BIOREACTOR PROCESSES BASED ON DISPOSABLE MATERIALS FOR THE PRODUCTION OF RECOMBINANT PROTEINS FROM MAMMALIAN CELLS

THÈSE N^{O} 3947 (2007)

PRÉSENTÉE LE 2 NOVEMBRE 2007

À LA FACULTÉ DES SCIENCES DE LA VIE

LABORATOIRE DE BIOTECHNOLOGIE CELLULAIRE

PROGRAMME DOCTORAL EN BIOTECHNOLOGIE ET GÉNIE BIOLOGIQUE

ÉCOLE POLYTECHNIQUE FÉDÉRALE DE LAUSANNE

POUR L'OBTENTION DU GRADE DE DOCTEUR ÈS SCIENCES

PAR

Matthieu STETTLER

Master of Science ETH

de nationalité suisse et originaire de Eggiwil (BE)

acceptée sur proposition du jury:

Prof. N. Stergiopulos, président du jury

Prof. F. M. Wurm, directeur de thèse

Dr T. Battle, rapporteur

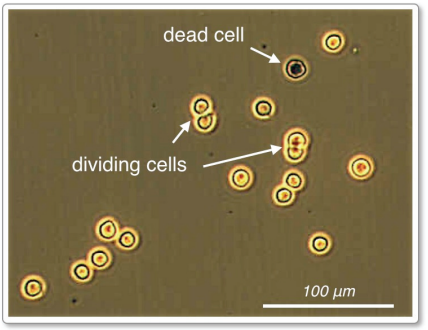
Prof. U. von Stockar, rapporteur

Prof. J. Weis, rapporteur

ÉCOLE POLYTECHNIQUE
FÉDÉRALE DE LAUSANNE

Suisse

2007



Single-cell suspension of Chinese hamster ovary cells

Abstract

The objective of industrial cell culture technology is to produce high value-added therapeutic proteins that are well suited for the treatment of infectious diseases, cancer, and autoimmune diseases. Nowadays, an important focus is the reduction of the time and costs from the discovery of the candidate molecule to the full-scale production. Relying on enabling technologies is critical for meeting this objective. Simultaneously, novel technologies are expected to increase the clinical and commercial success rates. In recent years, new concepts based on innovative disposable materials emerged and appeared to be promising alternatives to standard bioprocessing equipment. This thesis work was focused on the use of such disposable compounds in mammalian cell culture applications, particularly for the containment of the cells, and was aimed at demonstrating their benefit in terms of cost-effectiveness, ease-of-use and flexibility. First, a novel disposable packed cell volume tube was shown to be ideal for the quick and reliable assessment of biomasses. A characterization and validation of the measurement system demonstrated that it could replace time-consuming and less accurate cell counting methods. Then, to determine the appropriate growth conditions for cells in culture, small-scale single-use tubes were orbitally agitated with conventional lab shakers. This approach was found to be well-suited for multi-parameter optimization strategies. A systematic characterization of the liquid mass transfer in shake tubes proved that sufficient oxygen was available even at cell densities beyond 1 x 10^7 cells mL^{-1}. Non-invasive optical methods were used to assess the dissolved oxygen variations in various orbital shake vessels, from the milliliter to the multi-liter scale. This simple and yet powerful cultivation principle, orbital shaking, was scaled-up to pilot and production scales. To match the requirements in terms of oxygen transfer at larger scales, a given airflow was provided to replace the gas in the headspace. When required, the airflow was enriched with oxygen. At scales above 20 L, sterile disposable cell cultivation bags were used to contain the cells. Combining orbital shake technology and disposable cell culture bags with a cylindrical shape was promising, both in terms of efficiency and ease-of-use. Prototype shake bioreactors of 200 L and 1'500 L were designed, constructed and operated with maximal cell densities up to 5-7 x 10^6 cells mL^{-1} in batch cultivations of CHO cells. The benefits in terms of time and cost savings were even more obvious when novel shake bioreactors were compared with standard stirred-tank bioreactors. This was shown in a realistic optimization, scale-up and production sequence. Most importantly, this work established that orbital shake technology, unlike other disposable cell cultivation systems, can be used for a wide range of operating scales, from only a few milliliters for optimization purposes up to production scale.

Keywords: Animal cell technology, cell culture, disposable, single-use, transient transfection, recombinant protein, monoclonal antibody, orbital shake technology, process optimization, scale-up

Résumé

La culture cellulaire industrielle consiste à produire des protéines recombinantes à hautes valeurs ajoutées pour différentes thérapies, telles que le traitement des déficiences du système immunitaire et de certaines formes de cancer. Réduire le temps et les coûts de développement d'une nouvelle molécule, de son identification à la production industrielle, représente un défi majeur et une nécessité. L'utilisation de technologies appropriées est un aspect décisif pour atteindre cet d'objectif. En même temps, les probabilités de succès en phases cliniques et commerciales devraient être sensiblement améliorées. Récemment, de nouvelles technologies basées sur des matériaux à usage unique ont été développées et représentent désormais une réelle alternative par rapport aux technologies standards. L'objectif de ce travail consistait précisément à utiliser ce genre de matériaux innovants dans le contexte de la culture cellulaire et à démontrer le potentiel en matière de réduction des coûts, de simplification des procédures et de flexibilité. En premier lieu, un nouveau système d'évaluation de la biomasse a été testé et évalué. Il s'agit d'un microtube à centrifugation jetable qui permet de mesurer rapidement et de manière précise la biomasse présente dans un échantillon de culture cellulaire. Ce système peut potentiellement remplacer des méthodes de comptages conventionnelles qui sont à la fois lentes et peu précises. Ensuite, pour la culture de cellules en suspension à proprement parler, un nouveau concept efficace a été testé et évalué. Il s'agit de tubes de centrifugation modifiés, agités de manière orbitale sur une plateforme horizontale. Cette approche s'est révélée particulièrement adaptée à l'échelle de laboratoire pour l'optimisation de lignées cellulaires, lorsque de nombreux paramètres doivent être testés simultanément. Dans ces tubes à usage unique, l'oxygénation par agitation permet de soutenir des densités de cellules élevées, supérieures à 1×10^7 de cellules par mL. Une méthode optique de détection de l'oxygène dissout a été utilisée dans ces tubes ainsi que dans des récipients de volumes plus importants. Progressivement, ce système d'agitation orbital simple mais efficace a été dimensionné pour atteindre l'échelle de production. De manière à transférer suffisamment d'oxygène à travers l'interface des phases gazeuses et liquides, le volume d'air a été renouvelé de manière active en utilisant un flux d'air constant. Au besoin, l'air a été enrichi d'oxygène pure. De manière à contenir des volumes importants de cellules dans un environnement stérile, des poches préstérilisées ont été utilisées avec succès. Ces poches plastiques de formes cylindriques et à usage unique ont été dimensionnées jusqu'à un volume maximal de 1'500 L. Deux bioréacteurs prototypes de 200 et 1'500 L ont été conçus de manière à agiter orbitalement les cultures cellulaires à large échelle. Des densités cellulaire de l'ordre de $5\text{-}7 \times 10^6$ de cellules par mL ont été enregistrées en procédé batch. La comparaison en terme de coûts et de performances entre ce type de bioréacteur novateur et les bioréacteurs en acier inox conventionnels a mis en évidence le potentiel de cette technologie. De plus, et contrairement à d'autres systèmes récents basés sur des poches de culture à usage unique, le système présenté ici permet de couvrir un large éventail de volumes, de seulement quelques millilitres à l'échelle de production.

Remerciements

Il m'importe de témoigner ma reconnaissance, en préambule de ce document, à toutes celles et ceux qui ont contribués au succès de ce projet de thèse.

Ce travail de recherche n'aurait pas été possible sans l'appui scientifique et financier de la CTI (Commission pour la Technologie et l'Innovation), ainsi que des sociétés ExcellGene SA et Adolf Kühner AG. Merci pour leur engagement qui s'est révélé être décisif.

De plus, je tiens à remercier les membres du jury pour les questions pertinentes et les remarques intéressantes formulées lors de l'examen de thèse du 9 octobre 2007.

Je me dois d'exprimer ma sincère gratitude à l'égard du Prof. Florian M. Wurm, mon directeur de thèse, pour la confiance qu'il m'a témoignée en me proposant ce projet. Ses excellents conseils scientifiques, ses intuitions et son enthousiasme se sont révélés être essentiels à la réussite de ce projet.

De même, je souhaite adresser toute ma reconnaissance à Lucia Baldi et David Hacker, collaborateurs scientifiques au Laboratoire de Biotechnologie Cellulaire (LBTC), pour leur soutien inconditionnel. Je souhaite également remercier sincèrement Maria de Jesus et Martin Jordan que j'ai eu l'immense plaisir de côtoyer pendant quelques mois au LBTC au début de ma thèse et qui ont initié la plupart des sujets développés dans le cadre de ce projet. Merci à vous tous pour votre aide et toutes ces discussions constructives et stimulantes!

Egalement, je désire rendre hommage à toutes les personnes du LBTC qui ont contribués à un moment ou un autre à la réussite du projet: Xiaowei Zhang, Jean Cevey, Carine Meerschman, Sarah Wulhfard, Evi Engelhard, Cedric Burki, Natalie Muller, Claude Alves, Alexandre Super, Nicolas Jaccard, Yves Moser, Aurèle Horisberger, Raffael Favaretto, Anne-Laure Dessimoz et Meryem Abouhachim Alami. Un grand merci également à mes autres collègues doctorants pour avoir crée une excellente dynamique de travail au LBTC et pour les quelques bons moments passés à boire une bière en fin de semaine: Sophie Nallet, Fanny Delegrange, Agata Oberbek, Gaurav Backliwal et Sébastien Chenuet.

Je souhaite aussi remercier toutes les personnes de l'ISIC qui ont participé à la réussite du projet, en particulier les collaborateurs de l'atelier mécanique: André Fattet, Gil Corbaz et Jean-Claude Rapit.

Finalement, je remercie toute ma famille, mes parents surtout, ainsi que la personne qui compte le plus à mes yeux, ma femme, Caroline. Je la remercie de m'avoir accordé son soutien continuellement et pour son amour indéfectible!

Matthieu Stettler

Contents

Chapter 1

Introduction

Among the various expression systems for therapeutic recombinant proteins, mammalian host cells show the best ability to achieve appropriate posttranslational modifications, especially N-linked glycosylation (1). A majority of these proteins, particularly when considering full-length monoclonal antibodies, are expressed in mammalian cell lines, mainly Chinese hamster ovary (CHO) and murine myeloma (NS0) cells (2, 3). These cell lines offer several advantages for industrial applications, including extensive know-how, established procedures, and process scalability. The number of approved biopharmaceuticals produced from CHO and NS0 cell lines has increased rapidly over the past few years and the trend is on-going (4) (Fig. 1.1). Now, monoclonal antibodies represent more than half of the approximately 40 licensed biopharmaceuticals (therapeutic proteins) (5), some of them being blockbuster products (e.g. Rituxan®, Remicade®, Herceptin®, etc.).

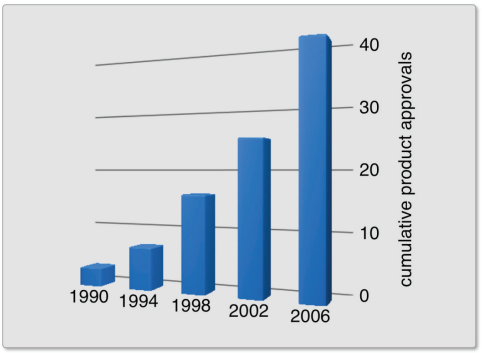


Fig. 1.1. Increase of licensed biopharmaceuticals (therapeutic proteins) from mammalian cells, data adapted from (4).

The high clinical dosage requirements for antibodies, grams rather than milligrams, result in larger production volume demands. To match this demand, research and development efforts from academia and industry resulted in important yield improvements for therapeutic recombinant proteins produced in mammalian cells (6). In this context, product quality, process robustness, and manufacturing capacity represent true challenges for sustained market supply (7, 8, 9). Also, cost issues have become more important due to more stringent market and government price control (10). This situation creates new opportunities to improve technologies for cell line optimization and scale-up (11, 12, 13).

Precisely, this thesis work focused on developments in new cell culture technologies. Innovative disposable tools for cell cultivation and biomass monitoring were characterized for the first time. Further, orbital shake bioreactors were scaled-up to reach pilot and production scale operations. Successful cell cultivation at different scales proved the performance and versatility of orbital shaking in animal cell technology. Instead of relying on different technologies for different processing volumes, which is the current situation, the benefits of using a single technology for process scale-up were demonstrated. The use of transient gene expression in scalable shake bioreactors and an evaluation of economic advantages related to single-use shake systems completed this thesis work dedicated to disposables in the context of animal cell technology. This introduction chapter provides a brief overview of the research field. First, recombinant cell line generation, media and process optimization and industrial product development are described (Section 1.1). Second, disposable cell culture technologies for optimization and development purposes are reviewed (Section 1.2). Finally, a detailed description of the research objectives of the thesis work is provided (Section 1.3).

1.1 Mammalian expression systems for recombinant proteins

Mammalian cells show an almost unlimited ability to take up foreign DNA via chemical or physical DNA transfer methods (14). The integration of foreign DNA into the genome of immortalized cultured mammalian cells has been exploited for decades to produce recombinant proteins for therapeutic use. One of the main drivers for studying gene transfer and amplification in mammalian cells is the need for productivity maximization and increased long-term stability (15) in therapeutic protein production. Nowadays, highly productive recombinant cell lines grown as single-cell suspensions in fed-batch mode achieve final product concentrations above 2 g L^{-1} (4) and sometimes up to 5 g L^{-1} and more (16, 17).

As mentioned before, CHO and NS0 cell lines are the most frequently used host cells in industrial bioprocessing. In these systems, the target gene can be transfected together with an amplifiable gene such as dihydrofolate reductase (DHFR) or glutamine synthetase (GS) (4, 6). The selective pressure is applied to the cells with an inhibitor of DHFR or GS. Exposure of the cells to gradually higher concentrations of the inhibitor increases the copy number of the transfected genes. These strategies are used to generate recombinant cell lines characterized by a stable integration of the target gene in the host cell chromosomes and stable production of the recombinant protein. These cell lines, as described later, are used in industrial bioprocessing to produce therapeutic proteins. As an alternative, the short-term transient expression of a target gene can be used for the production of milligram to gram quantities of recombinant protein at scales up to 100 L (18, 19, 20). Transient gene expression (TGE) shortens the time between DNA delivery and the production of candidate molecules and might be favored over stable expression in preclinical research.

The design of cell culture media is critical for high yield recombinant protein expression (21). For safety and cost reasons, modern cell culture media are free of animal-derived compounds (22, 23) and are often chemically defined, though they may contain plant protein hydrolysates (24, 25). Single compounds are statistically screened for concentrations and combinations that induce the optimal biological effect (26). Although productivity is the main driver for media design and optimization, other considerations are also important. Media compounds have an impact on product quality and should not interfere with downstream processing (i.e. product purification). Due to its potential for productivity and quality enhancement, medium development is

a highly strategic aspect for biotech companies. Also, medium development is cell line specific and therefore, the use of a common expression platform for different projects might be more rational.

In adequate cell culture media, recombinant cell lines usually grow up to 6-10 x 10^6 cells mL^{-1} within 4-5 days in batch cultivations. The limits for growth and protein expression are related to the depletion of nutrients such as glucose and glutamine and the accumulation of metabolic by-products like lactate, ammonium and CO_2. In fed-batch cultures, the growth and viability are usually extended for some time by addition of concentrated nutrients whereas in perfusion cultures, media is constantly replaced (27, 28). Fed-batch strategies, due to their simplicity, reliability, and flexibility for multi-purpose facilities, are industrially more relevant than perfusion processes. Also, in fed-batch cultures, compounds can be added during the process to control the metabolic activity, avoid inhibitory conditions, or improve protein glycosylation (29, 30). For example, the addition of selected amino acids was shown to reduce the growth inhibiting effect of higher ammonium concentrations or protect the cells from hyperosmotic conditions (31, 32).

In fed-batch cultures, cell proliferation is often followed by a phase in which cell division is arrested to allow the cells to attain higher specific productivities (4). In CHO cultures, hyperosmotic conditions were shown to enhance the antibody productivity (33). This might be due to the upregulation of certain glycolytic enzymes that could lead to an increased metabolic activity. Similarly, hypothermic conditions were shown to enhance the productivity of recombinant proteins (34, 35, 36). However, it seems that this effect is restricted to certain cell lines and products. The observed increase in productivity can be impaired by the reduction in growth and the longer process duration (37, 38).

Small-scale processes are scaled up stepwise to the process volumes that are required depending on the product demand. This is usually done in pilot and production scale stirred-tanks or airlift bioreactors. The volumes of some of these production scale bioreactors now exceed 10'000 L and represent true challenges in terms of process development and control (39, 40). To provide guidelines for process control, the regulatory authorities introduced procedures aimed to include quality concerns into the process design (41). Critical quality and performance attributes are defined to ensure final product quality. Sophisticated on-line process control and analytical tools are used to facilitate process understanding, develop risk-mitigation strategies and achieve continuous improvement (42, 43). Numerous contributions showed the impact of bioreactor process control on final product quality. For example,

a detailed study explored the effect of the frequency and duration of pH perturbations resulting from the addition of base for the pH control (44). Heterogeneities in large-scale bioreactors need to be kept within limits to avoid cell damage and product quality issues (45). A scale-down model of the production bioreactor can be used to investigate the effect of process parameter variations and might be an appropriate tool for post-approval modifications (46).

The industrial development of a new therapeutic protein is a long and complex process involving competence in cellular and molecular biology, process development and engineering. A few biotechnology companies have the capacity to develop a new therapeutical compound in-house, but others outsource part or all of the development steps. Contracting companies often do the very specialized activities like the management of clinical trials. A detailed view of a complete product development cycle is shown in Fig. 1.2.

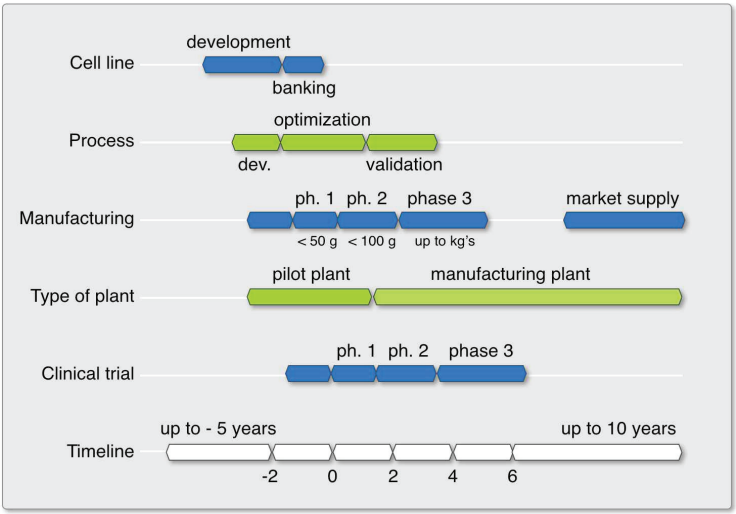


Fig. 1.2. Typical life cycle of a recombinant protein development, adapted from (47).

The coordination of all activities appears to be very critical (48). The manufacturing capability needs to be available years before a product gets the market approval. The time to market can be reduced by shortening the clinical development time and, most

importantly, the time it takes to design and construct a pilot plant and a manufacturing facility (49). In this context, flexible and high performing disposable cell cultivation systems are promising. Such systems are expected to reduce the elevated costs and risks associated with long development cycles, which should eventually result in reduced time to market.

1.2 Disposables in the context of upstream bioprocessing

Biotechnology companies face new challenges as therapeutic proteins, which are expensive to develop, account for a more important share of drug approvals. Recent trends in bioprocessing demonstrate an increased interest for disposable systems throughout the manufacturing facility (49, 50). The development of bioprocesses based on disposable materials is aimed at simplifying the technology for the production of biopharmaceuticals, resulting in several benefits. First, disposable systems increase the flexibility of bioprocesses (51). Compared to stainless steel equipment, the time required for changeovers between cell lines and batches is reduced, mainly because disposable systems require no cleaning and maintenance. Second, disposable systems reduce costs (52, 53). In particular, the initial investment necessary for equipping a research and development lab or a pilot plant is less, and capital costs are exchanged by consumable costs, resulting in a more balanced cost distribution over time. Improved cost-effectiveness is particularly important in the context of competition and growing governmental and market price controls (54). Accelerating the development process for a new therapeutic protein through increased flexibility and improved cost-effectiveness provides opportunities for achieving a competitive advantage.

While single-use technologies are now widespread in many process steps, including filtration, sterile liquid handling, media and buffer preparation, the standard equipment for cell cultivation remains non-disposable. Stirred-tank and airlift bioreactors were initially developed for microbial production systems and were designed to achieve high gas transfer properties using direct gas dispersion into the liquid phase. They constitute well-defined and well-controlled environments that allow efficient process monitoring. For mammalian cells however, the design and the position of impellers and spargers were modified to reduce the hydrodynamic shear conditions, resulting in less efficient gas transfer properties. Other drawbacks include the substantial capital investments required. The design and manufacturing of stainless steel bioreactors with sterilization in place (SIP) is expensive. Each new production batch requires a time-consuming sterility testing procedure. Similarly, equipment setup and cleaning during campaign changeovers in multi-product facilities require considerable time (48). Such downtimes result in poor overall manufacturing productivities. Finally, each new project is subjected to technical, clinical and commercial uncertainties (16, 54). In this respect, stainless steel bioreactors lack in flexibility and represent a risk in terms of future process volume estimations.

To overcome most of the disadvantages associated with stainless steel bioreactors, efforts were made to develop single-use cultivation systems. This trend was initiated a the millilitre scale with the development of disposable shake flasks and tubes for cell culture together with appropriate incubator shakers. These non-instrumented systems were found to be reliable and promising for cell line development applications (55). For larger processing volumes, disposable bioreactors based on single-use bags were developed. In 1998 Wave Biotech was the first company to commercialize a complete disposable cell cultivation system. The system included cell cultivation bags, filters, sampling system, aeration, agitation and monitoring (56). However, established biotechnology companies were successfully using standard procedures and conservative technologies. Many years were needed to gain wide acceptance. There were also concerns about maximal volumes and whether the regulatory authority would allow disposable bioreactors.

The system is now widely accepted and used for many applications at scales up to 500 L and as a cell expansion system to feed stirred-tank bioreactors (57). This success story created entirely new market opportunities for innovative cell culture systems. In fact, a number of other designs are quickly entering the market, such as single-use stirred-tank and air lift bioreactors based on disposable bag technology. Recently, major biotech equipment suppliers entered strategic or exclusive alliances with the initial manufacturers of Wave products. This trend reflects well the high degree of consideration gained by disposable bioreactor systems over the past few years. To summarize, the key arguments for disposable bioreactors are performance, flexibility, ease of handling, faster facility set-up, less maintenance and validation, reduced floor space and less capital investment.

Though disposable bioreactors seem to be well accepted and used, major issues are still not solved. For example, despite the perceived importance and potential of disposable bioreactors, few reports document the true advantage in terms of cost reduction or performance. This is due to the variety of possible applications and process specific productivities. Also, cost comparisons based on real situations are difficult to establish. Not many companies have the resources to run parallel optimizations with standard and disposable equipment. Another problem is the question of the largest possible operation scale. Unfortunately, current systems were not intended to reach production scales and might be limited to pilot scale applications. This is a drawback since the development and scale-up of a process currently relies on very different technologies when increasing the volumes from a few millilitres up to manufacturing scales. True opportunities exist to further develop and

improve disposable bioreactor processes. Innovative mixing and agitation principles might be developed to achieve better growth kinetics and higher final cell densities.

Disposable bags are widely used in the biotech industry mainly for the purpose of sterile liquid handling. They are gamma sterilized and validated to match GMP requirements. More recently, and due to improvements made in material properties, disposable bags were designed for cell cultivation in Wave bioreactors or in stirred-tank reactors with single-use contact parts. Such bags are equipped with sterile filters, connections and sampling ports. Normally, disposable bags are made with a polymeric film with at least three layers (58). The structural layer determines the overall mechanical behaviour of the film. Then, a barrier layer defines the structure's permeability. Finally, the fluid contact layer combines inertness and good sealing properties. To match the regulatory requirements, the validation procedures for a new film consist in testing a variety of material properties, including tensile properties, flex durability, permeability and possible interactions with the fluid. Further, to monitor the pH and the dissolved oxygen, innovative optical sensors can be integrated in the disposable cell culture bags. Sensor spots are immobilized on the inner layer of the bag in contact with the fluid. Using optical methods, the sensors can be assessed from the outside through the polymeric film. Optical sensors avoid contamination risks and can be discarded together with the cell culture bag. Response time and long term stability of optical sensors were improved to match process requirements (59, 60).

Taking advantages of breakthroughs in disposable technologies, appropriate and reliable bioreactor systems might be developed in the near future that would ideally allow high cell density cultures at scales beyond 1'000 L. This would definitely establish single-use bioreactor technology as a new standard for cost effective and flexible recombinant protein production.

1.3 Research objectives

The thesis work was divided in four distinct topics, all related to the characterization and development of novel disposable technologies in the context of recombinant protein production. The topics and research objectives are briefly outlined next:

Chapter 2: The objective was to characterize a newly developed biomass assessment tool for accurate packed cell volume (PCV) measurements. The present work consisted in demonstrating its application as a particularly convenient and sensitive means to precisely assess growth kinetics and cellular volume changes.

Chapter 3 and 4: Single-use bioreactors based on orbital shake technology were developed and characterized. The technology was scaled up from 50-mL shake tubes up to several hundred liters. To do this, the oxygen mass transfer in such systems was carefully investigated along with other process parameters such as working volume and agitation speed. The engineering principles of a production scale shake bioreactor were defined and a prototype was constructed and tested.

Chapter 5: The use of disposable shake bioreactors was combined with another promising tool for accelerating the product development of biotherapeutics: transient gene expression. Experiments showed that small-scale transfection protocols could be efficiently scaled up to considerably improve volumetric yields.

Chapter 6: Finally, the cost and time saving potential of disposable shake bioreactors over standard biomanufacturing equipment was investigated. A theoretical comparison was made to show precisely how the flexibility of disposable bioreactors allows a reduction in the time-to-market for therapeutic proteins.

1.4 References

(1) Walsh G, Jefferis R. 2006. Post-translational modifications in the context of therapeutic proteins. Nat Biotechnol. 24(10):1241-52.

(2) Chadd HE, Chamow SM. 2001. Therapeutic antibody expression technology. Curr Opin Biotechnol. 12(2):188-94.

(3) Walsh G. 2006. Biopharmaceutical benchmarks 2006. Nat Biotechnol. 24(7):769-76.

(4) Butler M. 2005. Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals Appl Microbiol Biotechnol. 68:283-291.

