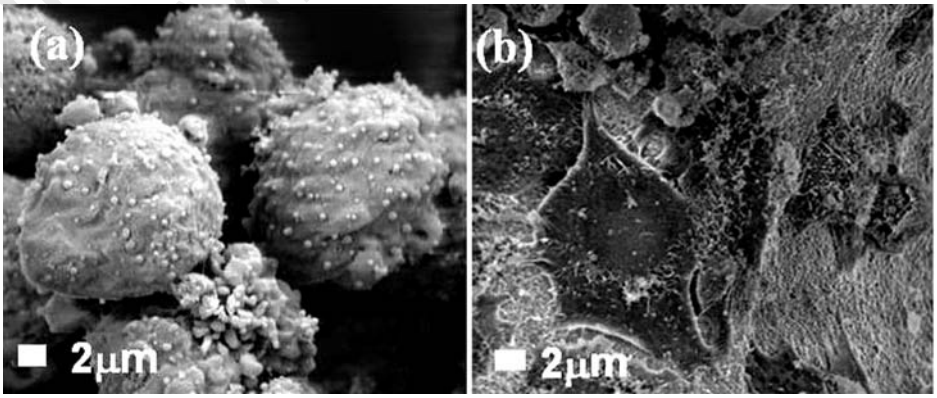

Figure 1. Examples for (a) cells for suspension growth (hybriodoma cells) and (b) adherent growing mammalian cells (HepG2 cells)

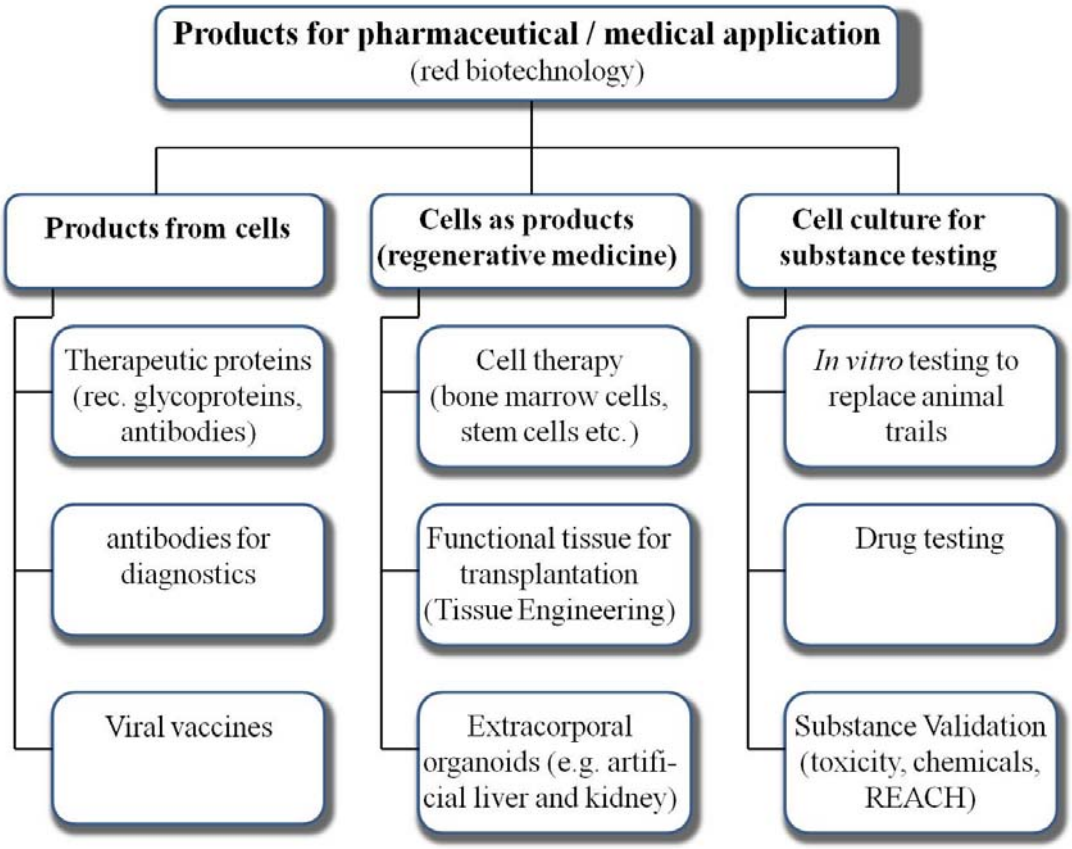

Figure 2. Fields of application for mammalian cells (from Eibl et al. 2009, modified, with kind permission from Springer Science+Business Media)

Mammalian cells synthesize a vast range of biopharmaceuticals ("products from cells" in Figure 2) that cannot be expressed by recombinant microbial techniques, including viral vaccines, antibodies for diagnostics, therapeutic proteins (rec. glycoproteins, antibodies).