(5) Pavlou AK, Belsey MJ. 2005. The therapeutic antibodies market to 2008. Eur J Pharm Biopharm. 59(3):389-96.

(6) Wurm FM. 2004. Production of recombinant protein therapeutics in cultivated mammalian cells. Nat Biotechnol. 22(11):1393-8.

(7) Werner RG. 2004. Economic aspects of commercial manufacture of biopharmaceuticals. J Biotechnol. 113(1-3):171-82.

(8) Farid SS. 2007. Process economics of industrial monoclonal antibody manufacture. J Chromatogr B Analyt Technol Biomed Life Sci. 848(1):8-18.

(9) Farid SS. 2006. Established bioprocesses for producing antibodies as a basis for future planning. Adv Biochem Eng Biotechnol. 101:1-42.

(10) Rathore AS, Latham P, Levine H, Curling J, Kaltenbrunner O. 2004. Costing issues in the production of biopharmaceuticals. BioPharm Int. February 2004.

(11) Coco-Martin JM. 2004. Mammalian expression of therapeutic proteins - A review of advancing technology. BioProcess Int. November 2004. pp. 32-40.

(12) Heath C, Kiss R. 2007. Cell culture process development: advances in process engineering. Biotechnol Prog 23(1):46-51.

(13) Eisenstein M. 2006. Thinking outside the dish. Nat Methods. 3(12):1035-1043.

(14) Wurm FM, Jordan M. 2003. Gene transfer and gene amplification in mammlian cells. In *Gene transfer and expression in mammalian cells*. Edited by Makrides, S. C., Elsevier Science. pp. 309-335.

(15) Barnes LM, Bentley CM, Dickson AJ. 2003. Stability of protein production from recombinant mammalian cells. Biotechnol Bioeng. 81(6):631-9.

(16) Thiel KA. 2004. Biomanufacturing, from bust to boom...to bubble? Nat Biotechnol. 22(11):1365-72.

(17) Charlebois TS, Leonard MW, 2007. Balancing speed, throughput and cell culture yield: can we have it all? Oral presentation, ESACT Conference 2007, Dresden, Germany.

(18) Schlaeger E-J, Legendre YJ, Trzeciak A, Kitas EA, Christensen K, Deuschle U, Supersaxo A. 1998. Transient transfection in mammalian cells. In *New developments and new applications in mammalian cells*. Edited by Merten *et al*., Kluwer Academic Publishers. pp.105-112.

(19) Wurm F, Bernard A. 1999. Large-scale transient expression in mammalian cells for recombinant protein production. Curr Opin Biotechnol. 10(2):156-9.

(20) Baldi L, Hacker DL, Adam M, Wurm FM. 2007. Recombinant protein production by large-scale transient gene expression in mammalian cells: state of the art and future perspectives. Biotechnol Lett. 29(5):677-84.

(21) Xie L, Wang DI. 1997. Integrated approaches to the design of media and feeding strategies for fed-batch cultures of animal cells. Trends Biotechnol. 15(3):109-13.

(22) Froud SJ. 1999. The development, benefits and disadvantages of serum-free media. Dev Biol Stand. 99:157-166.

(23) Rasmussen LK, Larsen YB, Hojrup P. 2005. Characterization of different cell culture media for expression of recombinant antibodies in mammalian cells: Presence of contaminating bovine antibodies. Protein Expr Purif. 41(2):373-7.

(24) Schlaeger EJ. 1996. The protein hydrolysate, Primatone RL, is a cost-effective multiple growth promoter of mammalian cell culture in serum-containing and serum-free media and displays anti-apoptosis properties. J Immunol Methods. 194(2):191-9.

(25) Chun BH, Kim JH, Lee HJ, Chung N. 2007. Usability of size-excluded fractions of soy protein hydrolysates for growth and viability of Chinese hamster ovary cells in protein-free suspension culture. Bioresour Technol. 98(5):1000-5.

(26) Kim EJ, Kim NS, Lee GM. 1998. Development of a serum-free medium for the production of humanized antibody from Chinese hamster ovary cells using a statistical design. In Vitro Cell Dev Biol Anim. 34(10):757-61.

(27) Chu L, Robinson DK. 2001. Industrial choices for protein production by large-scale cell culture. Curr Opin Biotechnol. 12(2):180-7.

(28) Kretzmer G. 2002. Industrial processes with animal cells. Appl Microbiol Biotechnol. 59:135-142.

(29) Chee Furng Wong D, Tin Kam Wong K, Tang Goh L, Kiat Heng C, Gek Sim Yap M. 2005. Impact of dynamic online fed-batch strategies on metabolism, productivity and N-glycosylation quality in CHO cell cultures. Biotechnol Bioeng. 89(2):164-77.

(30) Crowell CK, Grampp GE, Rogers GN, Miller J, Scheinman RI. 2007. Amino acid and manganese supplementation modulates the glycosylation state of erythropoietin in a CHO culture system. Biotechnol Bioeng. 96(3):538-49.

(31) Chen P, Harcum SW. 2005. Effects of amino acid additions on ammonium stressed CHO cells. J Biotechnol. 117(3):277-86.

(32) deZengotita VM, Abston LR, Schmelzer AE, Shaw S, Miller WM. 2002. Selected amino acids protect hybridoma and CHO cells from elevated carbon dioxide and osmolality. Biotechnol Bioeng. 78(7):741-52.

(33) Lee MS, Kim KW, Kim YH, Lee GM. 2003. Proteome analysis of antibody-expressing CHO cells in response to hyperosmotic pressure. Biotechnol Prog. 19(6):1734-41.

(34) Kaufmann H, Mazur X, Fussenegger M, Bailey JE. 1999. Influence of low temperature on productivity, proteome and protein phosphorylation of CHO cells. Biotechnol Bioeng. 63(5):573-82.

(35) Yoon SK, Song JY, Lee GM. 2003. Effect of low culture temperature on specific productivity, transcription level, and heterogeneity of erythropoietin in Chinese hamster ovary cells. Biotechnol Bioeng. 82(3):289-98.

(36) Shi M, Xie Z, Yu M, Shen B, Guo N. 2005. Controlled growth of Chinese hamster ovary cells and high expression of antibody-IL-2 fusion proteins by temperature manipulation. Biotechnol Lett. 27(23-24):1879-84.

(37) Yoon SK, Hong JK, Choo SH, Song JY, Park HW, Lee GM. 2006. Adaptation of Chinese hamster ovary cells to low culture temperature: cell growth and recombinant protein production. J Biotechnol. 122(4):463-72.

(38) Fox SR, Patel UA, Yap MG, Wang DI. 2004. Maximizing interferon-gamma production by Chinese hamster ovary cells through temperature shift optimization: experimental and modeling. Biotechnol Bioeng. 85(2):177-84.

(39) Varley J, Birch J. 1999. Reactor design for large scale suspension animal cell culture. Cytotech. 29:177-205.

(40) Andersen DC, Reilly DE. 2004. Production technologies for monoclonal antibodies and their fragments. Curr Opin Biotechnol. 15(5):456-62.

(41) Hinz DC. 2006. Process analytical technologies in the pharmaceutical industry: the FDA's PAT initiative. Anal Bioanal Chem. 384(5):1036-42.

(42) Junker BH, Wang HY. 2006. Bioprocess monitoring and computer control: key roots of the current PAT initiative. Biotechnol Bioeng. 95(2):226-61.

(43) Mandenius CF. 2004. Recent developments in the monitoring, modeling and control of biological production systems. Bioprocess Biosyst Eng. 26(6):347-51.

(44) Osman JJ, Birch J, Varley J. 2002. The response of GS-NS0 myeloma cells to single and multiple pH perturbations. Biotechnol Bioeng. 79(4):398-407.

(45) Lara AR, Galindo E, Ramirez OT, Palomares LA. 2006. Living with heterogeneities in bioreactors: understanding the effects of environmental gradients on cells. Mol Biotechnol. 34(3):355-81.

(46) Li F, Hashimura Y, Pendleton R, Harms J, Collins E, Lee B. 2006. A systematic approach for scale-down model development and characterization of commercial cell culture processes. Biotechnol Prog. 22(3):696-703.

(47) Brecht R, Sandig V, Koch S, Marx U, Riedel M. 2005. Boosting Mammalian Cell-line Manufacturing Pilot Plant - A Case Report BioPharm Int. July 2005.

(48) Lakhdar K, Zhou Y, Savery J, Titchener-Hooker NJ, Papageorgiou LG. 2005. Medium term planning of biopharmaceutical manufacture using mathematical programming. Biotechnol Prog 21(5):1478-89.

(49) Sinclair A, Monge M. 2004. Biomanufacturing for the 21st century: Designing a concept facility based on single-use systems. BioProcress Int. Octobre 2004. pp. 26-31.

(50) D'Aquino R. 2006. Bioprocessing systems go disposable. Chem. Eng. Prog. May 2006.

(51) Carson KL. 2005. Flexibility - the guiding principle for antibody manufacturing. Nat Biotechnol. 23(9):1054-8.

(52) Farid SS, Washbrook J, Titchener-Hooker NJ. 2005. Decision-support tool for assessing biomanufacturing strategies under uncertainty: stainless steel versus disposable equipment for clinical trial material preparation. Biotechnol Prog. 21(2):486-97.

(53) Sinclair A, Monge M. 2002. Quantitative economic evaluation of single use disposables in bioprocessing. Pharmaceutical Eng. 22(3):20-34.

(54) Novais JL, Titchener-Hooker NJ, Hoare M. 2001. Economic comparison between conventional and disposables-based technology for the production of biopharmaceuticals. Biotechnol Bioeng. 75(2):143-53.

(55) Morrow J. 2006. Disposable bioreactors gaining favor. Gen Eng News. June 2006.

(56) Singh V. 1999. Disposable bioreactor for cell culture using wave-induced agitation. Cytotech. 30:149-158.

(57) Marjanovic D, Greller G. 2007. Disposable bioreactors based on wave agitation technology. Parmaceutical Technology Europe. February 2007.

(58) Barbaroux M, Sette A. 2006. Properties of materials used in single-use flexible containers: requirements and analysis. BioPharm Int. November 2006.

(59) Wittmann C, Kim HM, John G, Heinzle E. 2003. Characterization and application of an optical sensor for quantification of dissolved O2 in shake-flasks. Biotechnol Lett. 25(5):377-80.

(60) Stark E, Hitzmann B, Schugerl K, Scheper T, Fuchs C, Koster D, Markl H. 2002. In-situ-fluorescence-probes: a useful tool for non-invasive bioprocess monitoring. Adv Biochem Eng Biotechnol. 74:21-38.

Chapter 2

Biomass assessment using disposable packed cell volume tubes[1]

2.1 Introduction

Biomass is a key parameter for the evaluation of bioprocesses. The precise monitoring of this variable is required to establish the specific growth rate and the maximal or final cell concentration. Moreover, the accurate assessment of biomass is a prerequisite for the determination of metabolic shifts and for the establishment of control strategies for fed-batch and continuous cultures. Numerous methods that allow the quantification of biomass have been suggested.

Optical density and the gravimetric determination of dry mass are widely used for the monitoring of growth kinetics in microbial systems, but these methods are not sensitive enough for cultures of mammalian cells due to the relatively low densities to which these cells grow in vitro. Microscopic cell counting using dye exclusion remains the most common approach in many academic laboratories. However, the reproducibility and accuracy of this method are limited, and the microscopic examination is highly subjective, tedious, and particularly time-consuming for multiple samples.

Automated particle counters offer a faster and more reproducible approach to cell counting. Such instruments use sampling devices, vital dyes, and a counting chamber combined with an integrated microscope or they operate without dyes and use

[1] A slightly modified version of this chapter was published in *Biotechnology and Bioengineering* (Stettler M, Jaccard N, Hacker D, De Jesus M, Wurm FM, Jordan M. 2006. New disposable tubes for rapid and precise biomass assessment for suspension cultures of mammalian cells. Biotechnol Bioeng 95(6):1228-33).

electrical or optical properties to distinguish living and dead cells. However, these systems often require the dilution of the cell suspension within a defined cell density range, the staining of cells, and the use of specific suspension buffers. Also, cell aggregates can induce major biomass determination errors. Although some of these systems are already applied in industrial bioprocesses, they represent a major cost factor. For instrumented bioreactors the rates of oxygen uptake or glucose consumption can be employed for the indirect on-line determination of mammalian cell concentration (1, 2, 3). Dielectric spectroscopy is also used to estimate the biomass concentration in bioreactors (4, 5). Common intracellular enzymatic assays have also been utilized to quantify the number of viable cells. These methods demand carefully controlled incubations and/or the establishment of a standard curve for each cell type.

Faced with the limitations of current biomass determination methods an alternative approach was tested that is widely employed for blood analysis. Typically, an aliquot (50 μL) of blood is loaded into a capillary and then centrifuged. The height of the cell pellet within the capillary is measured instrumentally and expressed as the packed cell volume (PCV) or hematocrit, meaning the fraction of blood occupied by the cells. Typical PCV values in this case range from 30-50%. For cultivated cells the PCV is much lower and as a consequence the sensitivity of the hematocrit tube is not sufficient. Therefore, a novel disposable microcentrifuge tube[2] was designed and tested that overcomes this limitation. The geometry of the tube was chosen so that up to 1 mL of cell suspension can be loaded into the upper chamber having an external diameter that is identical to a standard 1.5 mL microcentrifuge tube. The lower chamber is a graduated capillary with a volume of 5 μL. During a brief centrifugation, the cells in the sample are concentrated within the capillary. Thus the method uses the occupied volume of the cells and centrifugable cell fragments in a sample as a measure of biomass.

The method described here has the advantage of being more precise, more reproducible, and faster than microscopic cell counting. In the case of multi-parameter testing of a large number of process conditions, the PCV method allows a comparison of biomass proliferation in hundreds of different cultures at any given time. Typically, the assessment of 50 samples takes less than 30 min, which is clearly faster than any

[2] This single-use plastic tube was invented and designed by Prof. Florian M. Wurm together with his former co-worker Dr. Martin Jordan. VoluPAC is the commercial name of the product that was released in 2006 in collaboration with Sartorius AG (Göttingen, Germany), a worldwide supplier of lab and manufacturing equipment.

other currently available biomass assessment method. The precision and ease of this method even makes it convenient to measure and analyze with high precision the growth kinetics within a large cell culture vessel in a time interval of only a few hours.

This is the first description of the novel disposable VoluPAC tube for the biomass assessment of suspension cultures of mammalian cells. The study focused on the reproducibility, precision, and reliability of the PCV method as compared to manual cell counting. Also, examples are presented that describe the successful application of VoluPAC tubes in typical bioprocesses with Chinese hamster ovary (CHO), human embryo kidney 293 (HEK 293), and mouse NS0 myeloma cells.

2.2 Material and methods

2.2.1 Cells

Suspension adapted CHO DG44, HEK 293, and NS0 cells were grown in chemically defined ProCHO5-CDM (Lonza, Verviers, Belgium), EX-CELL 293 Serum-Free Medium (SAFC Biosciences, Andover, UK), and HyQ-CDM4NS0 (HyClone, South Logan, UT), respectively. Unless otherwise mentioned, the cells were cultivated in either 50-mL disposable tubes fitted with a filter cap for passive headspace aeration (CultiFlask 50, Sartorius AG, Göttingen, Germany) (6) or 500-mL round- or square-shaped bottles (7). The cells were incubated in a CO_2- and humidity-controlled atmosphere at 37°C (ISF4-X, AdolfKühner AG, Birsfelden, Switzerland) with orbital agitation. The cells were passed every 3-4 days at densities of 2-5 x 10^5 cells mL^{-1}.

Samples of CHO DG44 cells with different viabilities were produced by mixing viable and non-viable cells. To produce non-viable cells, a cell suspension was centrifuged at 200 *g* for 5 min. After removal of the supernatant, the cell pellet was incubated at 37°C for 4 h. Manual cell counting by Trypan blue exclusion method (see below) demonstrated that the cell lost their viability but retained their size and shape. Samples with different viability percentages were obtained by mixing aliquots of viable and non-viable cells at different ratios.

2.2.2 Cell counting and PCV determination

Cell density and viability were assessed with a hemocytometer using the Trypan blue exclusion method. The cell density included both viable and non-viable cells and was therefore referred to as total cell density. For PCV measurements, aliquots of a well-mixed suspension culture (100 - 1'000 μL in multiples of 100 μL) were transferred into VoluPAC tubes (Sartorius AG, Göttingen, Germany) (Fig. 2.1). The tubes were centrifuged in a micro-centrifuge (Model 5417C, Eppendorf AG, Hamburg, Germany) fitted with a swinging-bucket rotor (Model A-8-11, Eppendorf AG) for 1 min at 2'500 *g* (5'000 rpm) unless otherwise mentioned. The standard procedure for PCV assessment is schematically described in Fig. 2.2. For determining the height of the cell pellet, a visual assessment was made using the capillary graduation.

Alternatively, an in-house image analysis device was employed with non-graduated tubes provided by the manufacturer. An image of the lower part of the VoluPAC tube was acquired with a webcam (Watchport/V2 USB camera, Inside Out Networks, Austin, TX) and automatically analyzed the PCV with a program that was specifically developed for this purpose using Java technology. The software was optimized to measure the height of the cell pellet and to calculate the corresponding volume as a percentage of the volume loaded into the tube.

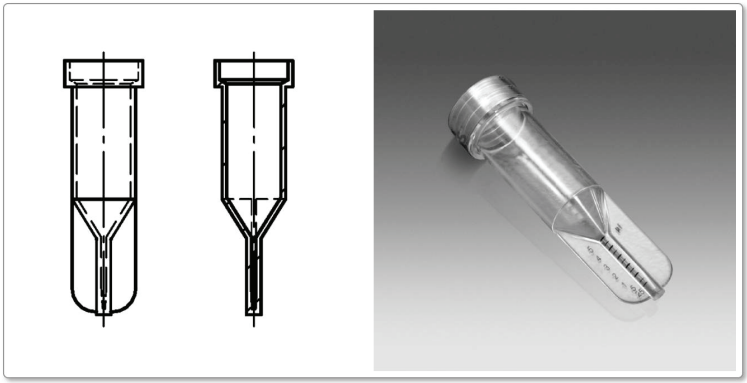


Fig. 2.1. VoluPAC tube geometry (left) and current commercial version (right) of the VoluPAC tube with graduation (technical drawing and photo courtesy of Sartorius AG, Göttingen, Germany). The transparent tube has a diameter of 13 mm and a height of 43 mm. The maximal volume of the capillary is 5 μL.

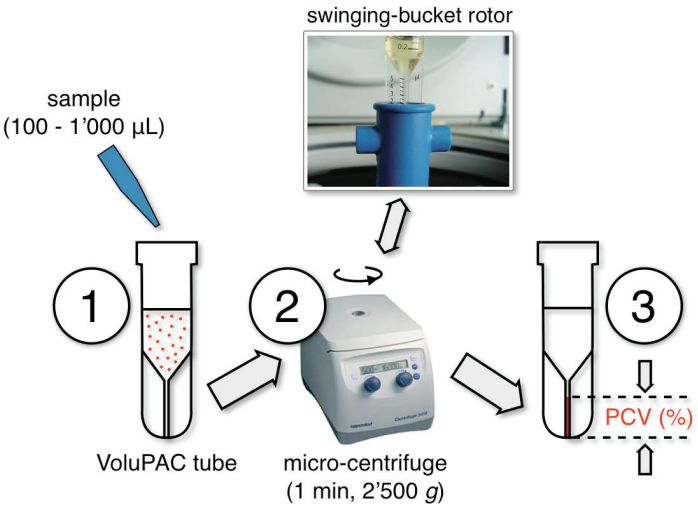


Fig. 2.2. Procedure for the packed cell volume (PCV) assessment using VoluPAC tubes. (1) A cell culture sample of known volume is loaded into a VoluPAC tube. (2) The tube is centrifuged for 1 min at 2'500 *g* in a micro-centrifuge with a swinging-bucket rotor. (3) The packed cell volume is calculated from the height of the cell pellet in the tube capillary. The percentage of PCV can be assessed either visually by reading the graduation or using an automated image analysis device.

2.2.3 20-L stirred-tank bioreactor

A 20-L pilot-scale bioreactor equipped with a marine impeller was used for the cultivation of stable cell lines in fed-batch cultures (Bioengineering AG, Wald, Switzerland). The DO was maintained at a level of 20% or more using bubble aeration and oxygen enriched air. CO_2 in the inlet airflow and NaOH were used to keep a physiological pH. Agitation speed was kept in a range of 150-250 rpm. Glucose, glutamine and bicarbonate were supplemented to avoid depletion of these compounds.

2.3 Results and discussion

2.3.1 Evaluation of optimal centrifugation conditions

The PCV biomass assessment method was validated by determining the optimum centrifugation conditions. First, samples from cultures of either CHO DG44 or HEK 293 cells were centrifuged in VoluPAC tubes at centrifugation forces ranging from 100 to 6'000 *g* while the centrifugation time was kept constant at 1 min. The PCV varied from approximately 1-2% depending on the centrifugal force with a sharp decrease as the force increased from 100 to 1'000 *g* (Fig. 2.3.A). At centrifugation forces greater than 2'500 *g*, the PCV tended toward a horizontal asymptotic value. At centrifugation forces less than 2'500 *g*, the profiles of the curves for CHO DG44 and HEK 293 cells were clearly different. At the lower centrifugation forces tested, even small differences in medium viscosity may explain such variation by acting on the drag forces. CHO DG44 and HEK 293 cultures, even at roughly the same biomass concentration, might have different viscosities due to different medium compositions and to the release of cell-line specific metabolic compounds. The packing of the cells at lower centrifugation forces may also have differed because of cell-line specific size distribution. This may have resulted in different void or liquid fractions between the packed cells, especially at low centrifugation forces. At higher forces, due to increased packing of the cell pellet, these differences appeared to be less important.

Next, the centrifugation force was kept constant at 2'500 *g* and the time of centrifugation was varied from 10 to 600 s. The resulting PCV values for CHO DG44 and HEK 293 cells varied from about 1-1.3% (Fig. 2.3.B). A centrifugation time of 1 min appeared to be a good compromise between the need for rapidity and the amount of variation in cell pellet size. From these observations, the standard conditions for PCV measurements were defined as a centrifugation force of 2'500 *g* for 1 min. Further increases in both variables reduced the level of the error because of a denser cell packing process.

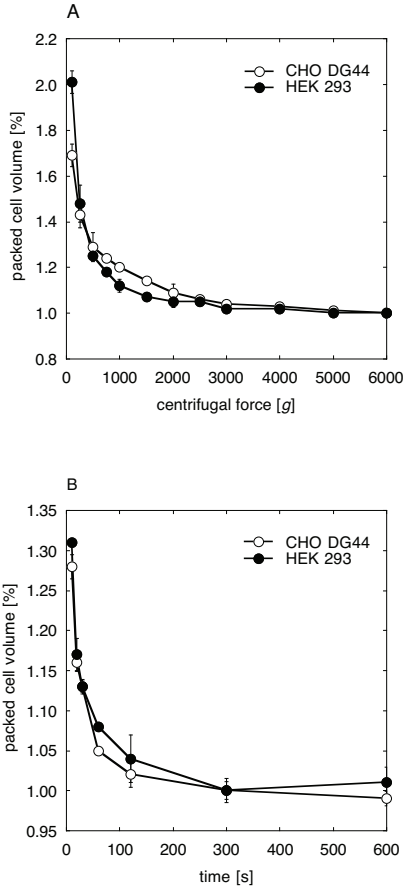


Fig. 2.3. Variation of the PCV as a function of the centrifugation force (A) and centrifugation time (B) for suspension CHO DG44 and HEK 293 cells. The cells were centrifuged for 1 min in VoluPAC tubes. Average PCV values are reported (n = 3).

It was observed that as the density of cell packing increased, a higher degree of post-centrifugation cell pellet expansion resulted (Fig. 2.4). Cell culture samples were centrifuged in VoluPAC tubes under two different conditions. Then the expansion of the packed cells was monitored over a period of 5 h. It was shown that after a 5 min centrifugation at 10'000 *g* the expansion of the cell pellet at 5 h post-centrifugation was 8.2%. This was almost twice as much as the expansion measured with the standard centrifugation conditions (4.7% at 1 min and 2'500 *g*). Moreover, at the higher centrifugation force and with the longer centrifugation time, the pellet continued to expand after 5 h at room temperature. The results also showed that most of the pellet expansion occurred within 10 min after the centrifugation (Fig. 2.4). During this time, the increase of the PCV due to pellet expansion was approximately 2%. For this reason the measurement of the PCV was typically performed immediately after centrifugation, thus increasing the reproducibility of the method. We assumed that the expansion of the cell pellet after centrifugation was due, at least in part, to the recovery of a more spherical cell shape which may have been deformed during the process.

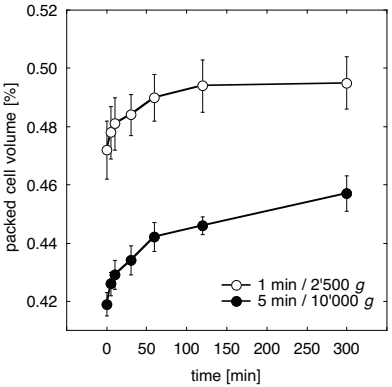


Fig. 2.4. Increase of the packed cell volume post-centrifugation in VoluPAC tubes at different time and speed combinations. PCV measurements were made from the same batch of CHO DG44 cells. Average PCV values are reported (n = 8).

2.3.2 Accuracy of packed cell volume measurements

The accuracy of the PCV measurement was evaluated by measuring 10 individual samples from the same CHO DG44 suspension culture. The PCV measurement was then repeated 1 h later with the same culture. First, the statistical analysis of the results showed that the standard deviation of the 10 independent PCV measurements was typically less than 1% of the average value, which indicated a high degree of reliability. Second, the increase of biomass within a period as short as 1 h was statistically relevant with the average PCV values of 0.484% and 0.492% for the two time points. The high degree of precision illustrated in this example opens new perspectives in cell culture growth monitoring. Previously, the measurement of cell growth within very short time periods was limited, mainly because of the low resolution of conventional methods of biomass analysis. Manual counting using a hemocytometer typically produces errors between 5 and 20%. For the PCV method described here, the most probable source for error was in sampling and the transfer of the material into the VoluPAC tube. However, the error was very low since only a single pipetting was required and no dilution step was necessary.

To verify the resolution of the PCV method the biomass increase of suspension growing cells was monitored within very short time intervals. Exponentially growing CHO DG44 and HEK 293 cells were cultivated in 500-mL square-shaped bottles and samples were taken every 30 min for 4 h. Results showed a measurable increase in biomass from a starting PCV value of 1.17% for both cell lines up to 1.49% and 1.36% for CHO DG44 and HEK 293 cells, respectively. By fitting the data with an exponential curve, the specific growth rates (μ) were determined to be 8.6×10^{-4} and 6.1×10^{-4} min^{-1} for CHO DG44 and HEK 293 cells, respectively (Fig. 2.5). These values corresponded to doubling times of approximately 13 and 19 h, respectively. This example showed that differences in growth kinetics between cell lines or in process conditions can be easily and reliably assessed with the PCV method even within brief time intervals. The same procedure is not feasible with manual cell counting due to the level of error associated with this method.