Viral vaccines against polio, hepatitis B, measles, influenza or mumps for human use, rubella, rabies, or food-and-mouth-disease (FMD) for veterinary use, among others, are produced very efficiently by means of a cell-based vaccine technology. Genetically engineered or DNA-vaccines are under development. For vaccine production mainly primary cells, diploid cells or permanent cell lines are applied, recently even recombinant established cell lines (see Section 2.2).

Monoclonal antibodies are widely applied in diagnostics as well as in therapy. The antibody technology was first based on hybridoma cells. As these mostly mouse-derived antibodies cause severe immunogenic problems during application in humans, nowadays chimeric, humanized or human antibodies are produced by means of established cell lines (see Section 2.2). In diagnostics, tens of thousands different monoclonal antibodies are available having a specific binding to target molecules. Antibodies are applied in therapy as well, e.g. organ transplantation, cancer diagnostic and treatment, rheumatoid arthritis, leukemia, asthma, or multiple sclerosis. For some

antibodies the production scales have reached kilogram quantities. Recent developments are dealing with new antibody formats such as fragmented antibodies (FAb´s) or bivalent antibodies.

Production of recombinant glycoproteins as human therapeutic agents by mammalian cells, has been well established and in use since the 1990s. Here valuable products such as cytokines (e.g. Interferons and Interleukins), hematopoietic growth factors (e.g. Erythropoietin for treatment of anemia), growth hormones, thrombolytic agents (e.g. tissue plasminogen activator [tPA]), coagulation factors (factor VII, factor VIII, factor IX etc.), and recombinant enzymes (DNAse) are just a few. Most of these proteins cannot be produced in bacterial or yeast cells, as only mammalian cells are able to provide the required specific, human-like glycosylation pattern, which is difficult to obtain in other host systems. During the 2000s the main drawbacks of mammalian cell culture, e.g. high shear sensitivity, cell adherence requiring a surface for cell growth and low product yield have been overcome at least for most cell lines used in large scale production. Alternative expression systems such as transgenic animals are still under development and do not compete against 'classical' mammalian cell culture at present.

Regenerative medicine or tissue engineering is devoted to cell therapy (bone marrow cells, stem cells etc.), functional tissue for transplantation or extracorporal organoids (e.g. artificial liver and kidney) (“Cells as Products” in Figure 2). Here the challenge is to develop artificial organs in bioreactor systems (tissue engineering of liver, kidney) and tissues (skin, cartilage, bone), or the expansion of hematopoietic cells for bone marrow transplantation or gene therapy.

In drug development and drug testing new challenges arise from new regulatory requirements, mainly as animal trials have to be replaced by cell culture assays, preferably by test systems with human material (“Cells for substance testing” in Figure 2). Therefore cell cultures are applied for substance testing, e.g. *In vitro* testing to replace animal trials, drug testing, substance validation (toxicity, chemicals, REACH program). As 2 D monolayer cultures are often unsatisfactory, tissue-like 3 D cultures are investigated as an alternative. The exciting prospects of these new techniques are outlined in Section 5.

2.2. Properties and Medium Requirements of Mammalian Cells

Mammalian cells relevant for industrial processes can be characterized as follows:

- Primary cells – cells taken from the tissue and further grown *in vitro*, without doubling.
- Cell strains - When these cells start to divide, you will get a cell strain, with finite life time (30-40 generations) and the cells are unchanged.
- Permanent or established cell lines – cells that have gone through some transformations (in-finite life-span, used for expression of recombinant proteins).
- Hybridoma cells – cells obtained through the fusion of Lymphocytes and tumor cells, expression of monoclonal antibodies.