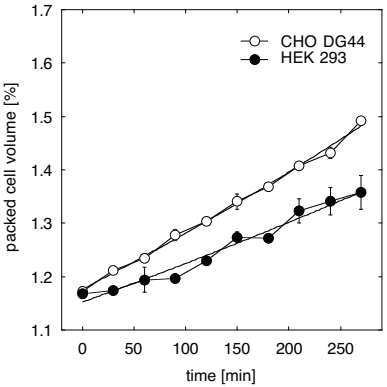


Fig. 2.5. Packed cell volume measurements at 30 min time intervals of suspension CHO DG44 and HEK 293 cells in exponential growth phase. Average PCV values are reported (n = 3).

2.3.3 Correlation between PCV and manually obtained cell density values

To determine the correlation between manually obtained cell density values and PCV in exponentially growing cultures, CHO DG44 and NS0 cells were maintained in agitated 50-mL tubes with subcultivation every 3-4 days. The PCV and cell density were assessed in duplicate twice every day for 30 days giving a total of 120 samples for each cell line. Each average PCV value was plotted as a function of the corresponding average cell density (Fig. 2.6). It is obvious from the results that the two cell lines have different individual cellular volumes. However, for both cell lines, up to a density of 4 x 10^6 cells mL^{-1}, the relationship between PCV and cell density was approximately linear (Fig. 2.6). At higher cell densities, however, the average cell volume was slightly reduced and the correlation was not linear (data not shown). This suggested that the cell volume changed when the batch cultures approached stationary phase. This may be explained by the fact that an increase in cell volume is not necessarily correlated with the progression through the cell cycle. In fact, while

the cell density increases, the availability of growth factors may be reduced, resulting in a lower maximal cell volume (8).

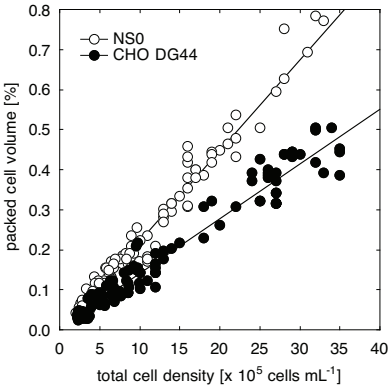


Fig. 2.6. PCV of CHO DG44 and NS0 myeloma cells as a function of total cell density assessed by manual cell counting. The cells were subcultivated every 3-4 days and samples were taken twice per day. The linear regression gives the correlation between the two biomass assessment methods for each cell line. Average PCV values and manual cell counts are reported (n = 2).

2.3.4 The osmolarity dependency of PCV measurements

Usually, cell growth includes the increase in both cell volume and number. However, the comparison of PCV and manual cell counts indicated that the two biomass assessment methods provide information which is intrinsically different. This aspect of biomass analysis was considered in an experiment in which the variation in cell volume due to osmolarity changes was assessed. An aliquot (200 μL) of a suspension of either CHO DG44 or HEK 293 cells was mixed with the same volume of phosphate-buffered saline (PBS) of various concentrations so that the osmolarity was varied from hypotonic (150 mOsm kg^{-1}) to strong hypertonic conditions (1'500 mOsm kg^{-1}). The resulting PCV values were assessed for each different osmotic condition and normalized to that obtained at 150 mOsm kg^{-1} (Fig. 2.7). Surprisingly, a

threefold difference in cell volume was observed between 150 and 800 mOsm kg^{-1} for both cell lines. However, little difference in PCV was observed from 800 to 1'500 mOsm kg^{-1} (Fig. 2.7). The osmolarity of most commercially available cell culture media is between 260 and 310 mOsm kg^{-1}. The data shown here indicated that small osmolarity shifts in this range affected the cellular volume quite dramatically.

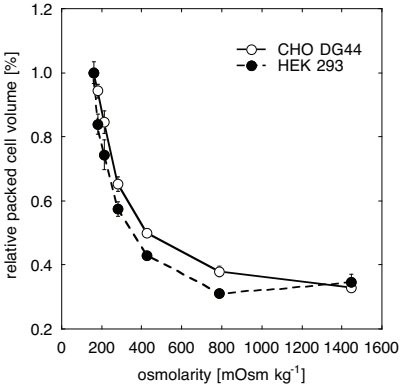


Fig. 2.7. Relative PCV of CHO DG44 and HEK 293 cells under different osmotic conditions. Samples (200 μL) were mixed with 200 μL of PBS at different concentrations and transferred to a VoluPAC tube for measurement. The samples were taken from the same initial cultures of CHO DG44 and HEK 293 cells, respectively. Average relative PCV values are reported (n = 3).

Similar experiments with osmolarity changes have been carried out previously with red blood cells and chondrocytes where it was shown that the Boyle-van't Hoff relationship predicts the cell volume under various osmotic conditions (9, 10). This ''ideal'' osmotic behavior of eukaryotic cells was exploited to distinguish between osmotically active (viable) and inactive (dead) cells. During batch cultivation of cells beyond 4 or 5 days the viability of a culture usually decreases, probably due to nutrient depletion and the accumulation of metabolic waste products. Dead cells were expected to lose their ability to adapt their volume to the osmotic environment. This assumption and its impact on the packed cell volume are schematically described in Fig. 2.8.

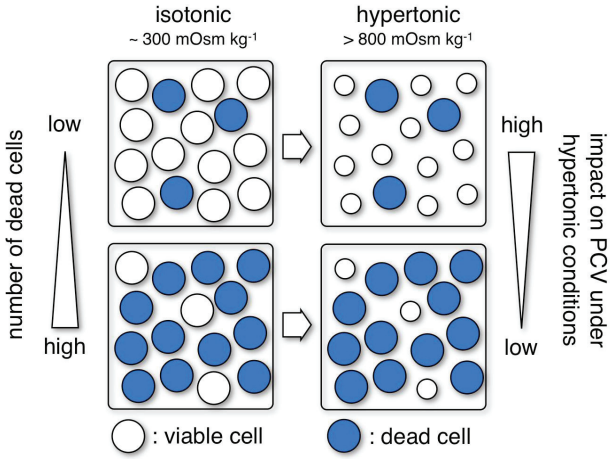


Fig. 2.8. Schematic model for the osmolarity dependency of viable (white dots) and dead cells (blue dots). Unlike viable cells, dead cells are expected to maintain their volume, independent from the osmolarity of the medium.

To simulate this behavior, samples of CHO DG44 cells with different viabilities were produced by mixing viable and non-viable cells. To produce non-viable cells, a cell suspension was centrifuged at 200 *g* for 5 min. After removal of the supernatant, the cell pellet was incubated at 37°C for 4 h. Microscopic cell counting by the Trypan blue exclusion method demonstrated that the cells lost their viability but retained their size and shape. By mixing aliquots of viable and non-viable cells at different ratios, samples with different viability percentages were obtained. Thus, aliquots of CHO DG44 cells with identical cell densities but ranging in viability from 0 to 99% were prepared and mixed with an equal volume of a concentrated PBS solution giving a final osmolarity of 1'300 mOsm kg^{-1}. The PCV values of the various cultures were then plotted as a function of the cell culture viability (Fig. 2.9). Whereas cells at 99% viability had a PCV value of 0.7% under these conditions, the same number of non-viable cells had a PCV value of more than 1.1% (Fig. 2.9). Thus, as assumed, non-viable cells (as monitored by Trypan blue exclusion) did not respond to the increase in osmolarity, which proves the validity of the model described in Fig. 2.8. These findings showed that the PCV method may also be used as a tool for the rapid assessment of cell condition (i.e., viability) during bioprocesses.

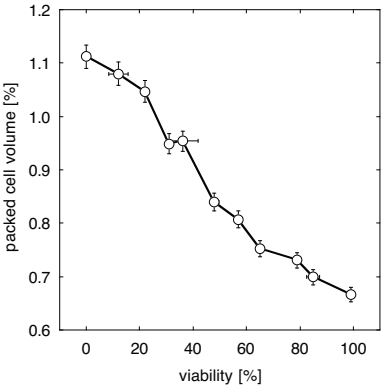


Fig. 2.9. Packed cell volume of CHO DG44 cells under hyperosmotic conditions (1'300 mOsm kg^{-1}) as a function of cell culture viability. The viability was assessed in duplicates by Trypan blue exclusion. Average PCV values are reported (n = 2).

2.3.5 Example of bioprocess monitoring using PCV

The PCV method was applied to the monitoring of recombinant protein production with an NS0 cell line at the 20-L scale in a stirred-tank bioreactor. The manual cell counts and the PCV values were used to determine the growth profile of the culture (Fig. 2.10). During the entire exponential growth phase, an excellent correlation between both measurement methods was observed. However, the average of the errors was lower with the PCV method (1.7%) as compared to manual cell counting (5%). After reaching the maximal cell density, the PCV remained relatively constant, whereas the total cell density decreased (data not shown). This probably reflected the accumulation over time of dead cells and cell fragments that were included in the PCV measurements but not in the manual cell counts. In the stationary and death phases of the culture, the manual cell counting was extremely inaccurate due to the presence of numerous particles, cell fragments, and aggregates. In contrast, because

all centrifugable material was packed in the pellet, the error for the PCV measurements remained very low even at the late phases of the batch cultivation.

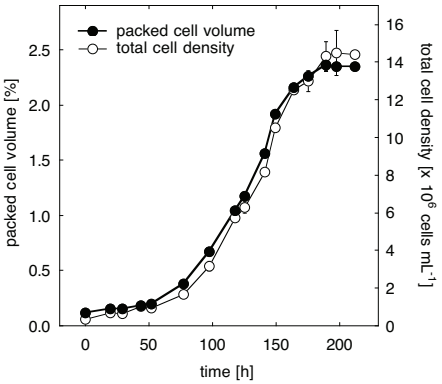


Fig. 2.10. PCV and total cell density of a recombinant NS0 myeloma cell line. The cells were cultivated in a 20-L fed batch bioreactor for the production of a monoclonal antibody. Average PCV and cell counts are reported (n = 2). The average errors for all the PCV measurements and the manual cell counts were 1.7% and 5%, respectively.

2.4 Conclusion

The biomass assessment method described here was found to provide a unique combination of speed and precision at low cost. The validation of the method allowed the identification of the optimal conditions for centrifugation. The study also demonstrated the potential use of the method under different conditions, with different cell lines, media and cultivation techniques. Unlike manual cell counting, the PCV method allows statistically reliable measurements of the total biomass including cell fragments and aggregates present in late phases of batch cultures. Due to the high sensitivity of the method, even slight variations in metabolic activity were monitored with a high degree of precision. The new method is another step in the introduction of low-cost, disposable technologies in modern bioprocessing.

2.5 References

(1) Lubenova V, Rocha I, Ferreira EC. 2003. Estimation of multiple biomass growth rates and biomass concentration in a class of bioprocesses. Bioprocess Biosyst Eng. 25(6):395-406.

(2) Ducommun P, Bolzonella I, Rhiel M, Pugeaud P, von Stockar U, Marison IW. 2001. On-line determination of animal cell concentration. Biotechnol Bioeng. 72(5):515-22.

(3) Schoenherr I, Stapp T, Ryll T. 2000. A comparison of different methods to determine the end of exponential growth in CHO cell cultures for optimization of scale-up. Biotechnol Prog. 16(5):815-21.

(4) Cerckel I, Garcia A, Degouys V, Dubois D, Fabry L, Miller AO. 1993. Dielectric spectroscopy of mammalian cells. 1. Evaluation of the biomass of HeLa- and CHO cells in suspension by low-frequency dielectric spectroscopy. Cytotechnology. 13(3):185-93.

(5) Cannizzaro C, Gugerli R, Marison I, von Stockar U. 2003. On-line biomass monitoring of CHO perfusion culture with scanning dielectric spectroscopy. Biotechnol Bioeng. 84(5):597-610.

(6) De Jesus MJ, Girard P, Bourgeois M, Baumgartner G, Jacko B, Amstutz H, Wurm FM. 2004. TubeSpin satellites: a fast track approach for process development with animal cells using shaking technology. Biochem. Eng J. 17:217-223.

(7) Muller N, Girard P, Hacker DL, Jordan M, Wurm FM. 2005. Orbital shaker technology for the cultivation of mammalian cells in suspension. Biotechnol Bioeng. 89(4):400-6.

(8) Conlon I, Raff M. 2003. Differences in the way a mammalian cell and yeast cells coordinate cell growth and cell-cycle progression. J Biol. 2(1):7.

(9) Guilak F, Erickson GR, Ting-Beall HP. 2002. The effects of osmotic stress on the viscoelastic and physical properties of articular chondrocytes. Biophys J. 82(2):720-7.

(10) Yang XS, Kamino K. 1995. Photometric determination of phenomenological correlation between osmotic behavior and hemolysis of red blood cells. Jpn J Physiol. 45(5):723-41.

Chapter 3

Characterization of small-scale shake cultivation systems

3.1 Introduction

In animal cell technology, the availability of an appropriate small-scale or scale-down system is essential for understanding and developing new cell culture processes. Recently, as an alternative to existing systems such as shake flasks and agitated multi-well plates (1), modified 50-mL centrifugation tubes[3] have been tested. The tubes are fitted with a ventilated cap allowing for passive gas exchange while preventing contamination. Using an adequate incubator and orbital shaker, several hundred single shake tubes can be operated simultaneously. Previous studies have partially defined the physico-chemical characteristics of these disposable 50-mL shake tubes (2, 3). Parameters such as optimal working volume, water evaporation, oxygen supply and release of carbon dioxide have been studied and optimized.

Apart from the ease-of-use and the potential for high throughput cell line development, 50-mL shake tubes were developed because of their bioreactor-like behavior (4). With working volumes of 5-25 mL, samples of a few microliters can be taken daily for monitoring purposes. The system is believed to fill a gap between very small-scale shake systems used for true screening approaches (multi-well plates) and larger systems such as lab- or pilot-scale bioreactors for process development. Here, a description of an efficient subcultivation strategy ensuring quality and consistency of

[3] The 50-mL shake tube with filter cap was invented and designed by Prof. Florian M. Wurm together with his former co-worker Dr. Martin Jordan. Now, the tubes are commercially available under the tradename CultiFlask 50 by Sartorius AG (Göttingen, Germany), a worldwide supplier of lab and manufacturing equipment. A second generation CultiFlask 50 tube with a modified geometry is expected to be released soon.

the seed train is provided. Also, an example of multi-parameter screening for growth optimization using 50-mL shake tubes is shown.

Apparently, the high cell densities observed in 50-mL shake tubes indicated that oxygen is transferred to the liquid phase more rapidly than it is taken up by the cells. To confirm this assumption, a systematic characterization of the oxygen transfer was done and results are shown in this chapter. The oxygen transfer rate (OTR) per unit of bioreactor volume is given in equation (3.1) (5).

$$OTR = k_L a \cdot (C_L^* - C_L) = k_L a \cdot (L \cdot p_G - C_L) \qquad (3.1)$$

where k_L [m h^{-1}] is the liquid mass transfer coefficient, a [m^{-1}] the specific interfacial area (gas to liquid phase) of mass transfer per liquid volume, C_L^* [mg L^{-1}] the oxygen concentration at saturation, C_L [mg L^{-1}] the measured dissolved oxygen concentration, L [mg L^{-1} bar^{-1}] the solubility of oxygen in the liquid phase and p_G [bar] the partial pressure of oxygen in the gas phase.

The specific liquid mass transfer coefficient *($k_L a$)* is often used to compare the efficiency of different bioreactors in terms of oxygen transfer both for microbial and animal cell bioprocesses (6, 7). The usual range of $k_L a$ values achieved in microbial cultivation systems is shown in Fig. 3.1. For animal cell cultivation, due to lower oxygen uptake rates compared to microbial systems, the required $k_L a$ values are more in a range between 1 and 25 h^{-1}. According to equation 3.1, an increased oxygen transfer results when either k_L or a are increased. In shake cultivation systems such as 50-mL shake tubes, the interfacial area a increases at higher agitation speed. Another possibility, though not feasible in passively aerated shake systems, is to increase the saturation concentration C_L^*, by either increasing the oxygen partial pressure in the gas phase *(p_G)* or its concentration (5).

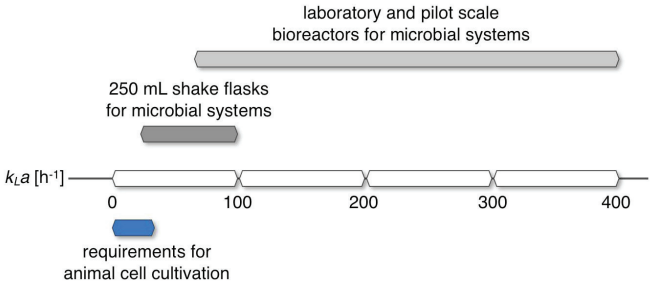


Fig. 3.1. Specific liquid mass transfer coefficient *(k_La)* in cultivation systems used for microbial bioprocesses and theoretical requirements for animal cells. Data adapted from (7).

Numerous studies recently evaluated the k_La in small-scale shake cultivation systems such as micro-well plates (8, 9, 10) and shake flasks (11, 12, 13). In these studies, the monitoring of dissolved oxygen (DO) was performed using novel optical sensors. The measurement principle is based on the quenching of luminescence caused by collision between molecular oxygen and luminescent dye molecules in the excited state. The system uses an immobilized fluorescence sensor made of two fluorophores. The first has a signal intensity that is related to the dissolved oxygen. The second is insensitive to oxygen and is used as a reference. Both fluorophores are excited at the same wavelength but emit light at two distinct wavelengths (14). Turbidity and changes in the shaking rate have no influence on the measurement. Also, no cross sensitivity exists for CO_2 and other compounds or towards pH variations. The non-invasive optical sensing of DO proved to be well suited for small-scale systems and is relatively easy to adapt to different vessel geometries and sizes.

Here, non-invasive oxygen sensing technology was used to evaluate the k_La of 50-mL shake tubes as a function of working volume and shaking speed. Then, the oxygen uptake rate of different commonly used cell lines sampled in culture conditions was experimentally measured using the same setup. These data were essential for considering the scale-up of shake cultivation vessels.

3.2 Material and Methods

3.2.1 Cells

Suspension adapted CHO DG44, CHO AMW (kindly provided by Dr. Maria de Jesus) and HEK 293 cells were grown in serum-free ProCHO5 medium (Lonza, Verviers, Belgium) and EX-CELL 293 medium (SAFC Biosciences, Andover, UK), respectively. Unless otherwise mentioned, the cells were cultivated in 250- or 500-mL round- or square-shaped bottles with passive air diffusion through the cap (15). The cells were incubated in a CO_2- and humidity-controlled atmosphere at 37°C (ISF-4-W, Adolf Kühner AG, Birsfelden, Switzerland) with orbital agitation. The cells were passed every 3-4 days at densities of 2-5 x 10^5 cells mL^{-1}. Cell density and viability were assessed with a hemocytometer using the Trypan blue exclusion method and total biomass was measured using the packed cell volume method (16, 17).

3.2.2 Small-scale orbital shake cultivation systems

Presterilized single-use 50-mL shake tubes with modified caps were used for small-scale cell cultivations (2) (Fig. 3.2). The air diffuses passively through a 0.22 μm pore size membrane. The membrane is located on the inner side of the cap and is protected from mechanical damages. The design of the tube was based on the geometry of a standard 50-mL centrifugation tube with an inner diameter of 27 mm. The tubes were agitated on holders designed to be used in large capacity incubator shakers with CO_2 and humidity control (ISF4-X, Adolf Kühner AG, Birsfelden, Switzerland) (Fig. 3.2).

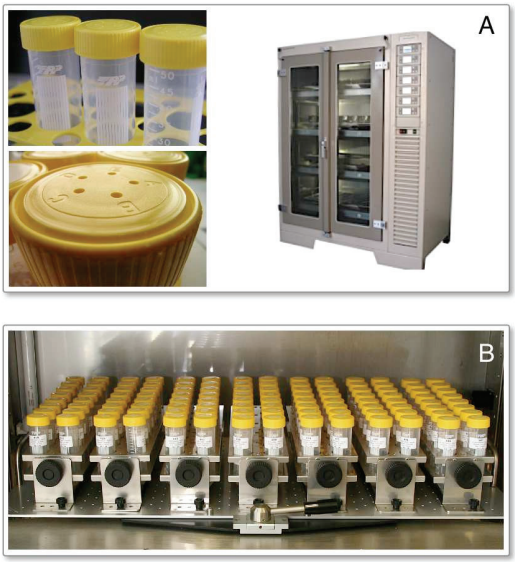


Fig. 3.2. (A) 50-mL shake tubes for the cultivation of mammalian cells (left). The tubes are fitted with a ventilated cap allowing for passive sterile gas exchange between tube headspace and environment (CultiFlask 50, Sartorius AG, Göttingen, Germany). ISF4-X shaker incubator with CO_2 and humidity control (right) (Adolf Kühner AG, Birsfelden, Switzerland). (B) Multi-parameter screening using 50-mL shake tubes. Working volume: 10 mL, agitation speed: 180 rpm. Up to 448 50-mL shake tubes can be operated simultaneously in one shaker incubator.

3.2.3 Optical system for dissolved oxygen sensing

Oxygen sensing spots were immobilized on the inner wall of 50-mL shake tubes (Fig. 3.3). A polymer optical fiber was used to guide the light signal between the OXY-4 module and the sensor spot (PreSens GmbH, Regensburg, Germany). According to the manufacturer, the response time of the oxygen sensor in stirred systems is very brief (less than 30 s) and was neglected in this work. Due to their small size, the sensor spots did not interfere with the fluid dynamics. The oxygen transfer measurements were carried out using a lab shaker fitted with a tube holder

specifically designed for multiple 50-mL shake tubes (ES-X-Adolf Kühner AG, Birsfelden, Switzerland). The shaker diameter was set at 50 mm. The oxygen monitoring system was calibrated by saturating the liquid phase with either air or N_2.

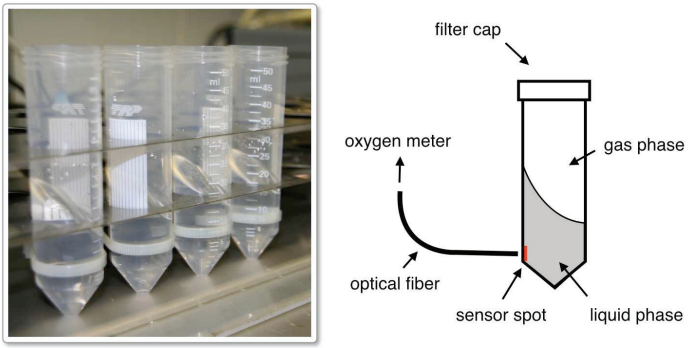


Fig. 3.3. 50-mL shake tubes with 20 mL liquid volume (water) and agitated at 200 rpm (left). Schematic description of the optical oxygen sensing setup (right). The sensor spot was placed right above the conical bottom part of the 50-mL shake tube. Diameter of the sensor spot: 3 mm.

The classic dynamic method was used for the evaluation of the specific liquid mass transfer coefficient *(k_La)*. The liquid phase was saturated with N_2 to reach a constant near zero value of the dissolved oxygen (DO). After N_2 supply was stopped, the gas phase was rapidly replaced by air. Immediately after this, a given agitation speed was applied. The resulting DO increase was monitored until saturation was reached. The k_La was calculated from the slope of the mass balance equation (3.2),

$$ln\frac{(C^* - C_1)}{(C^* - C_2)} = k_L a\,(t_2 - t_1) \qquad (3.2)$$

where C^* [mg L^{-1}] is the saturation DO[4], C_1 and C_2 [mg L^{-1}] are the DO at time t_1 and t_2 [h] respectively.

[4] 6.72 mg L^{-1} at 37°C, assuming normal pressure conditions (1013 mbar)

The following procedure was applied for the estimation of the cell-specific oxygen uptake rate from cells sampled in culture conditions. The cells were seeded at a density of 4-8 x 10^5 cells mL^{-1} and cultivated in 1-L square-shaped bottles (400 mL working volume). The cultures were incubated at 37°C in a CO_2- and humidity-controlled incubator (ISF4-X, Adolf Kühner AG, Birsfelden, Switzerland). The agitation speed was set at 110 rpm. Volumes of 20 mL were sampled and transferred to 50-mL shake tubes fitted with an oxygen sensing spot. The tubes were agitated at 200 rpm at 37°C and the DO was allowed to reach a stationary liquid phase equilibrium. The specific oxygen uptake rate *(qO_2)* [mmol $cell^{-1}$ h^{-1}] was calculated using the previously experimentally estimated k_La, according to equation (3.3),

$$qO_2 = \frac{k_L a(C^* - C)}{d} \qquad (3.3)$$

where d [cells mL^{-1}] is the viable cell density, C^* [mg L^{-1}] is the DO at saturation and C [mg L^{-1}] is the DO measured in the cell suspension at equilibrium with the gas phase.

3.3 Results and discussion

3.3.1 Subcultivation strategy in 50-mL shake tubes

Normally, suspension adapted cell lines are cultivated in shake flasks or spinner flasks for optimization and scale-up purposes. Here, the use of 50-mL shake tubes was investigated as an appropriate alternative for routine cell cultivation and passaging. To illustrate this aspect, CHO DG44 cells and a CHO-derived recombinant cell line (CHO AMW) were continuously subcultivated over a period of five months. CHO AMW cells express a human monoclonal antibody (IgG). Twice a week, the cells were centrifuged and resuspended at a defined initial cell density in new medium. The time interval was kept constant at 84 h ± 4 h. The biomass was precisely assessed using the packed cell volume (PCV) method before and after the subcultivation step (Fig. 3.4).

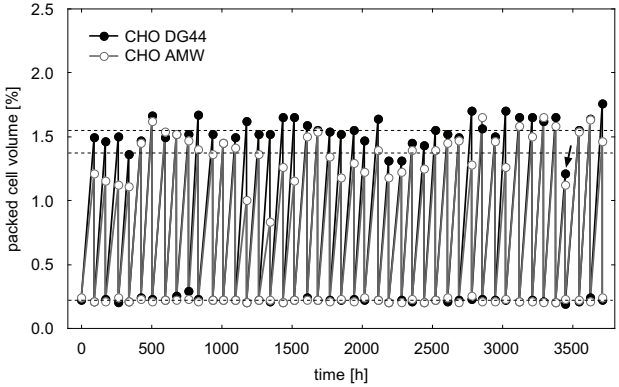


Fig. 3.4. PCV as a function of time of CHO DG44 and AMW cells that were subcultivated continuously twice a week over a period of 5 months in 50-mL shake tubes. The working volume was 20 mL and the orbital agitation speed was set at 200 rpm. The dotted lines represent the average of the PCV values at passage time for both cell lines.