During cultivation of mammalian cells *in vitro*, outside of a living organism, some distinct difficulties arise from the extraction of the cells from a "safe" tissue. Slow growth rates with doubling times between 18 and 28 hours, low productivity, a high sensitivity against shear stress due to the lack of a cell wall and high demands in respect to the growth medium, all challenges for the techniques required for mammalian cells. Furthermore, many cell lines can only grow when adherent, and a suitable surface for attachment must be provided for these cells to proliferate. As of the origin from multi cellular organisms, mammalian cells still hold the genetic program of inducing apoptosis or "programmed cell death". This can limit culture productivity in biotechnological processes. Another major problem is the finite life-span of cell strains, which die after several doublings *in vitro*. This problem was solved by transforming cell strains into immortal "permanent" or "established" cell lines. Examples are given in Table 1.

Permanent cell lines	Characteristics
Baby Hamster Kidney (BHK)	adherent cells, can be adapted to suspension, used for production of foot & mouth disease vaccine, rabies vaccine, recombinant proteins (Factor VIII).
Chinese Hamster Ovary (CHO)	adherent cells, can be adapted to suspension, used for production of recombinant proteins (HBstg, tPA, Factor VIII).
COS (monkey kidney)	used for transient protein expression
HEK-293 (human embryonic kidney)	used for transient protein expression
MDCK (canine kidney)	adherent cell line with good growth characteristics, animal vaccines
MRC-5 (human embryonic lung cells)	"normal" cells with a finite life span, vaccine production
NAMALWA (human lymphatic tissue)	used for production of Alpha-Interferon
NS0 and SP2/0 (mouse-myeloma from B-lymphocytes)	used for antibody production
PERC.6 (Human embryonic retina cells)	immortalized cell line, well characterized, produce high levels of recombinant proteins and viruses
Vero (long-tailed monkey kidney)	established cell line, but with some characteristics of the normal diploid cells.

WI-38 (human embryonic lung cells)	"normal" cells with a finite life span, Vaccine production

Table 1. Examples for permanent cell lines

Adherent cells cause severe problems in large scale production, as they require large quantities of surface. One breakthrough for the industrial application of mammalian cells were the invention of microcarriers (e.g. for vaccine production) to support growth of adherent cells (see below). In modern industrial production mostly cells adapted to grow in suspension, e.g. cell lines derived from Baby Hamster Kidney cells (BHK) or Chinese Hamster Ovary cells (CHO), are used.

Much effort has been put in the development of appropriate cell culture media to ensure growth and product formation. Media that used to contain up to 10% serum were continuously improved, and the cultivation in defined serum-free and even chemically defined, protein-free media is now common for most relevant industrial cell lines. A growth medium for mammalian cells has to supply all the necessary nutrients required for growth and product formation, it should have enough buffer capacity to stabilize the pH (pH optimum 7.0 – 7.3) and should provide an appropriate osmolality in order to avoid damage of the sensitive cell membrane. Several basal medium formulations are in use, e.g. Eagle´s minimal essential medium (MEM), Dulbecco´s enriched (modified) Eagle´s medium (DMEM), Ham´s F12, and RPMI 1640 among others. As an example the main compounds of a 1:1 mixture of IMDM* and Ham´s F12 (* Iscove´s Modification of Dulbecco´s Medium) are given:

- Salts:

  $CaCl_2$; $CuSO_4$; $Fe(NO_3)$; $FeSO_4$; KCl; $MgSO_4$; $MgCl_2$; NaCl; $NaHCO_3$; NaH_2PO_4; $ZnSO_4$

- Vitamins (biotine; D-Ca pantothenate; choline chloride; folic acid; i-inositol; nicotineamide; pyridoxal; riboflavin; thiamine; vitamine B12)
- Amino acids (L-alanine; L-asparagine; L-arginine; L-aspartic acid; L-cysteine; L-glutamic acid; L-glutamine; Glycine; L-histidine; L-isoleucine; L-lysine; L-methionine; L-phenylalanine; L-proline; L-serine; L-threonine; L-tryptophane; L-tyrosine; L-valine)
- Other compounds (D-glucose; hypoxantine (sodium salt); linoleic acid; lipolic acid; phenol red; Putrescine; Thymidine; Na-pyruvate)