Starting from an initial biomass concentration of 0.22% (corresponding to approx. 8 x 10^5 cells mL^{-1}), CHO cells reached an average maximal biomass concentration of 1.54 ± 0.11% (approx. 6 x 10^6 cells mL^{-1}) while CHO AMW cells reached lower average PCV values of 1.36 ± 0.19%. Clearly, the antibody producing cell line showed slightly lower growth kinetics than the parental cell line from which it was derived. This observation correlated well with the fact that CHO AMW cells were more prone to form small cell clumps. The reasons for this are not clear, but the overall growth of the population is obviously less efficient when cells form aggregates.

This subcultivation strategy was conceived to keep the cells for months in a well-defined and strictly controlled environment. The cells were purposely maintained in the exponential growth phase, thus maintaining the growth kinetics and viability as high as possible. This strategy might be used as a quality control for maintaining cells in cultivation over longer time periods and guaranteeing that the cells used for different purposes (e.g. transfection, optimization, expansion) have a well-documented track record and systematically reach maximal biomass concentrations within the defined tolerances. Also, small differences in growth kinetics between cell lines or clones can be precisely assessed using this method when subcultivating the cells over a certain number of passages. Similarly, if the PCV values for a given cell line varies strongly over passage number, it might be a useful indicator of poor performance in a production process.

A common assumption is that after a given number of passages, cells in culture should be discarded and replaced by new cells from the cell bank. That might be true if the cultivation system is not optimized, especially when the time between passages fluctuates or when the cells are allowed to move into stationary growth phase before passaging. In contrast, this example demonstrates that sustained growth performances, even over months and more than 40 passages, can be maintained or maybe even improved when using an appropriate cultivation strategy and an adequate medium.

Anomalies in growth behavior can be detected quickly when cells are maintained in strictly controlled conditions. One of these anomalies is highlighted in Fig. 3.4. In fact, a drop in maximal cell density occurred after 3450 h and affected both cell lines. This was due to a power cut that happened during this particular passage. For about 2 h, the shaker was stopped and the cells settled within the tube, thus reducing the growth kinetics. This kind of irregularity and others, such as problems with media formulations, equipment, or operator error can be detected and solved, thus reducing the impact of fortuitous events. Lastly, when maintaining the cells in culture with a

volume as low as 10 or 20 mL, the overall medium consumption is limited. In the example shown in Fig. 3.4, one cell line was maintained in culture over 5 months with a total consumption of less than 900 mL serum-free medium.

3.3.2 Cell growth optimization in 50-mL shake tubes

To illustrate the potential of 50-mL shake tubes as a powerful tool for multi-parameter screenings, a cell growth optimization experiment is briefly presented next. A second example is shown in Chapter 5 of this thesis. Here, the objective was to improve the growth of CHO DG44 cells in a chemically defined serum-free medium. The use of protein hydrolysates (peptones) from non-animal sources is an increasingly applied strategy for improving growth and productivity. Some commercially available media contain plant peptones in their formulation. Here, peptones, mostly from soy bean origin, were added to cells growing in a peptone-free cell culture media. The test included 6 peptones (Tab. 3.1) each added at 4 concentrations. Each condition was tested in triplicate.

Tab. 3.1. Peptones used in the screening experiment. All peptones originated from soy, except P02, which was a casein hydrolysate.

Code	Peptone	Supplier
P01	Soy Peptone A2 SC	Organotechnie
P02	Tryptone N1	Organotechnie
P03	Soy Peptone E110	Organotechnie
P04	HyPep 1510 soy	Quest
P05	ProPEP2 soy	Cambrex
P06	Difco CG Soypeptone UF	BD

First, 10 mL aliquots of a cell suspension with a density of 3 x 10^5 cells mL^{-1} were transferred into 72 tubes for the peptone screening test and into 8 additional tubes for controls. The peptones were then added as a concentrated sterile filtered aqueous solution (10%). The tubes were incubated in a CO_2 and humidity controlled incubator,

with an agitation speed of 180 rpm. The tubes were sampled on day 6 for measuring PCV.

The results show a benefit for each peptone and concentration tested (Fig. 3.5). However, the fold increase over the control cultures varied from only 1.2 (P05, 0.1%) up to 2.3 (P06, 0.5%). The cell density, as evaluated on day 6, was improved from approximately 4 x 10^6 cells mL^{-1} in peptone-free media to more than 1 x 10^7 cells mL^{-1} in the same media supplemented with peptone (P06, 0.5%).

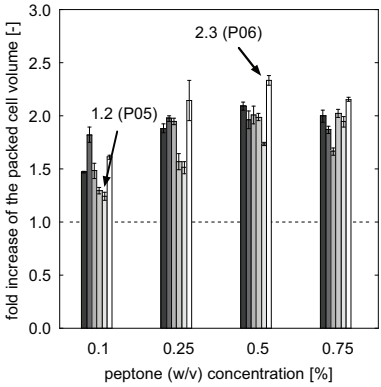


Fig. 3.5. CHO DG44 cells cultivated in peptone-free media supplemented with 6 different soy peptones (P01 to P06, from left to right) each added at 4 different concentrations (0.1, 0.25, 0.5 and 0.75%). The day 6 PCV values were plotted as the fold increase over the average day 6 PCV of the control cultures. The average day 6 PCV value in the control tubes was 0.98% (n = 8), which corresponds to approx. 4 x 10^6 cells mL^{-1}. The error bars represent the standard deviation of triplicates.

The complexity of cell culture processes requires extensive empirical testing capacity. For example, the outcome of the case shown here has a very limited application. Each cell line has its own response to a particular media. Multi-parameter testing in shake tubes combined with the precision of biomass assessment using the PCV method represents a true opportunity for efficient cell culture process optimizations. No matter the kind of process, it is of particular interest to rapidly identify the best possible culture conditions and ensure the robustness of the process. Standard tools

such as conventional shake flasks or spinner vessels were not developed to run such optimization procedures in an efficient way. In contrast, disposable 50-mL tubes are easy to handle and represent a well-defined environment that ensures reproducible testing conditions. They shorten the set-up time and allow to test up to hundreds of parameters simultaneously (3).

As demonstrated here, the use of peptones is promising and seems to be well accepted by the regulatory authorities for industrial applications. However, the positive impact on growth might be impaired by fluctuating properties. Variations in raw material or in production process are known to change the impact of peptones on cell metabolism (18). Therefore, relying on a high performance small-scale testing technology is critical, ensuring that such changes are detected and characterized readily. Disposable 50-mL shake tubes might well be used by media or peptone producers as an efficient tool for continuously assessing the quality of media compounds and ensuring consistent productivities.

3.3.3 Oxygen transfer in 50-mL shake tubes

The classic dynamic method was used for the evaluation of the specific liquid mass transfer coefficient *(k_La)* in 50-mL shake tubes. Pure water was used as a liquid phase. The tests were performed at a constant temperature of 37°C. Preliminary tests were done to estimate the impact of the gas transfer resistance *(k_Ga)* due to the filter cap on the overall oxygen mass transfer rate. As expected in short time tests, no measurable difference was observed between tubes fitted with a filter cap (closed) and tubes that were open. The rate of dissolved oxygen increase in pure water at 37°C was comparable in both systems (Fig. 3.6.A) resulting in very similar k_La values (approx. 18 h^{-1} for 20 mL and 200 rpm) (Fig. 3.6.B). This means that during the brief time required to reach saturation (12 min in this case) the gas partial pressure in the tube headspace was very similar in both closed and open tubes. Therefore, to simplify, subsequent k_La evaluations in 50-mL tubes were systematically performed without caps.

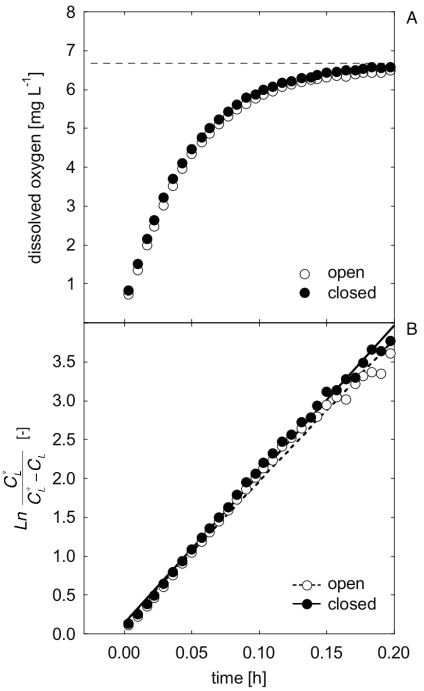


Fig. 3.6. (A) Dissolved oxygen increase in 50-mL shake tubes with 20 mL water at 37°C and an agitation speed of 200 rpm. The dotted line represents the saturation DO (c_L^*) at 37°C and normal pressure (6.72 mg L^{-1}). (B) The slope of the regression lines represents the calculated k_La according to equation 3.2. k_La values of 17.4 and 18.8 h^{-1} resulted for the open and closed systems, respectively.

To characterize the oxygen transfer effects in 50-mL shake tubes, the k_La was assessed for different combinations of agitation speed and working volume. The experiments were done with pure water at 37°C without a filter cap. The k_La was noticeably affected by the variation of the agitation speed (140-280 rpm). Values varied between 7 and 45 h^{-1} with 20 mL working volume and between 3 and 22 h^{-1} with 30 mL (Fig. 3.7.A). It was assumed that the k_La varied essentially in relation with

the increased gas-liquid interfacial area *(S)* that resulted at higher shaking speeds. This is schematically shown in Fig. 3.8.A.

The liquid mass transfer coefficient k_L was probably increased at higher agitation speed as well. k_L depends on the diffusivity of the gas in the liquid, which might be favored at increased agitation speed (5). Hermann *et al*. assessed the variations of the k_L in deep-well plates with liquid volumes of 200 μL (19). k_L was found to be more or less constant in deep-wells with a round cross-section (0.2 m h^{-1}). A small increase was observed at agitation speeds above 600 rpm.

In bubble aeration systems, such as stirred-tank and airlift bioreactors, the specific interfacial area is given by the number and size of the bubbles that are generated. In surface aeration systems, the oxygen transfer occurs over the liquid surface only. This should result in poor oxygen transfer rates, as it is the case in spinner systems. However, investigations made with shake flasks and now with 50-mL shake tubes show that generating a dynamic interface with increased exchange surface is sufficient to achieve optimal oxygen transfer properties in small-scale systems.

Next, the working volume was varied from 15 to 45 mL. A more than 2-fold k_La decrease at both 200 and 240 rpm was observed (Fig. 3.7.B). Noteworthy, even with a working volume of 45 mL (90% of the nominal tube volume), a k_La of approximately 8 h^{-1} resulted. The decrease of the k_La was correlated to the reduction of the specific interfacial area *a* (Fig. 3.8.B). At a constant agitation speed, the interfacial surface *(S)* is expected to be approximately the same for different volumes. Therefore, when using larger working volumes, the specific interfacial area *(a)* is less favorable than with smaller volumes. An increase in working volume should be compensated by a higher shaking speed, thus keeping the specific interfacial area constant.

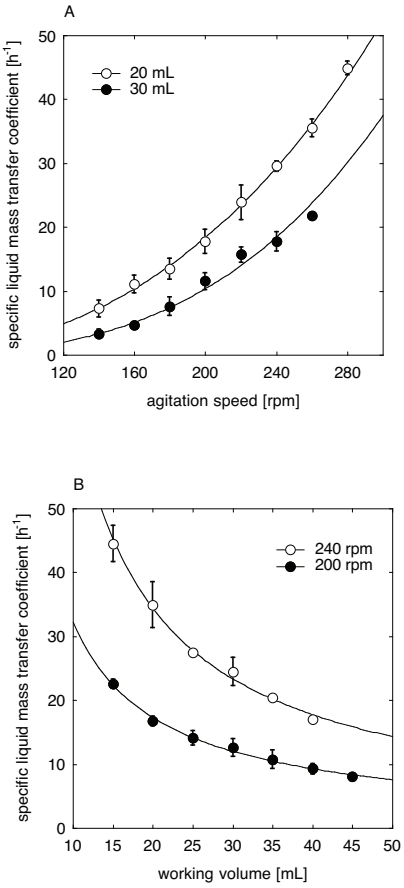


Fig. 3.7. Experimentally estimated specific liquid mass transfer coefficient *(k_La)* as a function of agitation speed with constant working volume (20 and 30 mL) (A) and as a function of volume with constant agitation speed (200 and 240 rpm) (B). Each data point represents the average of measurements performed simultaneously in 3 different 50-mL shake tubes (n = 3). The liquid phase was pure water. Experiments were carried out at 37°C.

$$a = \frac{S}{V}$$

a : specific interfacial area [m^{-1}], S : interfacial area [m^2]
V : liquid volume [m^3]

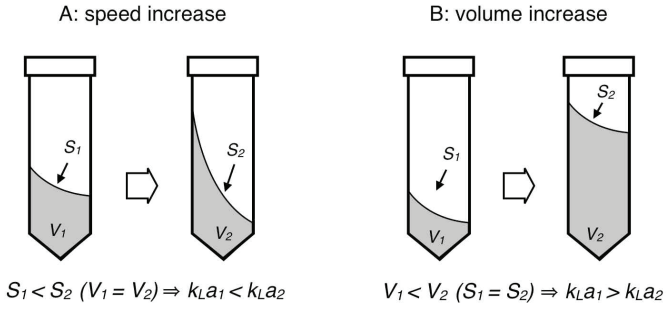


Fig. 3.8. Schematic description of the impact of variations in agitation speed (A) and working volume (B) on the specific liquid mass transfer coefficient *(k_La)* in 50-mL shake tubes.

The oxygen transfer characterization in 50-mL shake tubes revealed that almost any desired k_La values could be obtained when varying the shaking speed or the working volume. The upper limit of k_La values was close to 50 h^{-1} and might be higher when using lower working volumes, such as 5 and 10 mL. The position of the sensor spot might be optimized in that respect. If positioned closer to the tube bottom, which would require a more complicated setup, measurements with smaller working volumes could be made. Additionally, using cell culture media instead of pure water would result in an even better experimental estimation of the k_La. The salinity of cell culture media reduces the saturation DO.

3.3.4 Oxygen uptake rate of mammalian cells

Based on the experimentally assessed k_La values, the specific oxygen uptake rate *(qO_2)* was measured for different cell lines in the exponential growth phase. The absolute values, between 1 and 3 x 10^{-10} mmol $cell^{-1}$ h^{-1}, are in good agreement with data from earlier studies (9, 20, 21, 22). The data obtained here suggest a linear correlation between qO_2 and cell density (Fig. 3.9). The trend seems to be similar for

the different cell lines, with a reduced per cell oxygen consumption at higher densities. However, it is not known whether this effect is due to a rate-limiting factor or due to a metabolic shift occurring at higher cell density. The specific oxygen uptake of HEK 293 cells was approximately 1.5 times higher than the uptake measured in CHO cells. This is a logical consequence of the volume difference between both cell types. Also, the small qO_2 difference between CHO DG44 and CHO AMW cells seemed to be correlated with the growth rate differences between the two cell lines, as observed in long-term parallel subcultivation tests (see Fig. 3.4).

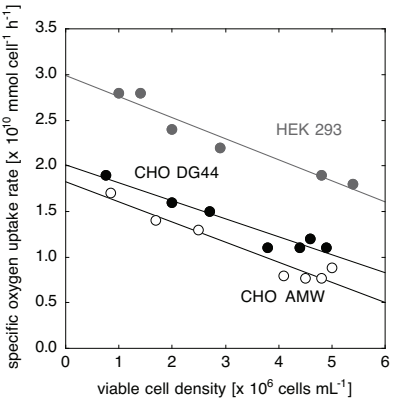


Fig. 3.9. Specific oxygen uptake rate *(qO_2)* of CHO DG44, AMW and HEK 293 cells as a function of viable cell density. qO_2 was measured in 50-mL shake tubes with 20 mL cell suspensions and an agitation speed of 200 rpm. The corresponding k_La was 18 h^{-1}.

An accurate knowledge of the cell line and process specific oxygen uptake is a prerequisite for a rational scale-up of bioprocesses. As shown in the previous section, there are methods for precisely assessing the qO_2. Most of the mammalian cells used in industrial bioprocesses have a qO_2 between 1 and 3 x 10^{-10} mmol $cell^{-1}$ h^{-1}. For example, knowing that the cell line has a qO_2 of 3 x 10^{-10} mmol $cell^{-1}$ h^{-1}, a k_La of at least 8.6 h^{-1} is theoretically required to achieve a cell density of 6 x 10^6 cells mL^{-1}. This correlation between qO_2, cell density and k_La is shown in Fig. 3.10 and is useful for adjusting a process to the cell specific oxygen requirements.

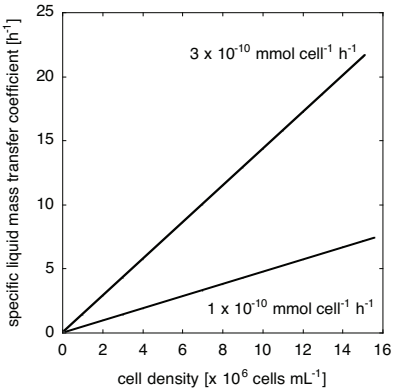


Fig. 3.10. Theoretical correlations between cell density and k_La. The lines represent two different specific oxygen uptake rates (1 and 3 x 10^{-10} mmol $cell^{-1}$ h^{-1}).

To avoid oxygen limitations when scaling up a bioprocess, this correlation might be used to predict the required k_La. However, the reliability of such a correlation is limited. The estimation of the qO_2 in 50-mL shake tubes was based on the experimentally determined k_La, which was assessed in a system with pure water (see previous Section). Medium salinity, viscosity increase and CO_2 release due to the cells are known to reduce the oxygen solubility.

3.4 Conclusion

50-mL shake tubes, as shown in this chapter, are ideal for maintaining cell lines in exponential growth phase over months in a strictly controlled and well-defined environment. Since each single tube reproduces the same physico-chemical growth conditions, cell line and media optimization are facilitated when using multiple tubes in parallel screening experiments. Thus, by varying a single parameter, the effect on growth is assessed with a high degree of confidence.

Further, 50-mL shake tubes were shown to continuously deliver sufficient oxygen to the cells growing in suspension, ensuring a non-limiting rate of oxygen transfer. This explains the high cell densities (more than 1 x 10^7 cells mL^{-1}) frequently observed in shake tubes. Finally, shake tubes equipped with a non-invasive DO sensing system were found to be useful and convenient for assessing the specific oxygen uptake rate of various cell lines.

Within the past two or three years, an increasing number of academic and industrial biotechnology labs successfully tested 50-mL shake tubes for different applications, mostly cell line and media optimization. Due to the reliability of the data obtained with 50-mL shake tubes, the technology presented here allows for a rapid, flexible, and cost-effective identification of optimal process parameters.

3.5 References

(1) Girard P, Jordan M, Tsao M, Wurm FM. 2001. Small-scale bioreactor system for process development and optimization. Biochem Eng J. 7(2):117-119.

(2) De Jesus MJ, Girard P, Bourgeois M, Baumgartner G, Jacko B, Amstutz H, Wurm FM. 2004. TubeSpin satellites: a fast track approach for process development with animal cells using shaking technology. Biochem Eng J. 17:217-223.

(3) Stettler M, De Jesus M, Ouertatani H, Engelhardt E-M, Muller N, Chenuet S, Bertschinger M, Baldi L, Hacker D, Jordan M and others. 2007. 1'000 non-instrumented bioreactors in a week - Novel disposable technologies for rapid scale-up of suspension cultures. In *Cell Technology for cell products*. Proceedings of the 19th ESACT meeting, Harrogate, UK. Edited by Rodney Smith, Springer. pp. 489-95.

(4) Muller N, Derouazi M, Van Tilborgh F, Wulhfard S, Hacker DL, Jordan M, Wurm FM. 2007. Scalable transient gene expression in Chinese hamster ovary cells in instrumented and non-instrumented cultivation systems. Biotechnol Lett. 29(5):703-11.

(5) Krahe M. 2002. Biochemical engineering. in *Ullmann's Encylopedia of Industrial Chemistry*, John Wiley & Sons. 5:171-240.

(6) Maier U, Buchs J. 2001. Characterisation of the gas-liquid mass transfer in shaking bioreactors. Biochem Eng J. 7(2):99-106.

(7) Zhang H, Williams-Dalson W, Keshavarz-Moore E, Shamlou PA. 2005. Computational-fluid-dynamics (CFD) analysis of mixing and gas-liquid mass transfer in shake flasks. Biotechnol Appl Biochem. 41:1-8.

(8) Kensy F, Zimmermann HF, Knabben I, Anderlei T, Trauthwein H, Dingerdissen U, Buchs J. 2005. Oxygen transfer phenomena in 48-well microtiter plates: determination by optical monitoring of sulfite oxidation and verification by real-time measurement during microbial growth. Biotechnol Bioeng. 89(6):698-708.

(9) Deshpande RR, Heinzle E. 2004. On-line oxygen uptake rate and culture viability measurement of animal cell culture using microplates with integrated oxygen sensors. Biotechnol Lett. 26(9):763-7.

(10) Deshpande RR, Koch-Kirsch Y, Maas R, John GT, Krause C, Heinzle E. 2005. Microplates with integrated oxygen sensors for kinetic cell respiration measurement and cytotoxicity testing in primary and secondary cell lines. Assay Drug Dev Technol. 3(3):299-307.

(11) Amoabediny G, Buchs J. 2007. Modelling and advanced understanding of unsteady-state gas transfer in shaking bioreactors. Biotechnol Appl Biochem. 46:57-67.

(12) Gupta A, Rao G. 2003. A study of oxygen transfer in shake flasks using a non-invasive oxygen sensor. Biotechnol Bioeng. 84(3):351-8.

(13) Wittmann C, Kim HM, John G, Heinzle E. 2003. Characterization and application of an optical sensor for quantification of dissolved O2 in shake-flasks. Biotechnol Lett. 25(5):377-80.

(14) John GT, Klimant I, Wittmann C, Heinzle E. 2003. Integrated optical sensing of dissolved oxygen in microtiter plates: a novel tool for microbial cultivation. Biotechnol Bioeng. 81(7):829-36.

(15) Muller N, Girard P, Hacker DL, Jordan M, Wurm FM. 2005. Orbital shaker technology for the cultivation of mammalian cells in suspension. Biotechnol Bioeng. 89(4):400-6.

(16) Stettler M, Jaccard N, Hacker D, De Jesus M, Wurm FM, Jordan M. 2006. New disposable tubes for rapid and precise biomass assessment for suspension cultures of mammalian cells. Biotechnol Bioeng. 95(6):1228-33.

(17) Luecke J. 2006. Fast, reproducible and reliable determination of biomass in suspension cell cultures with VoluPAC tubes. Nat Methods. published online: 21 September 2006.

(18) Gorfien S. 2007. Cell culture media development: customization of animal origin-free components and supplements. in *Cell culture and upstream processing*. Edited by M. Butler, Taylor & Francis Group. pp. 81-98.

(19) Hermann R, Lehmann M, Buchs J. 2003. Characterization of gas-liquid mass transfer phenomena in microtiter plates. Biotechnol Bioeng. 81(2):178-86.

(20) Ducommun P, Ruffieux P, Furter M, Marison I, von Stockar U. 2000. A new method for on-line measurement of the volumetric oxygen uptake rate in membrane aerated animal cell cultures. J Biotechnol. 78(2):139-47.

(21) Ruffieux PA, von Stockar U, Marison IW. 1998. Measurement of volumetric (OUR) and determination of specific (qO2) oxygen uptake rates in animal cell cultures. J Biotechnol. 63(2):85-95.

(22) Jorjani P, Ozturk SS. 1999. Effects of cell density and temperature on oxygen consumption rate for different mammalian cell lines. Biotechnol Bioeng. 64(3):349-56.

Chapter 4

Design and evaluation of pilot and production scale shake bioreactors

4.1 Introduction

Suspension cell cultivation using disposable shake bioreactors in volumes higher than 10 L is not new. Liu and Hong published pioneering work in 2001 (1). They reported the design of shake bioreactor systems adapted for animal cell cultures consisting of a shaker and a cylindrical shaped vessel of variable size with a height to diameter ratio of 3:2. In fed-batch, CHO cells were grown to 6 x 10^6 cells mL^{-1} in a 56 L shake vessel containing 18 L of cell suspension. The report stressed the potential utility of this simple and cost-effective bioreactor system in cell culture research. It also concluded that further characterization of oxygen transfer and mixing was required. More recently, Liu collaborated with another research group to successfully extend the use of the first system to insect and plant cells growing in suspension (2). In this study, the mixing time in a 50-L bioreactor with 35 L working volume varied between 100 and 10 s when the agitation speed increased from 80 to 120 rpm.

The possibility of using even larger disposable shake containers was suggested by Büchs and coworkers (3). The power consumption of large rotary shaking vessels with agitation speeds of 100-300 rpm was investigated. In large shake systems (20 L), the power consumption per unit volume was in the same range as that of small shaking flasks (less than 2 L). However, no study addressing the question of oxygen supply in larger orbital shake vessels has so far been published. Modelling approaches aimed at predicting the oxygen transfer in shake flasks were recently proposed (4, 5, 6, 7). Though developed for small-scale systems, such models might be useful for the development of large-scale shake bioreactors.

As shown in Chapter 3, the cell specific oxygen uptake rate *(qO_2)* of mammalian cells growing in suspension is 1-3 x 10^{-10} mmol cell^{-1} h^{-1}, depending on the cell line, the growth phase, and the process. Assuming a high qO_2 of 3 x 10^{-10} mmol cell^{-1} h^{-1}, the required specific oxygen transfer coefficient *(k_La)* would be approximately 8 h^{-1} at a cell density of 6 x 10^6 cells mL^{-1} (see Fig. 3.10), which corresponds to a maximal oxygen transfer rate (OTR_{max}) of 1.8 mmol L^{-1} h^{-1}. In large shake cultivation systems, the k_La has to be sufficient to match the oxygen demand at any time of the cell culture (Fig. 4.1).

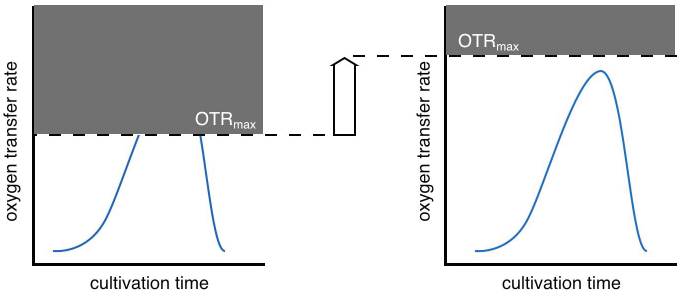


Fig. 4.1. Effect of oxygen limitation on an idealised cell culture process, adapted from (8). Process affected by oxygen limitation (left) and same process with appropriate OTR_{max}, ensuring that no oxygen limitation occurs (right).

When the k_La is limited, the oxygen transfer rate can be enhanced by increasing the saturation dissolved oxygen concentration *(C_L^*)* (see equation 3.1). This strategy is used in stirred-tank or in Wave bioreactors when the partial pressure of the oxygen above the liquid phase is increased or when the air inlet flow is enriched with oxygen (9, 10). This effect is schematically described in Fig. 4.2 (11). A similar aeration strategy was applied in this study when scaling up orbital shake bioreactors. Instead of passively exchanging air, like in 50-mL shake tubes, larger systems were actively aerated, meaning that a given airflow rate was provided to replace the gas phase. If needed, the airflow was enriched with pure oxygen to further increase the oxygen transfer rate.

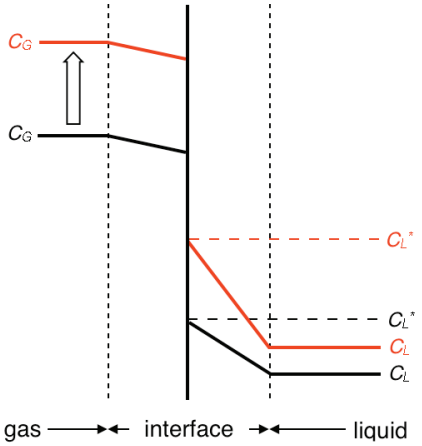


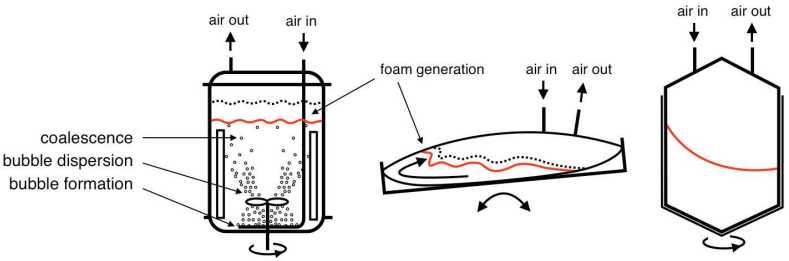
Fig. 4.2. Simplified concentration profiles at the gas-liquid interface and difference between passive (black line) and active (red line) aeration mode with oxygen enriched air. The passive aeration results in a given equilibrium dissolved oxygen concentration (C_L^*) that can be increased when providing an oxygen enriched airflow.

The shear sensitivity of animal cells in shake bioreactors is another important issue, though little is known about hydromechanical stress conditions at larger volumes. In stirred-tank bioreactors with radial or axial flow impellers, the shear stress is greatest near the impeller tip and declines rapidly when cells move away from the impeller. Expressions to estimate the maximum shear stress at the impeller blade are used to calculate the shear stress that can be expected when increasing the scale (12).

Unlike stirred-tank bioreactors, where the energy input is due to the impeller, for shake cultivation systems, the wetted contact area between the rotating liquid and the vessel is regarded as the “stirring element” (13). As a result, the power input is more evenly distributed than in stirred-tank bioreactors. The difference was recently quantified (14). At the same volumetric power consumption, the maximum energy dissipation rate in shake flasks was found to be 10 times lower than in stirred-tank bioreactors. Though promising, these investigations were limited to lab scale with

shake flasks of volumes no larger than 1 L. In wave-type bioreactors, shear stress conditions, though never characterized, are believed to be almost negligible (9).

Until recently, the major drawbacks of novel disposable shake bioreactors using single-use cell culture bags were the limitation in scale and problems in predicting the fluid dynamics at larger scales. The development of larger wave-type bioreactors was affected by such problems due to the complexity of the hydrodynamic behaviour and the almost endless number of combinations among bag geometry, filling volume, rocking speed, and rocking angle. A specificity of orbital shake systems is that the gas-liquid interfacial area remains nearly constant during shaking and is well-defined in contrast to all other bioreactor types, especially wave-type bioreactors (15) (Fig. 4.3). As a consequence, the scale-up of orbital shake systems might be simpler as compared to the Wave bioreactor. Similar fluid dynamics are expected to be reproduced at different scales, resulting in more predictable oxygen transfer rates. Additionally, less foaming is expected as compared to stirred-tank bioreactors and wave systems.



	stirred tank bioreactor	wave bioreactor	orbital shake bioreactor
mass transfer area	complex, difficult to determine	complex, difficult to determine	well-defined, predictable
foam formation	high	moderate	low

Fig. 4.3. Properties of the air-liquid mass transfer area in bioreactors for animal cell culture. The schematic description was adapted from (15).

To confirm the promising features of orbital shake technology at larger scales, various prototype shake bioreactors were designed and tested and the results are presented here. For nominal volumes of up to 20 L, autoclavable disposable polycarbonate containers were used. For larger volumes, presterilized disposable bags and

containers were tested. Despite a reduced specific area of the gas-liquid interface in larger systems, optimal conditions were identified, which resulted in growth kinetics approaching those observed in 50-mL shake tubes. This study shows that orbital shake technology might soon be applied from the millilitre scale (50-mL shake tubes) up to the production scale (1'000 L) for fast and reliable cell line optimization, process scale-up, and production of recombinant therapeutics.

4.2 Material and Methods

4.2.1 Cells

Suspension-adapted CHO DG44 and CHO AMW cells were grown in serum-free ProCHO5 (Lonza, Verviers, Belgium) or CHO PFM medium (SAFC Biosciences, St. Louis, MO). The cells were maintained in 50-mL disposable shake tubes fitted with a filter cap for passive headspace aeration (CultiFlask 50, Sartorius AG, Göttingen, Germany) (16). The cells were passed every 3-4 days at densities of 2-5 x 10^5 cells mL^{-1}. Round- or square-shaped shake bottles were used to expand the cells to larger volumes (17). The cells were incubated in a CO_2- and humidity-controlled atmosphere at 37°C with orbital agitation (ISF4-X, AdolfKühner AG, Birsfelden, Switzerland).

4.2.2 20- and 150-L stirred-tank bioreactors

20- and 150-L pilot scale bioreactors equipped with marine impellers were used for the cultivation of stable cell lines in batch and fed-batch cultures (Fig. 4.4) (Bioengineering AG, Wald, Switzerland). The DO was maintained at 20% or more using bubble aeration and oxygen enriched air. CO_2 in the inlet airflow and NaOH were used to maintain a physiological pH. Agitation speed was maintained in a range of 150-250 rpm in both bioreactors. Glucose, glutamine and sodium bicarbonate were supplemented to avoid depletion of these compounds.

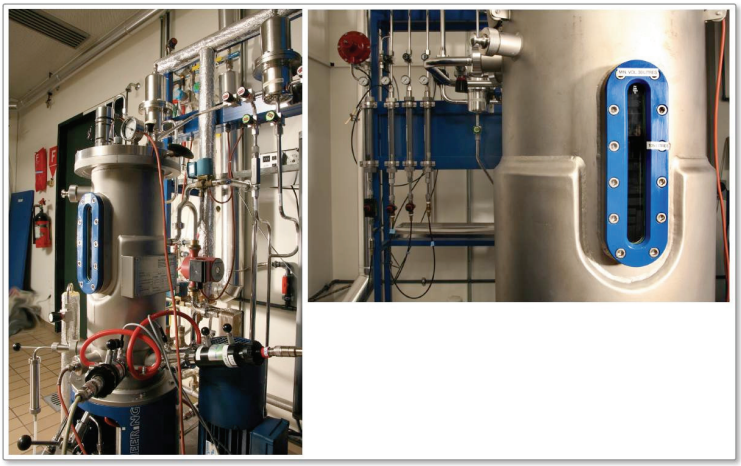


Fig. 4.4. 20- and 150-L instrumented stirred-tank bioreactors (photos courtesy of Alain Herzog).

4.2.3 5-, 10- and 20-L shake bioreactors

Square-shaped polycarbonate containers with nominal volumes of 5, 10 and 20 L were used (Biotainer Carboy, Cellon SA, Luxembourg) (Fig. 4.5). The working volume was half of the nominal volume unless otherwise mentioned. Though intended for single-use, the bottles were cleaned and autoclaved up to 10 times. Standard benchtop shakers with a rotational diameter of 50 mm (ES-X, Adolf Kühner AG, Birsfelden, Switzerland) were operated in CO_2-controlled incubators at 37°C. The bottles were fitted with a 3-port cap for active headspace aeration. One of the ports was connected to a low-cost "aquarium" membrane pump located in the CO_2-controlled incubator. A constant airflow of 1 L min^{-1} was pumped into the headspace through a sterile inlet filter. The gas outlet was fitted with a second sterile filter. For container with volumes up to 10 L, sampling was performed under a laminar flow hood. For containers with volumes of 20 L, samples were taken directly in the incubator through a sterile sampling port mounted on the cap of the shake bioreactor (Fig. 4.5).

Fig. 4.5. 20-L polycarbonate containers used as disposable shake bioreactors for the cultivation of mammalian cells in suspension. The sterile filtration of the cell culture media (left) and the *in situ* sampling (right) are shown.

4.2.4 Large-capacity prototype shakers

Kühner AG provided two large-capacity shakers (Fig. 4.6). First, a RC-X shaker was modified to hold two 200-L containers. This pilot scale shaker was operated in a warm room. The shaking diameter was set at 50 mm. Second, a production scale shaker was designed and constructed to hold containers up to 1'500 L. The agitation diameter was set at 100 mm. The prototype was operated on the ground floor of the pilot plant located in the chemical engineering building of the EPFL. Both shakers were equipped with Kühner's direct drive technology, meaning that the speed of the motor was identical to the speed of the container. The advantage was little noise, low power consumption due to less mechanical friction, and maintenance-free operation. A parallelogram mechanism ensured that the shaking movement on the platform was absolutely equal and orbital, independent of the load distribution.

Fig. 4.6. Pilot scale shaker based on a modified RC-X shaker (Adolf Kühner AG, Birsfelden, Switzerland) (left). Production scale prototype shaker designed and constructed by Adolf Kühner AG (right).

4.2.5 Single-use cell culture bags and containers

For cell culture volumes up to 200 L, standard bags and containers normally used for sterile liquid handling were tested (3D BPC, HyClone, South Logan, UT). The shape of the container was cylindrical with a diameter of 600 mm and a conical bottom. The bags were equipped with top and bottom connexions for inoculation, feeding and sampling. Inlet and outlet sterile air filters were connected to ports located on the top. The working volume was varied between 50 and 125 L. Since no appropriate standard bag was available for production scale tests, a 1'500 L cell culture bag was designed in collaboration with Lonza (Lonza SPRL, Verviers, Belgium). The sterile bags were equipped with ports and connections located on the top for inoculation, feeding, and sampling (Fig. 4.7). Inlet and outlet sterile air filters were connected to ports located on the top. The bags were designed to fit into a round open 1'500 L container made of linear low density polyethylene (LLDPE) with a diameter of 1'300 mm, a height of 1'250 mm, and a wall thickness of 8 mm (Plastomatic AG, Muttenz,

Switzerland). A metallic conical support was constructed to fit in the bottom of the container, resulting in a height difference of 180 mm between the center of the container and the wall.

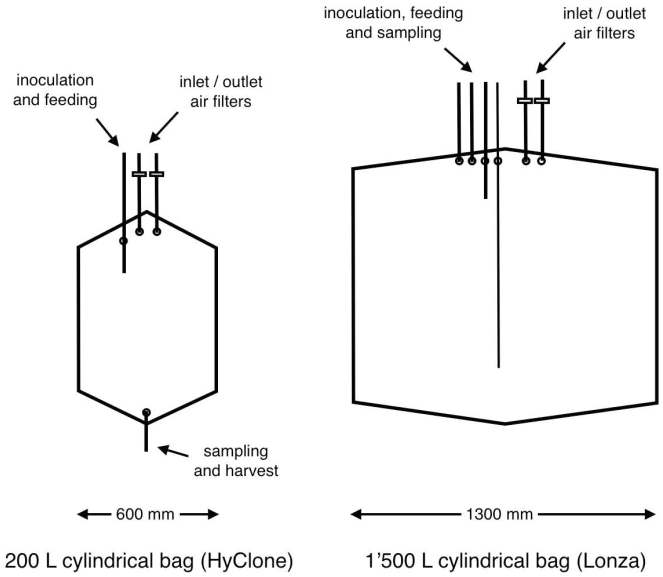


Fig. 4.7. Schematic drawing of the cylindrical disposable cell culture bags. The 200-L HyClone bag was standard item (left). The 1'500-L Lonza bag was designed and manufactured for this project (right). The ready-to-use bags were pre-sterilized by gamma irradiation.

Silicone heat elements were used to maintain the temperature of the cell culture suspension in the production scale shake bioreactor (1'500 L bag) (Prang + Partner AG, Pfungen, Switzerland). Two large half-circle silicon heaters were adjusted to the container conical bottom. The cell culture bag was placed in direct contact with the heating elements. A 10 mm thick neoprene insulation sheet was used to insulate the container wall from the outside environment. A PT-100 temperature probe was inserted between the container inner wall and the cell culture bag at a height of 300

mm from the bottom (Prang + Partner AG, Pfungen, Switzerland). A thermostatic temperature controller was used to maintain the temperature of the well-mixed bioreactor at ± 0.5°C of the set point. The temperature operating range was 22°C (room temperature) to 45°C.

4.2.6 200- and 1'500-L shake bioreactors

To expand the cells for pilot and production scale operations, shake bioreactor systems of increasing volumes were successively used. The scale-up sequence comprised shake bioreactors of the following nominal volumes: 50 mL, 1 L, 10 L, and 200 L. The latter one was used to inoculate the production scale shake bioreactor using a 1:10 dilution factor (Fig. 4.8).

Fig. 4.8. 200-L shake bioreactor and modified large-capacity shaker (left). The production scale bioreactor was inoculated with 75 L of cells expanded using the 200-L shake bioreactor (right).

Each of these systems was optimized to reach cell densities of 4 - 6 x 10^6 cells mL^{-1}. At pilot and production scale, the airflow rate through the headspace was actively controlled using a membrane pump. The pH was manually adjusted by varying the CO_2 concentration in the inlet airflow. The 1'500-L production scale shake bioreactor was supplied with the desired volume of cell culture medium using sterile connections and a sterile filtration step (0.22 μm). The medium was heated up overnight to 37°C at a moderate shaking speed. Then, the pilot scale bioreactor was connected to the 1'500-L system for the inoculation. The cells were cultivated at agitation speeds between 42 and 45 rpm, depending on the cell density. The airflow rate was varied between 10 and 50 L min^{-1}. At later stages of the exponential growth phase, an increasing proportion of O_2 was added to the airflow rate. The outlet air filter was heated up to avoid condensation. Samples were taken daily for the monitoring of cell density, viability, PCV, and pH. Glucose and sodium bicarbonate levels were measured and adjusted by regular feeds.

Fig. 4.9. (B) 1'500-L disposable shake bioreactor with a cell culture volume of 750 L and agitated at 43 rpm. Height: 2 m, floorspace: 4 m^2, weight empty: 1'000 kg.

4.2.7 Optical system for dissolved oxygen sensing

For oxygen transfer evaluations in large-scale shake bioreactors, an optical sensing set-up and methodology similar to the description in Section 3.2.3 was used. Oxygen sensor spots were fixed with silicone glue either on the lateral inner wall of polycarbonate containers or, in the case of cell culture bags, on the inside layer of the plastic film. In the 200 L bag, the spot was located in the conical bottom whereas in the 1'500 L bag, the spot was placed on the lateral inner wall at a height of 300 mm from the bottom (Fig. 4.10). Small openings were created in the containers to place the optical fiber in contact with the outer layer of the plastic film.

The classic dynamic method was used for the evaluation of the specific liquid mass transfer coefficient *(k_La)*, as described in Section 3.2.3. After saturating the liquid phase with N_2 bubbles and reaching a DO value near zero, the headspace was replaced with air and the shaker was started. A given airflow rate was applied to the bag, which resulted in an increased oxygen partial pressure in the headspace. The subsequent DO increase was monitored for the k_La estimation. Since sensor spots were inserted into non-sterile cell culture bags, this setup was used only for the purpose of oxygen transfer evaluations in pure water, not for the DO monitoring in cell cultures.

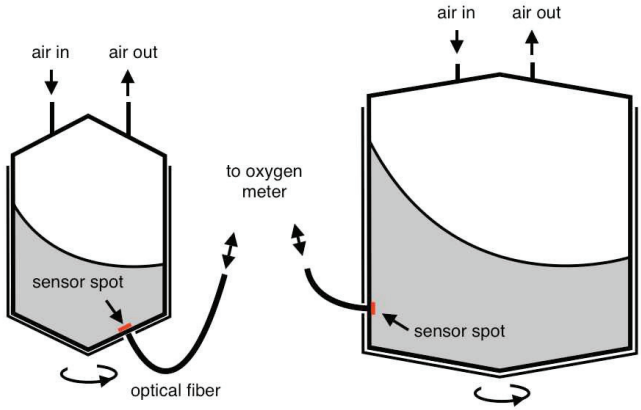


Fig. 4.10. Schematic description of the optical oxygen sensing set-up for the 200-L (left) and the 1'500-L (right) shake systems.

4.3 Results and discussion

4.3.1 Oxygen transfer in large-scale shake bioreactors

Preliminary tests consisted of evaluating the impact of active surface aeration on the oxygen transfer rate (OTR). Experiments were done at the 10-L scale with pure water at room temperature. A 10-L polycarbonate container was equipped with a sensor spot that was located on the inside wall, close to the bottom. An airflow rate of 1 L min^{-1} was applied for active aeration using sterile inlet and outlet air filters which resulted in a small air pressure increase in the container headspace. Passive aeration meant that the container was open for passive air exchange with the environment. At 60 rpm, a 2-fold k_La increase resulted when air was actively exchanged (Fig. 4.11). The difference was even more significant at 64 rpm where a k_La of 14.2 h^{-1} was found, corresponding to a 4-fold increase over the passively aerated system. As a comparison, this relatively high k_La obtained with a liquid volume of 5 L was similar to values obtained with passively aerated 50-mL shake tubes containing 25 mL and agitated at 200 rpm (Fig. 3.7). Due to an increased oxygen driving force resulting from the active aeration principle, comparable oxygen transfer properties were found despite a 200-fold liquid volume increase.

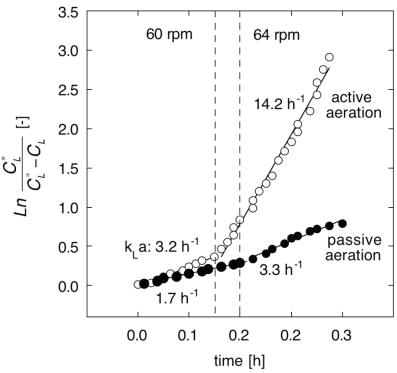


Fig. 4.11. Oxygen transfer in actively and passively aerated shake bioreactors. Tests were performed in a 10-L shake bioreactor with 5 L of pure water at room temperature (RT). Airflow rate: 1 L min^{-1}. The agitation speed was increased from 60 to 64 rpm. The dotted lines show the time at which the speed was increased for the active and for the passive system, respectively. C_L^* is the DO at saturation and C_L is the measured DO. C_L^*: 8.73 mg L^{-1} at RT assuming normal pressure conditions.

Next, the k_La was evaluated in cell culture bags at the 200 and 1'500 L scale (Fig. 4.12.A). Tests were performed at room temperature with pure water. At the 200 L scale, with working volumes of 100 and 120 L and an agitation speed of 70 rpm, k_La values close to 2 h^{-1} resulted. The airflow rate was set at 2.4 L min^{-1}. At the 1'500 L scale with a liquid volume of 750 L, k_La values of 1 h^{-1} were obtained at 43 rpm. The airflow rate was 10-15 L min^{-1}. The k_La values were compared to data obtained previously with 50-mL shake tubes described in Section 3.3.3 (Fig. 4.12.A). The k_La was reduced approximately 10 times when scaling up shake cultivation systems from the mL scale to pilot and production scales.

From the theoretic correlation shown in Section 3.3.4, the k_La values typically obtained in 200 and 1'500 L systems would limit the cell growth between 2 and 4 x 10^6 cells mL^{-1} assuming a low cell specific oxygen uptake rate. Enriching the inlet airflow with pure oxygen, and thus increasing the driving force for oxygen transfer is a possible strategy to overcome this rate-limiting situation. Experimental verifications were done at the 1'500 L scale with a working volume of 750 L and a speed of 43 rpm. Instead of air, oxygen was used to replace the bag headspace at a flow rate of

10-15 L min^{-1} (Fig. 4.12.B). As a result, the dissolved oxygen concentration at saturation (C_L^*) was increased approximately 5 times when using pure oxygen instead of air, demonstrating the potential of active aeration with oxygen enriched air.

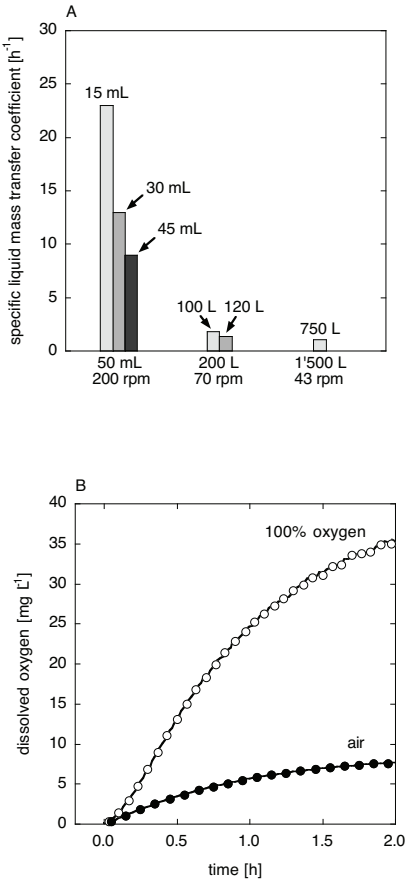


Fig. 4.12. (A) Specific liquid mass transfer coefficient *(k_La)* in 50-mL shake tubes, 200- and 1'500-L shake bioreactors. Tests in the 200- and 1'500-L systems were performed at RT with active aeration. (B) Dissolved oxygen (DO) increase when the headspace was actively aerated with either pure oxygen or air (10-15 L min^{-1}). Tests were performed at RT in the 1'500-L shake bioreactor with a liquid volume of 750 L and a shaking speed of 43 rpm.

4.3.2 Growth kinetics in 10- and 20-L shake containers

To verify the suitability of large-scale shake containers for animal cell culture and confirm the findings made by other research groups (1, 2), CHO DG44 cells were cultivated in 10 and 20 L square-shaped containers agitated on rotary benchtop shakers. Repeatedly, cell densities of 6 x 10^6 cells mL^{-1} and more were obtained at both scales in batch cultivations. An example is shown in Fig. 4.13. Typically, the viability was maintained above 90% during the exponential growth phase and declined rapidly when the stationary growth phase was reached. To ensure sufficient oxygen transfer, air was continuously added to the headspace at a flow rate of 1 L min^{-1}. The pH was manually adjusted by varying the CO_2 concentration in the inlet airflow. Normally, 5% CO_2 was added for 1 or 2 days. At higher cell densities, due to an increased cellular CO_2 release, no more CO_2 was added to the inlet airflow.

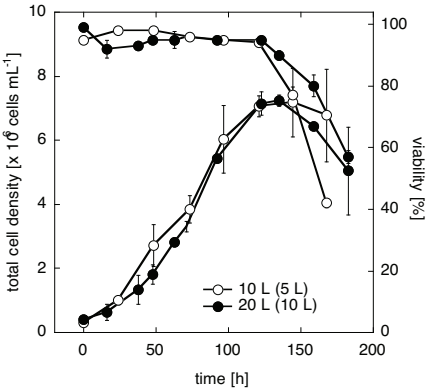


Fig. 4.13. CHO DG44 batch cultures in 10- and 20-L shake bioreactors operated with working volumes of 5 and 10 L and agitation speeds of 69 and 76 rpm, respectively. The airflow rate was set at 1 L min^{-1} in both systems. Average densities and viabilities are reported (n = 2).

The agitation speed was empirically set at values that ensured homogeneous mixing without producing excessive turbulence and foaming. Also, similar growth kinetics and viabilities were observed in square-shaped and cylindrical containers. To maintain the cells in suspension, higher agitation speeds were required in cylindrical

containers of the same volume (95-100 rpm instead of 74-76 rpm at the 20 L scale). Recently, a study demonstrated that at the same volumetric power consumption, the maximum local energy dissipation rate in baffled and in unbaffled shake flasks was very similar (14). Consequently, cultures in square- and round-shaped containers of the same volume might be exposed to comparable shear stress conditions when operated at different agitation speeds but at the same power consumption, resulting in similar growth kinetics.

Growth performance in a disposable shake bioreactor was compared with a stirred-tank bioreactor at the same scale. The comparative test was performed with a batch cultivation of recombinant CHO AMW cells that produce a human monoclonal antibody (IgG). When cell densities reached 5 x 10^6 cells mL^{-1}, the temperature was reduced to 31°C to extend the viability and enhance the IgG expression. The initial cell density was approximately 3 x 10^5 cells mL^{-1} in both systems. As shown in Fig. 4.14, very similar growth performances resulted in both bioreactor systems. Though a longer lag-phase resulted in the shake bioreactor, the overall growth kinetics were identical. The density at which the temperature was reduced was reached with a delay of less than 10 h in the shake bioreactor. This had a negligible consequence on the final IgG yield with concentrations above 100 mg L^{-1} assessed in both cases. A fine-tuning of the shaking speed at the very beginning of the cell culture may reduce the lag-phase observed in the shake bioreactor.

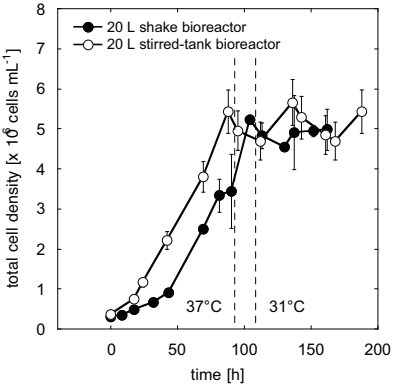


Fig. 4.14. CHO AMW batch cultures in 20-L stirred-tank and shake bioreactors with 10 L working volumes. The temperature was shifted from 37 to 31°C when densities reached 5 x 10^6 cells mL^{-1}. The dotted vertical lines represent the temperature shift for the stirred-tank bioreactor (97 h) and the shake bioreactor (106 h), respectively. Viability in both bioreactors was above 90% until the temperature was shifted and declined afterwards. Average densities are reported (n = 2).