Often these basic media are supplemented with approx. 5-10 % serum, mostly fetal calf serum [FCS] to provide specific growth and adherence factors and to protect the cells against shear stress. Serum containing media have some disadvantages such as indefinite composition and the batch inconsistency of the serum, serum costs, protein contamination during product purification, and the risk of virus contamination. Serum can be partially substituted by the addition of transferin, insulin, ethanol amine, albumin or eventually fibronectin as adherence factor. These media are serum-free but still contain proteins. In more advanced chemically defined, protein-free media, the animal derived proteins can be replaced by iron salts or iron complexes, IGF-1, chemically defined lipid concentrates, precursors or other simulating agents such as fatty acids,

biotin, choline, glycerole, ethanolamine, thiol–(s), hormones and vitamins. Often peptone or yeast extracts are added. To reduce the sensitivity of cells to shear stress, protecting characteristics agents, such as Pluronic F68 are in use.
As different cell lines require different medium compositions, adaptation of a cell line to grow without serum is quite time consuming and not all cell lines have been adapted to serum-free or protein-free media. Therefore, for lab scale, e.g. basic cell culture research or cultivation of primary cells, still mostly complex, serum-containing media are common. In industrial production with established, optimized cell lines serum-free, bovine-free and chemically defined media are state of the art.

2.3. Bioreactors for Mammalian Cells

2.3.1. Categorization

Great progress was achieved in the between 1990 and 2010 regarding the development of mammalian cell culture technology. Stirred tank bioreactors providing low shear environment by especially designed agitation and aeration systems were developed. Cultivation systems for immobilized cells such as hollow-fiber, fluidized-bed and fixed-bed–bioreactors are intended to protect the cells from stressful conditions. Nowadays mammalian cells can be cultivated in volumes up to 20000L to produce the necessary quantities of a desired protein. The available cell culture systems used for production of biopharmaceuticals can be divided in non-agitated (multiwall plates, dishes and flasks, culture bags) and agitated systems. The latter can be further distinguished due to the mode of agitation. This can be

- mechanical, either by external devices (shaker, roller unit, rocker unit) or by internal devices (rotating shaft stirrer, tumbling shaft stirrer, oscillating vibromixer)
- hydraulic (perfused hollow fiber bioreactor, fixed bed and fluidized bed bioreactors)
- pneumatic (air-lift-bioreactor)

In the design and selection of cell culture bioreactors special demands should be considered, such as gentle agitation and aeration without cell damage, a well controlled environment with respect to pH, temperature, dissolved oxygen, dissolved CO_2-concentration etc., low levels of toxic metabolites (ammonia, lactate), high cell and product concentrations, optimized medium utilization, surface for adherent cells, and scalability. Selection of a reactor systems depends to a large extent on the specific purpose, e.g. production of a certain amount of protein (small amounts for basic scientific studies up to kg-quantities for medical application), single-purpose or multi-purpose facility. In the following the different concepts are discussed and compared.

The mentioned systems support growth of mammalian cells in one or the other way. On lab scale mostly disposable flask, membrane or bag-systems are the method of choice. Small suspension reactors as well as fixed bed and fluidized bed reactors are mostly used for research or process development. For larger amounts of products only suspension reactors or, up to a certain scale, fixed bed or fluidized bed reactors have the required scalability. Important criteria relevant for selection of a cultivation system for mammalian cells are given in Table 2.

characteristics	criteria
cells	morphology, shear sensitivity, doubling time, adherent or growth in suspension, process parameters (pH, temp., oxygen, CO_2), genetic stability, medium
product	stability, quantity, production kinetics
process	automation, scale, operation mode (*batch*, *fed-batch*, perfusion), cleaning, inoculum
administrative	regulatory affairs and GMP requirements

Table 2. Criteria relevant for selection of cultivation systems for mammalian cells

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Biographical Sketches

Prof. Dr.-Ing. Milan K. Popović, born in Sremska Mitrovica, Serbia finished his study of Food Technology at the University Novi Sad, Serbia 1965 and obtained his Ph.D. degree in Technical Chemistry at the Technische Universität Berlin, Germany in 1976. He worked as university assistant (teaching and research) at the University Novi Sad, Serbia, as research assistant at the Versuchs- und Lehranstalt für Spirituosenfabrikation Berlin, Germany and as research associate at the University of Waterloo, Canada. After years as senior constructing engineer at Karl Fisher Industrieanlagen GmbH Berlin, Germany he became professor in Biochemical Engineering at the FH Giessen-Friedberg (now TH Mittelhessen) and from 1989 on at the University of Applied Sciences (BHT), Berlin. He retired in 2009. His research and teaching area are chemical and biochemical reactor engineering, environmental engineering and in last years the food and white biotechnology.

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