Actively aerated 10- and 20-L shake bioreactors were found to be ideal for rapid and efficient cell expansion. They required less setup time than stirred-tank bioreactors of the same volumes and the handling was simplified. Standard lab equipment was used to operate such systems, which was a distinctive advantage over Wave bioreactor systems. Every cell culture lab equipped with benchtop shakers and CO_2 controlled incubators can easily setup 10- and 20-L cell cultures. Though intended for single-use, polycarbonate containers can be cleaned, autoclaved and reused several times, thus further reducing the operating costs. Also, multiple shake bioreactors can be operated simultaneously (see Fig. 4.5), which is often desirable when optimizing and scaling up a process. Overall, the benefits of shake bioreactors at a scale up to 20 L are considerable when compared with other cultivation systems.

4.3.3 Growth kinetics in 200- and 1'500-L shake bioreactors

The promising data obtained with 10- and 20-L scale shake bioreactors were a strong driver for designing and testing systems for larger operating scales. The oxygen transfer tests initially performed at the 200 and 1'500 L scales and reported in Section 4.3.1 were useful to assess the reliability of the large-capacity shakers and to define combinations of working volumes and agitation speeds. Further, tests were performed with pure water as a liquid phase to assess the time required to heat up the shake bioreactors to 37°C. In stirred-tank bioreactors, the use of steam and heating jackets results in brief heat-up times. Similar heat exchange systems were inappropriate for large-scale disposable shake bioreactors. At the 1'500 L scale, a simpler and lightweight heating system was tested, as described in Section 4.2.5. For a liquid volume of 750 L and an agitation speed of 41 rpm, heat-up times of 10-12 h resulted. Tests with larger heat element contact surfaces and improved insulation might result in heat-up times of a few hours only.

Next, cell cultivation in a 200-L container was evaluated using CHO AMW cells, first with a working volume of 25 L and then with 100 L. As a control, a 150-L stirred-tank bioreactor was inoculated with 50 L and operated with a standard fed-batch procedure. The shake bioreactors and the control bioreactor were started with initial densities of 3-5 x 10^5 cells mL^{-1} (Fig. 4.15). In all cultures the CHO AMW cells grew to densities up to 6 x 10^6 cells mL^{-1} by 125 h after inoculation. During the exponential growth phase, the viability was maintained above 90% in all three cultures (Fig. 4.15). To ensure sufficient oxygen transfer when operated with 100 L, the airflow for the 200 L container was enriched with pure oxygen (20-50%) at higher cell densities (3 x 10^6 cells mL^{-1}).

The comparison with the 150-L stirred-tank bioreactor was promising, since similar growth trends and viabilities were observed in the 200-L container. As with observations made at the 20 L scale, a somewhat longer lag-phase in the shake bioreactor delayed the time when the maximal cell density was reached in the shake bioreactor as compared to the stirred-tank bioreactor (Fig. 4.14). Importantly, none of the systems tested here was systematically optimized in the way it would be normally done for production purposes.

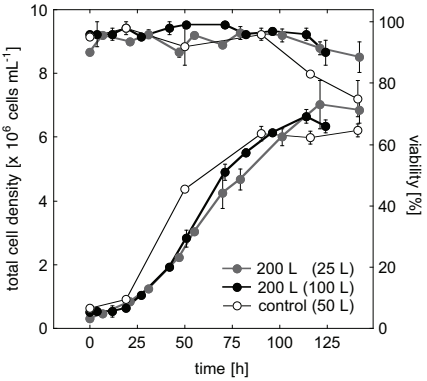


Fig. 4.15. CHO AMW batch cultures in a 200-L shake bioreactor with 25 and 100 L working volumes. The shaking speed was set at 50 and 65 rpm, respectively. The airflow rate was 1-2 L min^{-1} in both cases. At the 100 L working volume, when the density was above 3 x 10^6 cells mL^{-1}, the airflow rate was enriched with 20-50% oxygen. Cell growth was compared with a batch culture in a 150-L stirred-tank bioreactor (control) with 50 L working volume. The DO for this culture was maintained above 20% using oxygen enriched air. Average cell densities and viabilities are reported (n = 2).

The next step towards larger scales was more challenging in terms of required volumes of media and inoculum. Sterility issues were found to be particularly critical at production scales. Knowledge acquired during the successive scale-up of the system were useful in that respect. The first attempt to cultivate CHO cells at production scale in a disposable shake bioreactor is summarized next.

The cell expansion was accomplished in the 200-L pilot scale shake bioreactor with a working volume of 75 L. A relatively inexpensive serum- and protein-free medium was used for this purpose (CHO PFM). As expected, slower growth kinetics resulted with a maximal cell density of 4 x 10^6 cells mL^{-1} (data not shown). For the production scale test, serum-free ProCHO5 medium was used. As shown at smaller scales, this medium usually supports growth of CHO cells up to 6-8 x 10^6 cells mL^{-1} (see Fig. 4.13). First, 500 L medium were transferred into the 1'500 L cell culture bag using a sterile filtration step. The next day, when the temperature reached 37°C, the 1'500 L

shake bioreactor was inoculated at a density of 4 x 10^5 cells mL^{-1}. Then, medium was added to reach a final working volume of 750 L.

During the exponential growth phase, 3 g L^{-1} glucose and 25 mM $NaHCO_3$ were fed to sustain the growth and maintain a physiological pH. An airflow rate of 10-20 L min^{-1} was provided. At a cell density of 3 x 10^6 cells mL^{-1}, pure oxygen was used instead of air at a lower flow rate (5-10 L min^{-1}). On day 5 (120 h), a maximal total cell density of 4.8 x 10^6 cells mL^{-1} was assessed with a viability of 91% (Fig. 4.16).

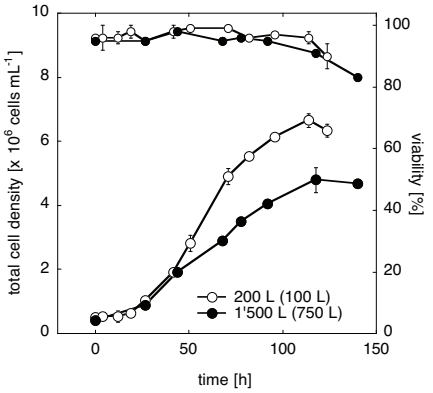


Fig. 4.16. CHO AMW batch culture in the 1'500-L shake bioreactor with 750 L working volume (43 rpm) and comparison with the pilot-scale system of 200 L with a 100 L working volume (65 rpm).

Though further investigations and validation steps are required to establish the technology and commercially exploit the concept, results from the 200-L and 1'500-L shake bioreactors confirmed the assumptions made previously that orbital shake technology is particularly well-suited for growing mammalian cells, even at the production scale. The use of non-invasive optical sensors will facilitate the monitoring and control of the dissolved oxygen and the pH. The design and manufacturing of sterile cell culture bags equipped with such sensors should be considered as a priority for further validation steps.

4.4 Conclusion

Disposable shake bioreactors were found to be ideal for cell culture applications at scales ranging from a few mL up to production scale operations. Here, efforts were made to empirically define optimal conditions for growth in containers for volumes up to 20 L and cell culture bags with volumes up to 1'500 L.

Head-to-head performance comparisons with instrumented stirred-tank bioreactors proved that excellent growth can be obtained in orbitally shaken bioreactors with much less setup time than necessary with stirred-tank reactors. Though surface aeration with air was found insufficient to transfer enough oxygen to the liquid phase at larger scales, the addition of oxygen in the airflow was shown to be ideal to overcome this limitation.

A cell density up to nearly 5 x 10^6 cells mL^{-1} was assessed in a cell suspension volume of 750 L. However, the process parameters need to be adjusted very precisely, which was certainly not the case in this first attempt at production scale. The fine-tuning of interacting parameters such as agitation speed, inlet airflow rate, and resulting dissolved oxygen will be critical for achieving reliably higher cell densities.

4.5 References

(1) Liu C, Hong L. 2001. Development of a shaking bioreactor system for animal cell cultures. Biochem. Eng. J. 7(2):121-125.

(2) Raval K, Liu C, Büchs J. 2006. Large-scale disposable shaking bioreactors. BioProcess Int. January 2006:46-50.

(3) Kato Y, Peter CP, Akgün A, Büchs J. 2004. Power consumption and heat transfer resistance in large rotary shaking vessels. Biochem. Eng. J. 21:83-91.

(4) Zhang H, Williams-Dalson W, Keshavarz-Moore E, Shamlou PA. 2005. Computational-fluid-dynamics (CFD) analysis of mixing and gas-liquid mass transfer in shake flasks. Biotechnol Appl Biochem. 41:1-8.

(5) Amoabediny G, Buchs J. 2007. Modelling and advanced understanding of unsteady-state gas transfer in shaking bioreactors. Biotechnol Appl Biochem. 46:57-67.

(6) Nikakhtari H, Hill G. 2005. Modelling oxygen transfer and aerobic growth in shake flasks and well-mixed bioreactors. Can J Chem Eng. 83:493-499.

(7) Büchs J, Maier U, Lotter S, Peter CP. 2007. Calculating liquid distribution in shake flasks on rotary shakers at waterlike viscosities. Biochem Eng J. 34:200-208.

(8) Maier U, Büchs J. 2001. Characterisation of the gas–liquid mass transfer in shaking bioreactors. Biochem Eng J. 7:99-106.

(9) Singh V. 1999. Disposable bioreactor for cell culture using wave-induced agitation. Cytotech. 30:149-158.

(10) Pierce LN, Shabram PV. 2004. Scalability of a Disposable Bioreactor from 25L – 500L Run in Perfusion Mode with a CHO-Based Cell Line: A Tech Review. BioProcessing J. 3(4):1-6.

(11) Flumerfelt RW, Glover CG. 2002. Transport phenomena. in *Ullmann's Encylopedia of Industrial Chemistry*, John Wiley & Sons. 37:331-430.

(12) Chisti Y. 2001. Hydrodynamic damage to animal cells. Crit Rev Biotechnol. 21(2):67-110.

(13) Büchs J, Zoels B. 2001. Evaluation of maximum to specific power consumption rate in shaking bioreactors. J. Chem. Eng. Jpn. 34(5):647-653.

(14) Peter CP, Suzuki Y, Buchs J. 2006. Hydromechanical stress in shake flasks: correlation for the maximum local energy dissipation rate. Biotech. Bioeng. 93:1164-76.

(15) Büchs J. 2001. Introduction to advantages and problems of shaken cultures. Biochem Eng J. 7:91-98.

(16) De Jesus MJ, Girard P, Bourgeois M, Baumgartner G, Jacko B, Amstutz H, Wurm FM. 2004. TubeSpin satellites: a fast track approach for process development with animal cells using shaking technology. Biochem. Eng J. 17:217-223.

(17) Muller N, Girard P, Hacker DL, Jordan M, Wurm FM. 2005. Orbital shaker technology for the cultivation of mammalian cells in suspension. Biotechnol Bioeng. 89(4):400-6.

Chapter 5

Transient gene expression in shake cultivation systems[5]

5.1 Introduction

Transient gene expression in suspension cultures of transfected mammalian cells is a rapid approach for the generation of mg to g quantities of recombinant proteins for biochemical and preclinical studies (1, 2, 3). In stirred-tank bioreactors, recombinant monoclonal antibodies have been produced in both CHO and HEK 293 cells at volumes up to 100 L (4, 5, 6). Transient gene expression in these two cell lines has also been performed in Wave bioreactors up to the 20 L scale (7, 8). The use of orbital shake bioreactor systems for transient gene expression has been shown to be feasible to nominal volumes up to 1 L (4). Here, existing polyethylenimine-mediated transfection protocols for CHO host cells (4, 5) were optimized to obtain up to 30-60 mg L^{-1} recombinant antibody under reduced temperature conditions. Optimization and scale-up to 30 L of working volume were exclusively done in disposable shake bioreactors.

Polyethylenimine (PEI) is considered as one of the most efficient non-viral gene delivery agents. It is an organic macromolecule with a high cationic charge density potential, resulting in efficient plasmid DNA (pDNA) condensation. The excess positive charge of the PEI/pDNA complex promotes the interaction with the negatively charged surface of the cellular membrane. It was shown that these complexes are taken up by the cells into acidified endosomal compartments which are then released

[5] A slightly modified version of this chapter was accepted for publication in *Biotechnology Progress* (Stettler M, Zhang X, Hacker D, De Jesus M, Wurm FM. 2007. Novel orbital shake bioreactors for transient production of CHO derived IgGs. Biotech Progress).

into the cytoplasm via the so-called “proton sponge effect” (9, 10). Various studies compared the efficiency of linear and branched PEI of different molecular weights (5, 11, 12). Results showed that linear PEI with a molecular weight of 25 kDa resulted in the highest efficiency at the lowest toxicity.

Previous studies reported that cultivation of stable CHO-derived cell lines at reduced temperatures of 31-33°C enhanced production levels of a variety of recombinant proteins (13, 14, 15, 16, 17). It was shown that despite a decrease in specific growth rate, the improved cell viability and reduced protease activity at low temperature was in part responsible for the higher product yield. Recently, this simple strategy was applied on transiently transfected cells resulting in comparable benefits. The transient expression of a recombinant monoclonal antibody in CHO cells was found to be up to 3-fold higher at 32°C as compared to 37°C (18). However, these experiments were performed at volumes of less than 100 mL. As shown next, these findings were confirmed at scales of operation up to 30 L of working volume.

Protein hydrolysates (peptones) of non-animal sources are used to supplement cell culture media for enhanced growth and yields (19). Similarly, the addition of peptones post-transfection in transient processes was recently suggested as a possible strategy to enhance protein expression (20, 21). Pham *et al*. reported a two-fold increase in secreted alkaline phosphates transiently expressed by HEK 293 host cells when a casein peptone was added 24 h post-transfection. This effect was shown to be time and concentration dependent. To date, no report addressed this issue in the case of transiently transfected CHO cells. Here, the impact of different plant peptones on the expression of an antibody in CHO cells was evaluated in small-scale screening experiments using 50-mL shake tubes.

The present study illustrates how effectively and reliably a PEI-mediated transient transfection protocol can be optimized and scaled-up when using appropriate cultivation strategies. This contributed to the demonstration of the highly promising potential of both small-scale optimizations with disposable shake tubes and large volume shake bioreactors in terms of flexibility, cost-effectiveness, ease of use, and reliable performance.

5.2 Material and methods

5.2.1 Cells

Suspension-adapted CHO cells (dihydrofolate reductase-deficient strain DG44) (*22*) were cultured in serum-free ProCHO5 medium (Lonza Verviers SPRL, Verviers, Belgium), unless otherwise specified. The media was supplemented with 0.68 g L^{-1} hypoxanthine and 0.194 g L^{-1} thymidine (HT) (SAFC Biosciences, St. Louis, MO). The cells were maintained at 37°C in 0.5- and 1-L square-shaped bottles (23) agitated at 110 rpm in a CO_2- and humidity-controlled incubator with a shaker having a rotational diameter of 50 mm (ISF-4-W, Adolf Kühner AG, Birsfelden, Switzerland). The cells were subcultured every 3-4 days at a seeding density of 2-5 x 10^5 cells mL^{-1}. Cell density and viability were determined by the Trypan blue exclusion method. Packed cell volume (PCV) was determined using VoluPAC tubes (Sartorius AG, Göttingen, Germany) and expressed as the % of the total culture volume as described elsewhere (*24*). Cell size was measured using a CASY1 Counter (Schärfe System GmbH, Reutlingen, Germany).

5.2.2 Plasmid DNA preparation

The construction of the human anti-Rhesus D IgG light and heavy chain gene expression vectors pKML and pKMH, respectively, has been described (5). Plasmid DNA was extracted using a commercial purification kit (NucleobondAX, Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol and stored at a concentration of 1 mg mL^{-1} in sterile TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4).

5.2.3 Transient transfections

Linear 25 kDa PEI (Polysciences, Eppenheim, Germany) was prepared in water at a final concentration of 1 mg mL^{-1} (pH 7.0) and sterilized by filtration. For each mL of culture, 2.5 μg of DNA and various amounts of PEI were added separately to 50 μL of 150 mM NaCl. Prior to transfection, the PEI/NaCl solution was added to the DNA/NaCl solution and allowed to stand at room temperature for 10 min before addition to the culture. Transfections were performed with a 1:1 (w/w) mixture of pKML and pKMH unless stated otherwise.

5.2.4 Shake bioreactor systems

For transfections in 50-mL disposable shake tubes, the cells were centrifuged and resuspended in ProCHO5 at a cell density of 2 x 10^6 cells mL^{-1} (a PCV of 0.5%). A 5 mL aliquot of culture was added to each 50-mL tube fitted with a filter cap for passive headspace aeration (CultiFlask 50, Sartorius AG, Göttingen, Germany) (*25*). Following transfection the tubes were agitated at 37°C at 140 rpm in an ISF-4-W shaker incubator with a rotational diameter of 50 mm (Kühner AG, Birsfelden, Switzerland) in an atmosphere with 5% CO_2 and 85% relative humidity. After 4 h, 5 mL of ProCHO5 were added to each tube, the agitation speed was increased to 180 rpm (Tab. 5.1), and the temperature was reduced to 31°C or maintained at 37°C. IgG expression was determined by sandwich ELISA as described elsewhere (*26*).

For transfections at the 5 and 20 L scale, the cells were centrifuged and resuspended in ProCHO5 at a density of 3-4 x 10^6 cells mL^{-1} on the day before transfection (Fig. 5.2). The next day, the cells were centrifuged and resuspended in ProCHO5 medium at 2 x 10^6 cells mL^{-1} (a PCV of 0.5%). The appropriate volumes (Tab. 5.1) of cell suspension were then transferred into 5- or 20-L square-shaped polycarbonate containers (Biotainer Carboy, Cellon SA, Luxembourg) (Fig. 5.1). Prior to transfection, the cultures were maintained at 37°C and agitated at either 80 or 70 rpm for the 5- and 20-L containers, respectively (ISF-4-W shaker incubator, Adolf Kühner AG, Birsfelden, Switzerland). At 4 h post-transfection, the cells were diluted with one volume of ProCHO5 media with supplements. The agitation diameter was kept constant at 50 mm and the agitation speed was adjusted to the final working volume

(Tab. 5.1). The incubator temperature was reduced to 31°C or maintained at 37°C. The shake bioreactors were fitted with a three-port cap. One of the ports was connected to a membrane pump, and a constant airflow of 0.5-1.0 L min^{-1} containing an adjustable concentration of CO_2 (1-10%) was pumped into the headspace through a 0.22 μm inlet filter. The level of CO_2 in the inlet air was manually adjusted to maintain a physiological pH. The shake bioreactor gas outlet was fitted with a second sterile filter. Samples were taken directly in the incubator through a sterile sampling port mounted on the cap of the shake bioreactor. The culture was sampled daily for off-line pH measurement (340 pH Meter, Mettler-Toledo, Greifensee, Switzerland) and for analysis of metabolites and antibody quantification. Glucose, lactate, glutamine and ammonia were measured with a BioProfile 200 Analyzer (Nova Biomedical, Waltham, MA).

For transfections at the 50 L scale, disposable bioprocess bags with top and bottom ports and the corresponding container for mounting the bags on the shaker were used (BioProcess Container™ Systems, HyClone, South Logan, UT) (Fig. 5.1). The outer container, with a cylindrical shape and a conical bottom, was fitted on a modified benchtop shaker (Certomat RM, Sartorius AG, Göttingen, Germany) that was installed in a 15 m^3 warm room. The rotational diameter of the shaker was 50 mm. The volume of ProCHO5 medium required for transfection was pumped into the cell culture bag one day prior to transfection to allow the pH and temperature equilibria to be reached. For cell expansion prior to transfection, 5-L shake bioreactors were filled with 2.5 L of CHO cell suspension at a seeding density of 2-5 x 10^5 cells mL^{-1}. The agitation speed was set at 90 rpm and 5% CO_2 was added to the inlet airflow (1 L min^{-1}). The medium was exchanged one day prior to the transfection and on the day of transfection as described above. The 50-L shake bioreactor was then inoculated with the appropriate volume of cell suspension to reach an initial density of 2 x 10^6 cells mL^{-1}. Transfection was performed as described above. The temperature was reduced to 31°C at the time of transfection. At 4 h post-transfection, the cell suspension was diluted with one volume of ProCHO5 medium (Tab. 5.1). The airflow rate and CO_2 level were adjusted to keep the pH between 6.8 and 7.0. Due to a small overpressure resulting from the airflow, the bag was completely inflated allowing the bag to adapt its shape to the geometry of the outer container. Samples were taken from the bottom port of the bioprocess bag.

Tab. 5.1. Shaking speed and working volume for different disposable shake bioreactor systems.

Nominal volume	Geometry	Shaking speed [rpm]		Working volume	
		At time of transfection	Post-transfection (4 h)	At time of transfection	Post-transfection (4 h)
50 mL	Round, conical	140	180	5 mL	10 mL
5 L	Square	78	89	1.25 L	2.5 L
20 L	Square	70	78	5 L	10 L
50 L	Round, conical	45	55	15 L	30 L

The rotational diameter was kept constant (50 mm) for all bioreactor volumes

Fig. 5.1. 20-L square-shaped shake bioreactors (left) and 50-L shake bioreactor with a modified benchtop shaker (right).

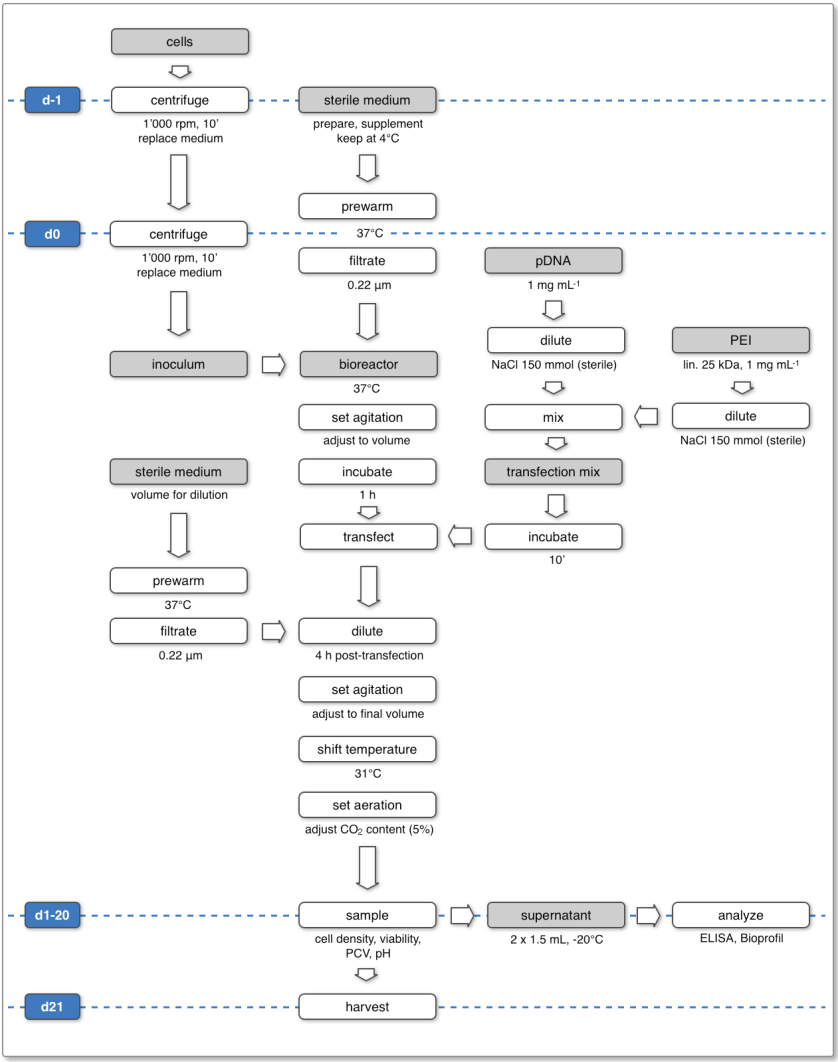


Fig. 5.2. Process flow chart of large-scale PEI-mediated transfection. This procedure was used to transfect CHO DG44 host cells for the expression of a monoclonal IgG at the 5, 20 and 50 L scale.

5.3 Results and discussion

5.3.1 Small-scale optimizations in 50-mL shake tubes

Preliminary experiments consisted in determining the optimal ratio of the IgG light and heavy vectors for transient recombinant antibody expression in CHO cells at 31°C and 37°C. The cells were transfected with different mass ratios of pKML and pKMH in 50-mL shake tubes at a DNA:PEI ratio of 1:4 (w/w). 24 h post-transfection, the tubes were transferred to 31°C or maintained at 37°C. Antibody concentration and PCV were assessed on day 6 post-transfection. The highest antibody concentration was obtained at a pKML to pKMH mass ratio of 1:1 at both temperatures (Fig. 5.3). The temperature shift to 31°C induced an increase in antibody expression over the control at each plasmid ratio tested. The biomass for transfections at 31°C was reduced as compared to the control transfections at 37°C with PCV values around 1.0% (corresponding to approx. 4 x 10^6 cells mL^{-1}) at 31°C and around 1.5% (6 x 10^6 cells mL^{-1}) at 37°C.

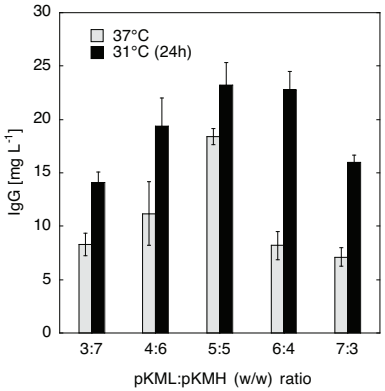


Fig. 5.3. Antibody expression level as a function of various light chain (pKML) to heavy chain (pKMH) ratios (w/w) and a constant DNA:PEI ratio of 1:4 (w/w). Total pDNA concentration was kept constant at 2.5 μg mL^{-1} (prior to dilution). Transfections were performed in 50-mL shake bioreactors. At 24 h post-transfection, the transfected cultures were transferred to 31°C or maintained at 37°C. Average IgG expression levels at day 6 post-transfection are reported (n = 3).

To determine the optimal DNA:PEI ratio for transient recombinant antibody production at 31°C and 37°C, various DNA to PEI ratios were tested in 50-mL shake tubes. Keeping the DNA amount at a constant level of 2.5 μg per mL of cell culture (prior to dilution), the amount of PEI was varied from 2.5 to 12.5 μg per mL of cell culture. The pKML to pKMH ratio was maintained at 1:1 (w/w) for all transfections. 24 h post-transfection, the cells were either shifted to 31°C or maintained at 37°C.

The optimal DNA to PEI ratio was found to be temperature-dependent. For transfections at 31°C, the highest antibody yields were obtained with DNA/PEI complexes formed at ratios of 1:3, 1:4 and 1:5 (w/w) (Fig. 5.4.A). For the transfected cultures maintained at 37°C, the highest antibody yields were achieved at DNA:PEI ratios of 1:4 and 1:5 (w/w) as previously reported (5). While lower PEI concentrations (ratios of 1:2 to 1:4) resulted in similar PCV values at day 6 post-transfection, an increase in the PEI concentration (DNA:PEI ratio of 1:5) negatively affected cell growth more significantly (Fig. 5.4.B). The inhibitory effects with higher amounts of PEI were greater at 31°C than at the control temperature, resulting in PCV values less than half of those observed at 37°C (Fig. 5.4.B).

The comparison of antibody yield and PCV for different DNA:PEI ratios showed that mass ratios of 1:3 and 1:4 were optimal for transfections at 31°C and 37°C, respectively. For extended cultivation periods (more than 9 days), these DNA:PEI ratios were advantageous for preserving the cell culture viability and consequently for yielding higher cumulative antibody levels.

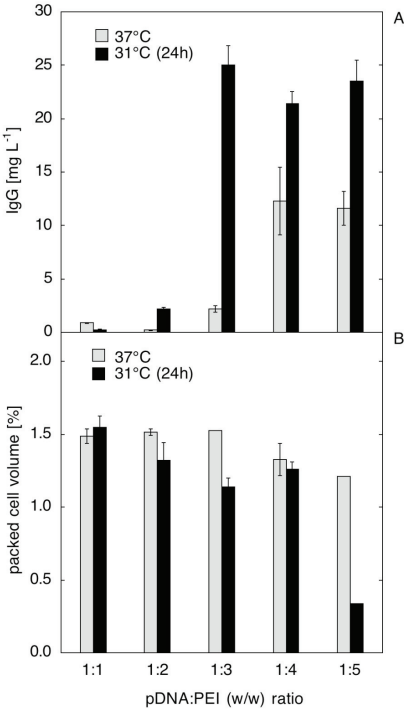


Fig. 5.4. Antibody expression level (A) and packed cell volume (B) as functions of various pDNA to PEI ratios (w/w) and a constant pDNA concentration of 2.5 μg mL^{-1} (prior to dilution). Transfections were performed in 50-mL shake bioreactors. At 24 h post-transfection, the transfected cultures were transferred to 31°C or maintained at 37°C. Average IgG expression levels and PCV values of day 6 post-transfection are reported (n = 3).

Next, transient gene optimizations were done with 50-mL shake tubes using a screening approach. Previously, the addition of protein hydrolysates (peptones) to the cell culture media was found to enhance the cell growth (see Section 3.3.2). Concentrations between 0.25 and 0.75% (w/v) of various soy peptones increased the growth by a factor of 2 in some cases. The possible impact of peptones on transiently transfected CHO cells was tested using a similar strategy. Six peptones, mostly from soy origin, were selected for this purpose (Tab. 5.2).

Tab. 5.2. Peptones used in the study. All peptones originated from soy, except P06, which was a potato hydrolysate.

Code	Peptone	Supplier
P01	Soy Peptone A2 SC	Organotechnie
P02	ProPEP1 soy	Cambrex
P03	ProPEP2 soy	Cambrex
P04	Difco CG soy UF	BD
P05	Soy Peptone RXDG UF	Organotechnie
P06	Plant peptone E1 (potato)	Organotechnie

The transfections were done in 5-L shake bioreactors as described in Section 5.2.4. 24 h post-transfection, cell suspension volumes of 10 mL were distributed into multiple 50-mL shake bioreactors agitated at 180 rpm. Then, a peptone concentration of 0.5% (w/v) was added to each 50-mL shake tube and the temperature was shifted to 31°C. IgG yield and PCV were assessed on days 3, 6, 9 and 12 post-transfection (Fig. 5.5.A and B). In most cases, the IgG concentration in the culture medium increased strongly until day 6 and reached a somewhat constant value from day 6 to 12 (Fig. 5.5.A). Interestingly, the PCV value increased constantly even until later stages of cultivation (day 12).

Overall, IgG levels were not improved as compared to the control. The exception was P04, whose addition resulted in a nearly 2-fold increase in IgG (Fig. 5.4A). Surprisingly, no PCV increase was observed with this peptone (Fig. 5.5.B). In some cases, the addition of soy peptones had a negative impact on growth and/or IgG expression (P06) (Fig. 5.4A and B). IgG levels at 31°C were noticeably improved for all peptones tested compared to the controls kept at 37°C (data not shown).

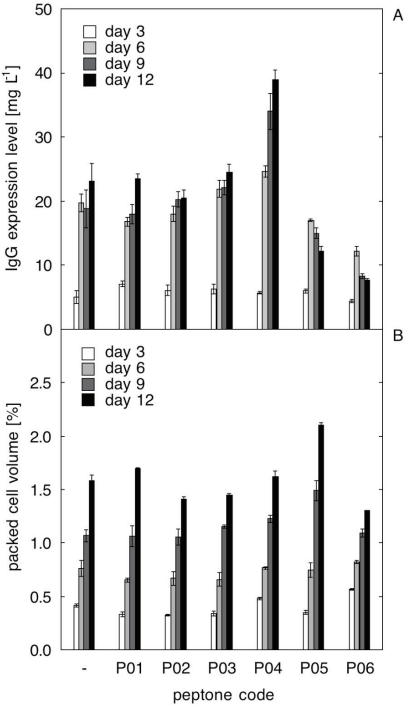


Fig. 5.5. IgG levels (A) and packed cell volume (B) as a function of different peptones added to the medium (ProCHO5) at a concentration of 0.5% (w/v). The cells were transfected at day 0 and distributed in 50-mL shake tubes 24 h later. At day 1, the peptones were added and the temperature was shifted down to 31°C. Average values of different 50-mL shake tubes are reported (n = 3).

The same screenings were reproduced in several different chemically defined media optimized for CHO cells. The cells were transfected with the same conditions in the different media in 5-L shake bioreactors as described. One day after transfection, the cells were distributed in 50-mL shake bioreactors. Then, the peptones were added and the temperature shifted to 31°C. IgG yield and PCV were assessed on day 9 and plotted as a function of different peptones and media (Fig. 5.6.A and B).

For transient gene expression, the ProCHO5 medium and the peptone-free ProCHO5 formulation were clearly superior to the PowerCHO medium series from the same supplier (Lonza Verviers SPRL, Verviers, Belgium). These two media allowed growth of the cells over 9 or 12 days while maintaining a good viability (more than 90%) at 31°C (Fig. 5.6.B) The addition of 0.5% (w/v) of peptone P04 enhanced IgG production in all media tested (Fig. 5.6.A). IgG levels around 40 mg L^{-1} were obtained in the best case, which was nearly twice as much as in the control transfection.

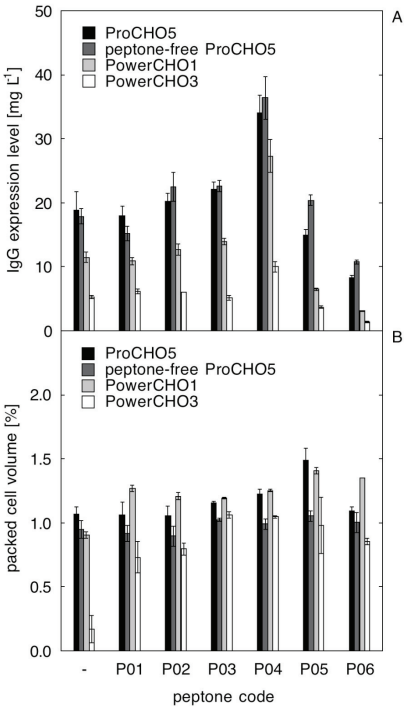


Fig. 5.6. IgG levels (A) and packed cell volume (B) as a function of different peptones added to the media at a concentration of 0.5% (w/v). The cells were transfected in 4 different chemically defined media at day 0 and distributed in 50-mL shake tubes 24 h later. At day 1, the peptones were added and the temperature was shifted down to 31°C. Average day 6 values of different 50-mL shake tubes are reported ($n = 3$).

Small-scale optimizations using 50-mL shake tubes stressed the importance of relying on an appropriate testing technology. In the case that was shown here, soy peptones that increase either the antibody expression level (P04) or the cell growth (P05) were identified with a high degree of confidence and confirmed the findings made by other research groups (20, 27). However, it should be noted that the validity of such a screening is particularly dependent upon the cell line, the transfection method, the expressed protein and the process. Peptones that positively influenced a given process might have inhibitory effects in an other process. Variability in raw material and in the hydrolysis process might also change the impact on yield and growth. Therefore, each new optimization process with given frame conditions should be considered as a unique opportunity to optimally match the nutrient requirements of the host cells for improved protein expression. The use of an adequate screening technology is critical in that respect.

5.3.2 5-L shake bioreactor

The impact of 3 different conditions was assessed for transient recombinant antibody production in CHO cells by transfection with pKML and pKMH at a DNA:PEI ratio of 1:4 (w/w). The temperature was maintained at 37°C throughout the cultivation period or shifted to 31°C either at the time of transfection or at 24 h post-transfection. Each of these conditions was tested simultaneously in duplicate in 5-L shake bioreactors. Maximal final antibody yields up to 50-60 mg L^{-1} were observed for cultures maintained at 31°C, while the yield was only a few mg L^{-1} for the cultures at 37°C (Fig. 5.7.A).

The growth kinetics at 37°C were noticeably different from those at 31°C (Fig. 5.7.B). At 31°C, the maximal biomass was reached with a delay of about 260 h (11 d) as compared to the cultures at 37°C (Fig. 5.7.B). The maximum PCV for the shake bioreactors shifted to 31°C at 24 h post-transfection was approximately 1.5-fold higher than the maximum PCV obtained when the temperature was reduced immediately post-transfection (Fig. 5.7.B).

Typically, the viability of cultures that were transfected and maintained at 31°C was above 70% for up to 400 h (16-17 d) post-transfection (Fig. 5.7.C). Then, the viability decreased. After the viability started to decrease, the antibody expression levels continued to increase (from 40-50 to nearly 60 mg L^{-1}) for the transfections at 31°C (Fig. 5.7.A). By comparison, the viability of the cultures at 37°C was above 70% for approximately 190 h (8 d).

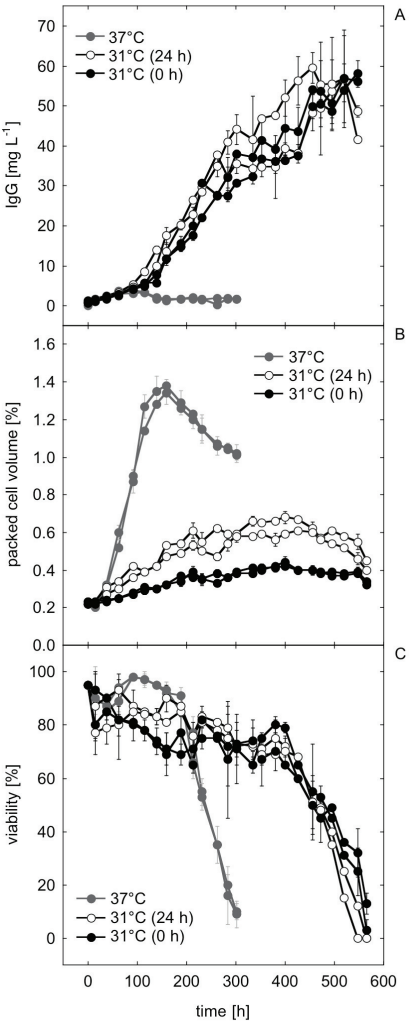


Fig. 5.7. Antibody expression level (A), PCV (B), and viability (C) as functions of time in six 5-L shake bioreactors transfected under identical conditions. The temperature was maintained at 37°C or shifted to 31°C either 24 h post-transfection (24 h) or at the time of transfection (0 h). Average IgG (n = 2), PCV (n = 3), and viability (n = 2) values are reported.

As expected, cell growth rates were reduced at 31°C as compared to 37°C. It is important to note, however, that the increased biomass (PCV) over time for cultures at 31°C was not necessarily correlated with increased cell densities. Instead, the increase in PCV indicated a temperature dependent cell volume increase under hypothermic culture conditions. CHO cells at 31°C were found to be up to 1.4-fold more voluminous than the same cells kept under normal cultivation conditions (approx. 2.8 pL at 31°C and 1.9 pL at 37°C). Thus, the volume of CHO cells at 31°C was dramatically expanded. Apparently, the cells adapted their metabolic activity to a different environment.

5.3.3 20-L shake bioreactor

Square-shaped polycarbonate containers were used for transient gene expression experiments at the 20 L scale (10 L of working volume) by transfecting cells with pKML and pKMH at a DNA:PEI ratio of 1:4 (w/w). As in experiments at the 5 L scale, an early temperature shift at the time of transfection was compared to a shift at 24 h post-transfection. Here, the earlier temperature shift resulted in an increased antibody yield over time (Fig. 5.8.A). After a cultivation period of more than 340 h (14 d), antibody concentrations of 30 and 50 mg L^{-1} were observed for the cultures shifted to 31°C at 24 h post-transfection and at the time of transfection, respectively (Fig. 5.8.A). These values were similar to transient antibody yields at the 5 L scale. However, the experiments at the 20 L scale were started at a higher initial cell density with PCV values of approximately 0.3% (instead of 0.2% for the 5-L shake bioreactors). Distinct growth kinetics were observed for the two cultures with maximum PCV values of 0.85% and 1.45% for the cultures shifted at the time of transfection and at 24 h post-transfection, respectively (Fig. 5.8.B). Cell viabilities over 90% were maintained for more than 200 h of cultivation in both cases (Fig. 5.8.C). Residual glucose levels of more than 1 g L^{-1} and glutamine levels between 2 and 3 mmol L^{-1} were assessed in the supernatant towards the end of the production period, indicating that the main nutritional compounds were not rate limiting.

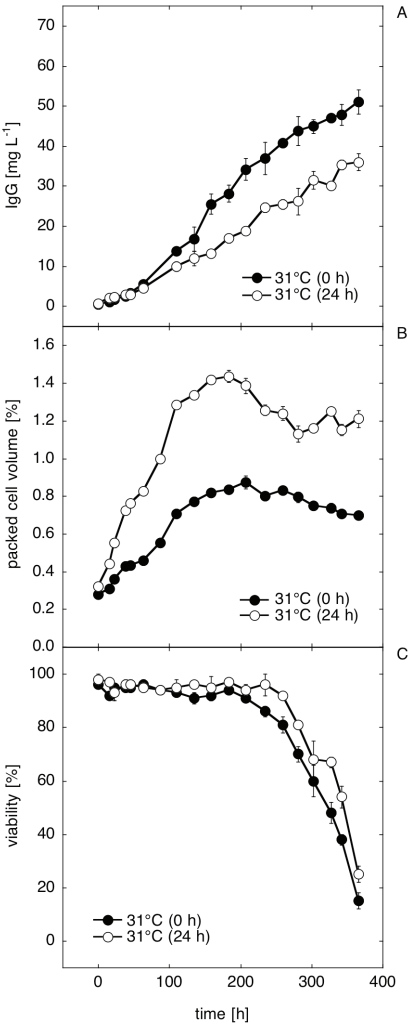


Fig. 5.8. Antibody expression level (A), PCV (B), and viability (C) as functions of time in two 20-L shake bioreactors transfected under identical conditions. The temperature was shifted to 31°C either 24 h post-transfection (24 h) or at the time of transfection (0 h). Average IgG (n = 2), PCV (n = 3), and viability (n = 2) values are reported.

5.3.4 50-L shake bioreactor

A further increase in operational scale was achieved using disposable cell culture bags instead of polycarbonate containers. An outer container was used to hold the bag and its content (Fig. 5.1). A large-capacity orbital shaker that was modified for this purpose and installed into a warm room provided acceptable shaking power for liquid volumes of up to 50 L. The agitation speed was carefully selected to keep the cells in a homogeneous suspension while avoiding excessive foam formation. CHO cells were transfected with pKML and pKMH as described for the transfections at the 5 and 20 L scales, and the temperature was reduced to 31°C at the time of transfection. Almost one gram of human IgG (non-purified) was produced within 14 d by achieving an antibody concentration of about 30 mg L^{-1} at 340 h post-transfection in a total volume of 30 L (Fig. 5.9.A). Similarly to data obtained with the 5- and 20-L shake bioreactors under comparable conditions, the PCV only slightly increased post-transfection (Fig. 5.9.B). The PCV reached a maximal value of 0.6% (corresponding to a total cell density of 2.5 x 10^6 cells mL^{-1}). The cell viability, however, decreased more rapidly than for cultures in the 5- and 20-L containers. Viabilities above 80% were recorded before a rapid decrease occurred approximately 200 h post-transfection (Fig. 5.9.B).

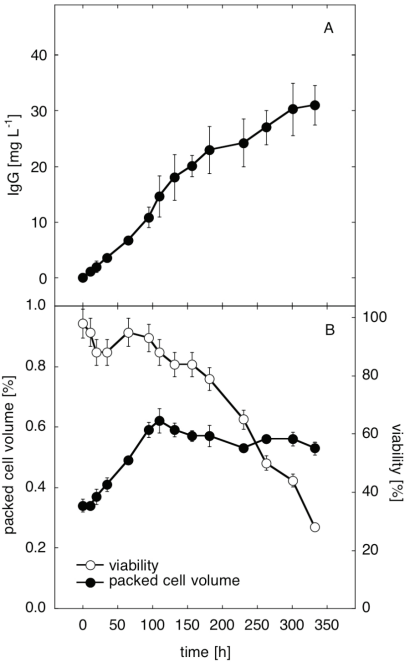


Fig. 5.9. Antibody expression level (A) and PCV and viability (B) as functions of time in a 50-L shake bioreactor with a working volume of 30 L. The temperature was shifted to 31°C at the time of transfection. Average IgG (n = 2), PCV (n = 3), and viability (n = 2) values are reported.

The 2-fold decrease in maximum IgG yield (Fig. 5.9.A) and the lower accumulation of biomass (Fig. 5.9.B) observed as the result of the scale-up from 5 to 50 L may have been due to suboptimal process conditions. For the 50 L scale test shown here, the viability was maintained above 80% for only about 180 h, whereas for the same process in the 5- and 20-L bioreactors the viability was maintained above 80% for 300 h and 250 h, respectively (Fig. 5.7.C and Fig. 5.8.C). The results may be an indication of inappropriate shear stress, insufficient air supply, or non-optimized shaking speed for the transfection at 50 L. Further investigations at the 50 L scale and beyond will focus on these parameters to try to improve cell viability and product yield.

5.4 Conclusion

This study demonstrated the potential of shake bioreactor systems for fast and reliable transient production of recombinant proteins in suspension cultures of mammalian cells. Multi-parameter experiments with small-scale vessels (50-mL shake tubes) allowed rapid identification of suitable process parameters with a high degree of confidence. For example, this system was used to show that the optimal DNA:PEI ratio for transfection was temperature dependant. Screening experiments were carried out to identify protein hydrolysates that favorably influenced the antibody expression level. Afterwards, the scalability of shake bioreactors up to a nominal volume of 50 L was verified. As a result, a 1'000-fold scale-up for transient recombinant antibody production in CHO cells was accomplished using orbital shake technology.

Importantly, cell cultivation and transfection in the prototype 50-L shake bioreactor described here relied on the same basic principles as those for the 50-mL shake bioreactors, ensuring smooth process development from laboratory to pilot scale. This represents a major advantage over established technologies such as spinner flasks, stirred-tank or Wave bioreactors because these cultivation systems do not allow operation of multi-parameter screening at small scale (less than 25 mL). Apart from typical scale effects such as a longer lag-phase and reduced growth kinetics, the transfected cultures in shake bioreactors showed very similar trends in terms of protein productivity at all scales tested.

In summary, large-scale disposable shake bioreactors at nominal volumes of up to 50 L were found to be promising in the context of drug discovery programs where milligram or even gram quantities of recombinant proteins are required within short timeframes. This is a significant advance because most commercially available mammalian cell cultivation systems in the 1-50 L volume range are costly and often complicated to operate and maintain. Here, conventional laboratory equipment was used to assemble high performing shake bioreactors. Thus, this study represents an important step towards flexible and low-cost disposable technologies in modern bioprocessing.

5.5 References

(1) Baldi L, Hacker DL, Adam M, Wurm FM. 2007. Recombinant protein production by large-scale transient gene expression in mammalian cells: state of the art and future perspectives. Biotechnol Lett. 29(5):677-84.

(2) Pham PL, Kamen A, Durocher Y. 2006. Large-scale transfection of mammalian cells for the fast production of recombinant protein. Mol Biotechnol. 34(2):225-37.

(3) Wurm F, Bernard A. 1999. Large-scale transient expression in mammalian cells for recombinant protein production. Curr Opin Biotechnol. 10(2):156-9.

(4) Muller N, Derouazi M, Van Tilborgh F, Wulhfard S, Hacker DL, Jordan M, Wurm FM. 2007. Scalable transient gene expression in Chinese hamster ovary cells in instrumented and non-instrumented cultivation systems. Biotechnol Lett. 29(5):703-11.

(5) Derouazi M, Girard P, Van Tilborgh F, Iglesias K, Muller N, Bertschinger M, Wurm FM. 2004. Serum-free large-scale transient transfection of CHO cells. Biotechnol Bioeng. 87(4):537-45.

(6) Girard P, Derouazi M, Baumgartner G, Bourgeois M, Jordan M, Jacko B, Wurm FM. 2002. 100-liter transient transfection. Cytotech. 38:15-21.

(7) Geisse S, Jordan M, Wurm FM. 2005. Large-scale transient expression of therapeutic proteins in mammalian cells. Methods Mol Biol. 308:87-98.

(8) Haldankar R, Li D, Saremi Z, Baikalov C, Deshpande R. 2006. Serum-free suspension large-scale transient transfection of CHO cells in WAVE bioreactors. Mol Biotechnol. 34(2):191-9.

(9) Boussif O, Zanta MA, Behr JP. 1996. Optimized galenics improve in vitro gene transfer with cationic molecules up to 1000-fold. Gene Ther. 3(12):1074-80.

(10) Akinc A, Thomas M, Klibanov AM, Langer R. 2005. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. J Gene Med. 7(5):657-63.

(11) Wightman L, Kircheis R, Rossler V, Carotta S, Ruzicka R, Kursa M, Wagner E. 2001. Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo. J Gene Med. 3(4):362-72.

(12) Schlaeger E-J, Christensen K. 1999. Transient gene expression in mammalian cells grown in serum-free suspension culture. Cytotech. 30:71-83.

(13) Kaufmann H, Mazur X, Fussenegger M, Bailey JE. 1999. Influence of low temperature on productivity, proteome and protein phosphorylation of CHO cells. Biotechnol Bioeng. 63(5):573-82.

(14) Hendrick V, Winnepenninckx P, Abdelkafi C, Vandeputte O, Cherlet M, Marique T, Renemann G, Loa A, Kretzmer G, Werenne J. 2001. Increased productivity of recombinant tissular plasminogen activator (t-PA) by butyrate and shift of temperature: a cell cycle phase analysis. . Cytotechnology. 36:71-83.

(15) Yoon SK, Song JY, Lee GM. 2003. Effect of low culture temperature on specific productivity, transcription level, and heterogeneity of erythropoietin in Chinese hamster ovary cells. Biotechnol Bioeng. 82(3):289-98.

(16) Fogolin MB, Wagner R, Etcheverrigaray M, Kratje R. 2004. Impact of temperature reduction and expression of yeast pyruvate carboxylase on hGM-CSF-producing CHO cells. J Biotechnol. 109(1-2):179-91.

(17) Shi M, Xie Z, Yu M, Shen B, Guo N. 2005. Controlled growth of Chinese hamster ovary cells and high expression of antibody-IL-2 fusion proteins by temperature manipulation. Biotechnol Lett. 27(23-24):1879-84.

(18) Galbraith DJ, Tait AS, Racher AJ, Birch JR, James DC. 2006. Control of culture environment for improved polyethylenimine-mediated transient production of recombinant monoclonal antibodies by CHO cells. Biotechnol Prog. 22(3):753-62.

(19) Burteau CC, Verhoeye FR, Mols JF, Ballez JS, Agathos SN, Schneider YJ. 2003. Fortification of a protein-free cell culture medium with plant peptones improves cultivation and productivity of an interferon-gamma-producing CHO cell line. In Vitro Cell Dev Biol Anim. 39(7):291-6.

(20) Pham PL, Perret S, Cass B, Carpentier E, St-Laurent G, Bisson L, Kamen A, Durocher Y. 2005. Transient gene expression in HEK293 cells: peptone addition posttransfection improves recombinant protein synthesis. Biotechnol Bioeng. 90(3):332-44.

(21) Hildinger M, Baldi L, Stettler M, Wurm FM. 2007. High-titer, serum-free production of adeno-associated virus vectors by polyethyleneimine-mediated plasmid transfection in mammalian suspension cells. Biotechnol Lett. Manuscript accepted.

(22) Urlaub G, Kas E, Carothers AM, Chasin LA. 1983. Deletion of the diploid dihydrofolate reductase locus from cultured mammalian cells. Cell. 33(2):405-12.

(23) Muller N, Girard P, Hacker DL, Jordan M, Wurm FM. 2005. Orbital shaker technology for the cultivation of mammalian cells in suspension. Biotechnol Bioeng. 89(4):400-6.

(24) Stettler M, Jaccard N, Hacker D, De Jesus M, Wurm FM, Jordan M. 2006. New disposable tubes for rapid and precise biomass assessment for suspension cultures of mammalian cells. Biotechnol Bioeng. 95(6):1228-33.

(25) De Jesus MJ, Girard P, Bourgeois M, Baumgartner G, Jacko B, Amstutz H, Wurm FM. 2004. TubeSpin satellites: a fast track approach for process development with animal cells using shaking technology. Biochem. Eng J. 17:217-223.

(26) Meissner P, Pick H, Kulangara A, Chatellard P, Friedrich K, Wurm FM. 2001. Transient gene expression: recombinant protein production with suspension-adapted HEK293-EBNA cells. Biotechnol Bioeng. 75(2):197-203.

(27) Pham PL, Perret S, Doan HC, Cass B, St-Laurent G, Kamen A, Durocher Y. 2003. Large-scale transient transfection of serum-free suspension-growing HEK293 EBNA1 cells: peptone additives improve cell growth and transfection efficiency. Biotechnol Bioeng. 84(3):332-42.

Chapter 6

Cost issues related to disposable shake bioreactors

6.1 Introduction

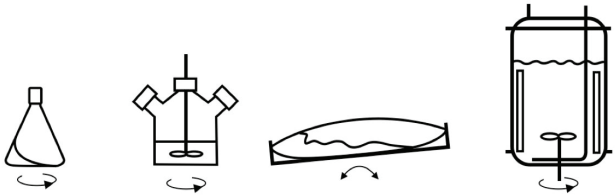
Any new technology, as opposed to standard processes, is expected to reduce the costs, be more efficient in terms of productivity, and/or increase the product quality. Often, these three criteria are intrinsically related and, when improved, result in shorter production times. In the context of biotechnology research and development, a technology that is less expensive to acquire or to operate might result in more experiments being performed in less time. This should favorably influence both the quality of the product and the time-to-market.

To illustrate this in the context of cell line optimization for recombinant protein production, the use of an adequate technology might have multiple benefits. For instance, the multi-parameter screening technology based on 50-mL shake tubes (described in Section 3.3.2) can be used to select appropriate process and medium conditions. When compared to conventional methods (shake flasks, spinners or small-scale instrumented bioreactors), this technology can be used to significantly increase the testing capacity and thus the number of the experimental variables. The result is that more tests can be done within a shorter time frame and certainly at lower costs. Also, the output should be of higher quality, since more variables can be tested simultaneously and repeatedly, if necessary. The 50-mL shake tubes are disposable, resulting in less handling and shorter set-up times. These are clear benefits that have opened new perspectives in fast and reliable small-scale cell line and process optimization (1).

However, what about the cost-efficiency of shake systems operated at larger scales compared to standard equipment? Can similar benefits be expected as shown in the case of multi-parameter screening using 50-mL shake tubes? To address these questions, the cost advantages and the key features of disposable shake bioreactors will be explained in more detail next. Then, a comparative cost study will be made to identify and quantify the cost advantage of disposable shake bioreactors over more conventional equipment (6.2).

6.2 Key features of disposable shake bioreactor systems

There are several good reasons why shake technology is expected to reduce the cost for cell culture process optimizations, scale-up, and production of biopharmaceuticals. In fact, this rational "one technology" approach might potentially replace a variety of different technologies. Nowadays, process optimization and scale-up of single cell cultures are done with a combination of different systems like shake and spinner flasks, stirred-tank bioreactors, or Wave bioreactors. None of these technologies is fully scalable, meaning that a process could be scaled up seamlessly with one technology from a few mL to production scales (Fig. 6.1). For example, stainless steel stirred-tank (or airlift) bioreactors of up to 25'000 L are successfully operated for production purposes (2). However, performing optimization studies using the same technology, even at the 1 L scale, is extremely costly in terms of investment and operating costs.

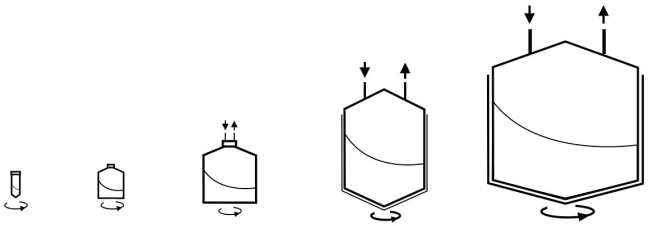


	Shake flask	Spinner vessel	Wave bioreactor	Stirred-tank bioreactor
Min. nominal volume	100 mL	250 mL	1 L	0.5 L
Max. nominal volume	5 L	10 L	1'000 L	25'000 L
Max. working volume	30 - 40%	30%	50%	75%

Fig. 6.1. Minimal and maximal nominal volumes of different standard cell cultivation systems, including the maximal working volumes as a percentage of the nominal volumes.

In contrast, the shake cultivation systems described here are expected to be scalable from a few mL up to production-scale. Shaken multi-well plates are successfully used in combination with standard liquid-handling robotics for high throughput screening (HTS) experiments (3). The present work demonstrated that production scale operations could be expected soon. The consequence is that a single technology might be used at different scales, from the lab to production (Fig. 6.2). This would

result in more straightforward scale-up or scale-down procedures. Additionally, it would provide a better guarantee that improvements can be reproduced at different scales without concern about process parameters that are typical for a specific technology at a given scale. This might result in a high cost-saving potential for any new product development project.



nominal volume	50 mL	1 L	10 L	200 L	1'500 L
agitation speed [rpm]	180	110	65	60	40
aeration	passive		active (air and oxygen)		

Fig. 6.2. Disposable shake bioreactors: possible scale-up sequence from mL scale to production scale. 50-mL and 1-L shake bioreactors are passively aerated, whereas larger systems are actively aerated. The arrows represent the inlet and outlet airflows.

The use of disposable cell culture bags is the other key feature that makes shake bioreactors potentially very cost effective. Though the arguments were described in Section 1.1.2, they are briefly summarized next. Compared to stainless steel bioreactors, the upfront costs for systems based on disposables are significantly reduced (4, 5, 6). Additionally, the use of disposables increases the flexibility in the volume of the process, reduces the risk of cross-contaminations, and minimizes the need for complex cleaning, sterilization, and validation procedures. As suggested in the present work, the employment of single-use sensors for the on-line monitoring of DO and pH is another aspect to be considered. Optical sensors are now sufficiently reliable to be considered as an alternative to the invasive probes used in stirred-tank bioreactors.

The cost-effectiveness due to an easier scale-up from a few mL to production volumes and to the use of single-use components represents a major advantage over established technologies. The next section shows the results of a more detailed comparison between shake bioreactors and well-established bioreactor systems.

6.3 Cost comparison between disposable and standard production systems

To illustrate the advantage of scalable shake bioreactors over standard bioreactors, the following commercially relevant situation was assumed. The aim of the project was to produce a total of 10'000 L of cell culture for the expression of a monoclonal antibody in a fed-batch process. The optimization, scale-up and production sequence is detailed in Fig. 6.3. First, 60 small-scale independent runs were required for growth and productivity optimizations. Then, to scale up the process, 4 runs at the 50 L scale and 2 production scale runs were needed. Finally, after optimization and scale-up, the example considered a total production volume of 10'000 L achieved in 10 successive full-scale runs. The growth cycle was 7 days.

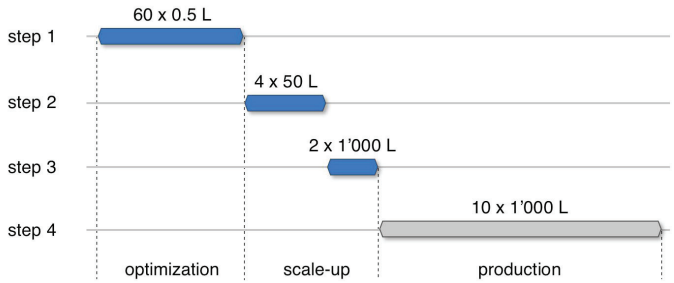


Fig. 6.3. Working volumes required at each step of the project, from optimization to production.

To simplify, the productivity was assumed equivalent for the standard production system using stirred-tank bioreactors and the disposable variants with shake technology. Also, the downstream processing (the purifications steps) was not considered in this example because it will not be affected by the technology chosen for upstream processing. The details of the equipment for both options, disposable and standard, are listed in Tab. 6.1.

Tab. 6.1. Disposable and standard equipment for an optimization, scale-up and production sequence.

Description of the equipment	Working volume	Standard	Disposable
1 x multi-bioreactor of 6 x 1 L	6 x 0.5 L	60'000	
2 x 100 L stirred-tank bioreactor	2 x 50 L	270'000	
1 x 1'500 L stirred-tank bioreactor	1'000 L	250'000	
Total for standard equipment (€)		580'000	
1 x incubator shaker with a capacity of 60 x 1 L	60 x 0.5 L		60'000
1 x shake bioreactor with a capacity of 2 x 200 L	2 x 50 L		45'000
1 x 2'000 L production shake bioreactor	1'000 L		60'000
Total for disposable equipment (€)			165'000

The capital investment for the option with disposable shake bioreactors is 3.5 times lower than the conventional strategy using stirred-tank bioreactors. The costs listed for the multi-bioreactor unit and for the stirred-tank bioreactors are current standard prices for such equipments (as communicated by Bioengineering AG, Wald, Switzerland). They include the probes, the gas mixing system and the monitoring and control unit. It is important to note that such prices might vary considerably depending on the supplier or the need for optional features. The prices for disposable shake bioreactors were estimated based on indications from Kühner AG, which constructed the large-capacity prototype shakers described in Section 4.2.4. Reliable CO_2- and humidity-controlled incubator shakers are commercially available (ISF4-X, AdolfKühner AG, Birsfelden, Switzerland). For both standard and disposable equipment, the prices did not include installation costs and supply of electricity, gas, and steam.

Next, the time required for process optimization, scale-up and production was detailed (Tab. 6.2). The disposable alternative was particularly efficient for small-scale optimizations (step 1). With a single incubator shaker, up to 60 1-L bottles were in operation simultaneously on three different shakers. The same optimization process with a multi-bioreactor system, which represented approximately the same investment as a shaker incubator (Tab. 6.1), had to be repeated 10 times. It can be argued that a multi-bioreactor allows for on-line monitoring of important process parameters, which is of course an advantage. However, 1-L shake bottles can be sampled daily and the supernatant can be analyzed off-line for pH, dissolved CO_2 and

oxygen. Using the packed cell volume method, the biomass increase can be assessed quickly day-by-day. Also, because all tests can be done simultaneously with the same initial cell pool the output of the optimization process might be more significant.

In the subsequent steps (2-4), the reduced time when using disposable shake bioreactors was due to the absence of cleaning, sterilization, and validation procedures. The time used for these operations when working with conventional stirred-tank bioreactors is a major constraint. Overall, this project could be completed in less than half the time normally required with such bioreactors.

Tab. 6.2. Detailed description of the time required for each steps from process optimization to production.

		Standard	Disposable
optimization and scale-up	Step 1: Optimization with 6 x 0.5 L or 60 x 0.5 L		
	Number of small volume test runs	10	1
	Setup time for total number of runs (day)	5	1
	Required time (day)	70	7
	Cleaning and maintenance (day)	5	1
	Step 2: Scale-up using 2 x 50 L		
	Number of test runs	2	2
	Setup time for total number of runs (day)	4	1
	Required time (day)	14	14
	Cleaning and maintenance (day)	8	0
	Step 3: Scale-up to 1'000 L		
	Number of test runs	2	2
	Setup time for total number of runs (day)	2	1
	Required time (day)	14	14
	Cleaning, maintenance and validation (day)	4	1
	First production run possible after (day)	126	40
production	Step 4: production runs for a total of 10'000 L		
	Number of production runs	10	10
	Setup time for total number of runs (day)	20	10
	Required time (day)	70	70
	Cleaning, maintenance and validation (day)	50	2
	Total production time (day)	140	82
	Total project time (day)	266	122

To compare the costs for both options, a depreciation calculation was done based on the investment costs listed in Tab. 6.1. The depreciation was calculated for the actual run days only, not for the period that equipment is idle (Tab. 6.3.A). These values together with estimations for various resources (labor, floor space, fixed and variable costs) were used to calculate the costs for the entire optimization, scale-up and production sequence (Tab. 6.3.B). The estimations were based on a cost comparison reported recently (7). Noteworthy, the costs for cell culture media and supplements were not taken into account, as they are identical in both strategies.

As a result, the calculated costs with disposable shake bioreactors were more than half the ones resulting from the use of standard equipment. Considering the fact that twice as much product could be manufactured in the same time, the final cost saving potential was more than 4-fold. The time and costs for both technologies are graphically summarized in Fig. 6.4.

Tab. 6.3. Equipment depreciation (A) and cost comparison for both options (B).

A

			Standard	Disposable
Optimization and scale-up	Total costs (€)		580'000	165'000
	Period equipment is used (day)		126	40
	Depreciation per year (€)	10 years	58'000	16'500
	Depreciation (€)		20'022	1'808
Production	Total costs (€)		520'000	105'000
	Period equipment is used (day)		140	82
	Depreciation per year (€)	10 years	52'000	10'500
	Depreciation (€)		19'954	2'359

B

		Standard	Disposable
optimization and scale-up	Steps 1-3:		
	Process operators (130 €/day)	16'380	5'200
	Depreciation	20'022	1'808
	Costs floorspace (220 €/m²)	2'200	2'200
	Fixed costs (overhead costs)	3'000	1'500
	Variable costs (disposables)	0	1'500
	Total operating costs (€)	41'602	12'208
production	Step 4:		
	Process operators (130 €/day)	18'200	10'660
	Depreciation	19'945	2'359
	Costs floorspace (220 €/m²)	2'444	4'410
	Fixed costs (overhead costs)	3'333	3'075
	Variable costs (disposables)	0	5'000
	Total operating costs (€)	43'923	25'604
	Total project costs (€)	85'525	37'812

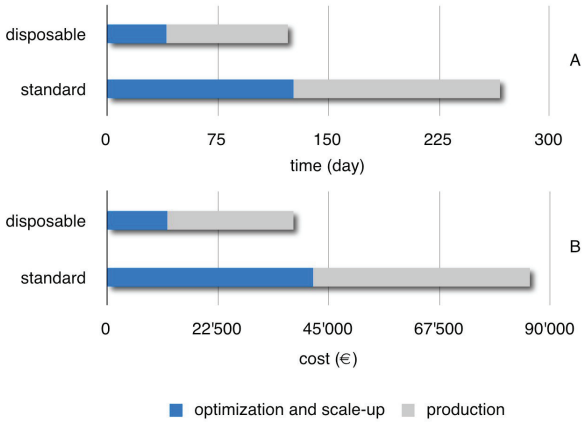


Fig. 6.4. Time (A) and cost (B) saving potential when using disposable shake bioreactors for an optimization, scale-up and production sequence.

This example could be extrapolated to many different manufacturing projects and demonstrates the cost-saving potential of a facility based on disposable shake technology. Also, the cost calculation, though convincing in itself, does not account for other important benefits resulting from the use of disposable technologies. Depending on the needs, disposable shake bioreactors can be easily moved in the production facility and included within other production units. They can be used for other products and applications, without risking a cross-contamination, which increases the flexibility of production planning. Also, alternative technologies such as Wave bioreactors, though widely used, would not be suited for the project described here. Wave bioreactors do not allow small-scale optimizations as shown in this case and they are not conceived for larger production volumes as required here.

6.4 Conclusion

After providing experimental evidence for the promising performance features of shake bioreactor technology at various scales in the previous chapters, strong arguments for its cost-effectiveness were presented here on a qualitative and quantitative basis. Shake bioreactors, as developed in this work, present advantages that go far beyond the fact that they use disposable bioprocessing components. Of course, when cleaning, sterilization, and validation procedures are minimized or eliminated, clear benefits result, as shown in the time and cost comparison described above. However, the fact that a single technology might be used from the mL scale to the production scale with similar performance features is new and could represent a distinct advantage over other alternative technologies, such as wave-type bioreactors.

6.5 References

(1) Stettler M, De Jesus M, Ouertatani H, Engelhardt E-M, Muller N, Chenuet S, Bertschinger M, Baldi L, Hacker D, Jordan M and others. 2007. 1000 non-instrumented bioreactors in a week - Novel disposable technologies for rapid scale-up of suspension cultures. In *Cell Technology for cell products*. Proceedings of the 19th ESACT meeting, Harrogate, UK. Edited by Rodney Smith, Springer. pp. 489-95.

(2) Chu L, Robinson DK. 2001. Industrial choices for protein production by large-scale cell culture. Curr Opin Biotechnol. 12(2):180-7.

(3) Heath C, Kiss R. 2007. Cell culture process development: advances in process engineering. Biotechnol Prog. 23(1):46-51.

(4) Farid SS, Washbrook J, Titchener-Hooker NJ. 2005. Decision-support tool for assessing biomanufacturing strategies under uncertainty: stainless steel versus disposable equipment for clinical trial material preparation. Biotechnol Prog. 21(2):486-97.

(5) Novais JL, Titchener-Hooker NJ, Hoare M. 2001. Economic comparison between conventional and disposables-based technology for the production of biopharmaceuticals. Biotechnol Bioeng. 75(2):143-53.

(6) Sinclair A, Monge M. 2002. Quantitative Economic Evaluation of Single Use Disposables in Bioprocessing. Pharmaceutical Eng. 22(3):20-34.

(7) Houtzager E, van der Linden R, de Roo G, Huurman S, Priem P, Sijmons PC. 2005. Linear scale-up of cell cultures - the next level in disposable bioreactor design. BioProcess Int. June 2005. pp. 60-66.

Chapter 7

Summary

Previously, the biomanufacturing of recombinant proteins was mainly driven by science and technology, with little cost constraints. Now, the focus is on reducing the product development cycles and maximizing returns on expensive facilities. This mindset shift in the biotech industry opened totally new opportunities for technology development. Concepts that were initially considered with distrust, such as the Wave bioreactor, have now gained wide acceptance. It is now recognized that technologies based on disposables have the potential to reduce the time and the costs associated with the development and production of new protein products.

The present work consisted in investigating technologies that match these new constraints. As shown in the comparison between disposable bioreactors based on orbital shake technology and standard bioprocess equipment, the costs are significantly reduced while maintaining or improving the performance. This was the main outcome of the thesis work, though other aspects of interest were studied as well.

First, an interesting side aspect of the topic was the evaluation of a new disposable biomass assessment tool. In contrast to conventional methods, PCV measurements using VoluPAC tubes were found to be fast and accurate. The procedure involves loading a sample of known volume into the measurement tubes followed by centrifugation for 1 min at 2'500 *g*. The height of the cell pellet in the capillary of the VoluPAC tube was assessed by image analysis and expressed as percentage of biomass. The characterization and validation of the measurement system demonstrated that it could optimally replace time-consuming and less accurate cell counting methods.

Then, disposable 50-mL shake tubes were found particularly well suited for maintaining cells in strictly controlled and well-defined culture conditions. Also, 50-mL shake tubes were shown appropriate for multi-parameter screenings. This is due to

the robustness and reliability of the cultivation system with apparently no rate-limiting conditions. To further characterize the properties of the shake tubes, the oxygen transfer was evaluated. As expected, the specific liquid mass transfer coefficient *(k_La)* varied with the agitation speed and the working volume. Further, knowing the k_La, the cell specific oxygen uptake rate of CHO and HEK 293 cells was experimentally evaluated. This knowledge was important for predicting the k_La required in larger shake bioreactors.

Further, disposable shake bioreactors were scaled stepwise to production volumes. Each step was evaluated in cell culture conditions with CHO cells growing in batch mode. To ensure sufficient oxygen supply, air (or oxygen enriched air) was provided at flow rates that increased the oxygen partial pressure in the bioreactor headspace. At scales up to 20 L, disposable polycarbonate shake containers were found both convenient and efficient for cultivating CHO cells. At scales beyond 20 L, cylindrical disposable bags were used in combination with lightweight containers. Large-scale shakers were constructed to deliver sufficient agitation capacity. The proof of principle involved cultivating CHO cells in a total working volume of 750 L at cell densities that are relevant for industrial processes. Promising results were obtained, though further testing and fine-tuning of culture conditions will be necessary before envisioning a commercial development.

Finally, disposable shake bioreactors of various volumes were used to optimize a transient gene expression protocol. Both technologies, when combined, were found adequate to shorten the time between the DNA delivery and the production of candidate molecules in preclinical research. In a single batch experiment, nearly one gram of IgG was expressed from 30 L of transiently transfected CHO cells.

To conclude, the disposable shake bioreactors developed within this study were found promising as a cost-effective alternative to expensive conventional technologies. Noteworthy, the use of disposable shake bioreactors increases the flexibility in volumes to be processed, reduces the risk of cross-contamination, and minimizes the need for complex cleaning, sterilization, and validation procedures. For the first time, the same technology, orbital shaking, might be used from the milliliter scale for process optimization up to production volumes. This should result in simpler and more straightforward scale-up or scale-down procedures.

Chapter 8

Outlook

Shake technology applied to animal cell technology proved to fulfill the requirements in terms of oxygen transfer, shear stress, flexibility in handling, cost-effectiveness and scale-up. Investigations demonstrated that it can be used for stable expression of recombinant proteins as well as for transient gene expression. Also, disposable bioreactors based on this technology are believed to address the needs of research, development and even production in a variety of possible applications. To achieve this, more research and development efforts are necessary. Also, the question of the maximal scale is still open. Next, some critical aspects that need to be investigated are highlighted:

Evaluation and prediction of mixing time at different scales: Though the mixing time is known to be a critical process parameter, it is not yet fully understood how it is related to geometry and shaking speed. Mixing time should be evaluated using a non-invasive pH sensing system.

Computational fluid dynamics (CFD): This technique should be used to establish a numerical model. This model should predict process parameters such as specific mass transfer coefficient (k_La), mixing time, and shear stress for different geometries, filling volumes, and rotational speeds and diameters. The model should also be able to predict critical agitation speeds for turbulent mixing and its impact in terms of shear stress.

Vessel geometry: Modified geometries might be used to successfully increase the mixing time and improve the homogeneity of the cell suspension. For example, a helical track system was used in preliminary experiments at different scales. It was already shown that such a system significantly improves the oxygen transfer rate.

Optical sensing technology: It should be evaluated how novel sensing technology could be combined with cell culture bags for the monitoring of pH and DO. The

response time and the reliability of such systems should be evaluated, especially with larger operation scales.

Cell culture and productivity: The present work almost exclusively focused on CHO cell lines. Other relevant mammalian cell lines for research and industrial purposes should be cultivated in disposable shake bioreactors, such as NS0, SP2/0, BHK and HEK 293 cells. This would contribute to establish novel shake bioreactors for a wider range of cell culture applications and ensure that high productivities are achievable.

Curriculum Vitae

Personal information

Date of Birth	December 5, 1977
Nationality	Swiss
Languages	French, German and English

Education

Nov. 2004 - present	Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne PhD candidate at the Life Science Faculty, Institute of Biotechnology and Bioengineering, Lab. of Cellular Biotechnology, Prof. Dr. Florian M. Wurm
April 2002	Swiss Federal Institute of Technology (ETH), Zurich Master of Science, Master thesis at the Lab. of Food Engineering Topic: Scale-down of a dispersing device, production of food emulsions

Continuing education

March 2005 - present	EPFL Doctoral school, Lausanne PhD Program in Biotechnology and Bioengineering
April - June 2007	EPFL MoT Biotech Modules, Lausanne Strategic Alliances, Partnership and Outsourcing, 3 days Marketing of Biotech/Medech/Pharma, 3 days Clinical Trial Management and Regulatory Affairs, 3 days
June 2005	EPFL Service des Relations Industrielles, Lausanne Cours de Management de l'innovation technologique (Mint), 3 days
May 2005	University of Minnesota, Minneapolis (USA) Advanced Course in Cellular Bioprocess Technology, 4 days

Scientific contributions

Workshop presentation

Stettler M, Wulhfard S, De Jesus M, Hacker D, Hildinger M, Wurm FM. 2006. Large-scale transient gene expression in shaking bioreactors of 1 to 50 Liter volumes. Fourth European BioTechnology Workshop (17-19 September 2006), Ittingen, Switzerland.

Publications

Stettler M, Zhang X, Hacker D, De Jesus M, Wurm FM. 2007. Novel orbital shake bioreactors for transient production of CHO derived IgGs. Biotech Progress. Manuscript accepted.

Hildinger M, Baldi L, **Stettler M**, Wurm FM. 2007. High-titer, serum-free production of AAV vectors by polyethyleneimine-mediated plasmid transfection in mammalian suspension cells. Biotech Letters. Manuscript accepted.

Ehrmann K, Pataky K, **Stettler M**, Wurm FM, Brugger J, Besse PA and Popovic R. 2007. NMR spectroscopy and perfusion of mammalian cells using surface microprobes. Lab Chip. 7(3):381-3.

Ehrmann K, Saillen N, Vincent F, **Stettler M**, Jordan M, Wurm FM, Besse PA, Popovic R. 2007. Microfabricated solenoids and Helmholtz coils for NMR spectroscopy of mammalian cells. Lab Chip. 7(3):373-80.

Stettler M, De Jesus M, Ouertatani H, Engelhardt EM, Muller N, Chenuet S, Bertschinger M, Baldi L, Hacker D, Jordan M, Wurm FM. 2007. 1000 non-instrumented bioreactors in a week - Novel disposable technologies for rapid scale-up of suspension cultures. In *Cell Technology for cell products*. Proceedings of the ESACT conference 2005, Harrogate, UK. Edited by Rodney Smith, Springer. pp. 489-95.

Stettler M, Jaccard N, Hacker D, De Jesus M, Wurm FM, Jordan M. 2006. New disposable tubes for rapid and precise biomass assessment for suspension cultures of mammalian cells. Biotech Bioeng. 95(6):1228-1233.

Seignez C, Adler N, Thoeni C, **Stettler M**, Péringer P, Holliger C. 2004. Steady-state and transient-state performance of a biotrickling filter treating chlorobenzene-containing waste gas. App Microbiol Biotechnol. 65:33-37.

Posters

Stettler M, Zhang X, Anderlei T, De Jesus M, Hacker D, Wurm FM. 2007. Large-scale disposable orbital shake bioreactors. CTI Medtech Event 2007 (4 September 2007), Bern, Switzerland.

Stettler M, Zhang X, Anderlei T, De Jesus M, Hacker D, Wurm FM. 2007. Towards a large-scale disposable orbital shake bioreactor - suitable for high-density batch/fed batch bioprocesses. ESACT Conference 2007, Dresden, Germany.

Stettler M, Zhang X, Anderlei T, De Jesus M, Hacker D, Wurm FM. 2007. Development of pilot-scale orbital shake bioreactors – ideal for cost-effective and efficient transient gene expression. ESACT Conference 2007, Dresden, Germany.

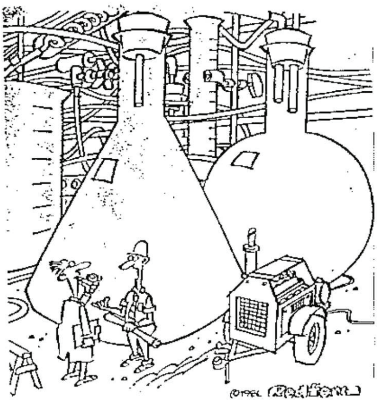
Zhang X, **Stettler M**, Reif O, Kocourek A, De Jesus M, Hacker D, Wurm FM. 2007. Helical tracks in shaken cylindrical bioreactors improve oxygen transfer and increase maximum cell density obtainable for suspension cultures of mammalian cells. ESACT Conference 2007, Dresden, Germany.

Zhang X, **Stettler M**, Hacker D, Reif O, Kocourek A, Kühner M, De Jesus M, Hacker D, Wurm FM. 2007. Gas transfer is far superior in a 30 liter helical track bioreactor than in a stirred vessel. European Downstream Technology Forum 2007, Sartorius College, Goettingen, Germany.

Stettler M, Muller N, Jaccard N, Wulhfard S, De Jesus M, Hacker D, Wurm FM. 2006. Transient gene expression of a human IgG in CHO DG44 cells - Evaluation of a multi-parameter process optimization strategy using shaking bioreactors. Cell Culture Engineering X 2006, Whistler BC, Canada.

Stettler M, Jaccard N, Wulhfard S, De Jesus M, Hacker D, Wurm FM. 2006. Transient gene expression of a human IgG in CHO DG44 cells. CTI Medtech Event 2006 (30 August 2006), Bern, Switzerland.

De Jesus M, Bonnet D, Décaillet S, Burki C, **Stettler M**, Wurm FM. 2005. The TubeSpin system - an innovative concept allowing rapid process development screening programs with increased process yields. ESACT 2005, Harrogate, UK.



"Got a few problems going from lab scale up to full-scale commercial."