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Review Article

Manufacturing recombinant proteins in kg-ton quantities using animal cells in bioreactors

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ABSTRACT

Mammalian cells in bioreactors as production host are the focus of this review. We wish to briefly describe today's technical status and to highlight emerging trends in the manufacture of recombinant therapeutic proteins, focusing on Chinese hamster ovary (CHO) cells. CHO cells are the manufacturing host system of choice for more than 70% of protein pharmaceuticals on the market [21]. The current global capacity to grow mammalian cells in bioreactors stands at about 0.5 million liters, whereby the largest vessels can have a working volume of about 20,000 l.

We are focusing in this article on the upstream part of protein manufacturing. Over the past 25 years, volumetric yields for recombinant cell lines have increased about 20-fold mainly as the result of improvements in media and bioprocess design. Future yield increases are expected to come from improved gene delivery methods, from improved, possibly genetically modified host systems, and from further improved bioprocesses in bioreactors. Other emerging trends in protein manufacturing that are discussed include the use of disposal bioreactors and transient gene expression. We specifically highlight here current research in our own laboratories.

1. Introduction

Stably transfected mammalian cells were first generated by non-viral gene delivery in the early 1980s [39,24,40]. Immortalized Chinese hamster ovary (CHO) cells were among the first hosts to be used for stable gene transfer because of the availability of auxotrophic (metabolic) CHO mutants deficient in dihydrofolate reductase (DHFR) activity. Using these, cells became feasible after two critical developments: (i) the molecular cloning of genes into plasmid DNAs [13] and (ii) the chemically mediated delivery of plasmid DNA into cultured mammalian cells [20,46,1]. The first CHO-derived cell lines included those that produced recombinant interferons and tissue-type plasminogen activator (tPA) [40,30,25]. In 1987, a tPA molecule became the first FDA-approved recombinant therapeutic protein (Activase®-tPA). The production of tPA by the company Genentech required the cultivation of suspension culture-adapted mammalian cells in large-scale stirred-tank bioreactors, a technology modified from the large-scale cultivation of bacteria in stirred-tank bioreactors [6]. In many ways, the technology has not dramatically changed in the last 25 years; CHO cells remain the major host for the generation of recombinant cell lines, and most manufacturing processes with recombinant cell lines are still performed in large-scale stirred-tank bioreactors. What then is the difference between today's technology and that of 25 years ago?

In the late 1980s, a typical batch culture production run lasted about 7 days with a maximum cell density of 1–2 million cells/ml, and the usual yield was 50–100 mg/l [54]. By comparison, today's fed-batch production runs can last up to 21 days with a maximum cell density of 10–15 million cells/ml. These bioprocesses typically have specific and volumetric productivities in the range of 50–60 pg/cell/day – only about two times higher than in the eighties – but 1–5 g/l in product concentration for antibodies and antibody-like molecules at harvest – i.e., about 20 times higher than in the early phases of the use of CHO cells in bioreactors [54]. Surprisingly, this dramatic yield increase has come about mainly due to improvements in the media compositions, including of complex feeds (medium concentrates), and in bioprocess modifications, resulting in more and healthier cells over a longer cultivation period. Obviously, these process modifications have been developed over years of research by the producer companies who are understandably reluctant to make the details of their bioprocesses and media compositions public.

Are further improvements in specific and volumetric productivity of recombinant mammalian cells possible or has a biological limit been reached? While clearly a biological limit seems to be achieved, at about 100 pg/cell/day in the specific productivity area,

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in our opinion, volumetric yields can still be pushed higher, maybe even 2–5-fold, by further increasing cell densities in the bioreactor. However, this requires even richer cell culture media that are not easy to identify due to the complexity of their composition. High-throughput cell cultivation systems for the rapid and cost-effective screening of media compositions and bioprocess conditions will be necessary and used, ultimately with the goal to allow for additional cell population doublings, but also for the identification of specialized feeds that assure the maintenance of a healthy, high-density cell population for longer time. This review will also discuss two additional topics, disposable containers for large-scale cell culture and transient gene expression (TGE), as emerging trends in protein manufacturing.

2. Generation of CHO-derived cell lines

The CHO cells used in protein manufacturing originated in 1957 as an immortalized cell from a primary culture of ovarian cells from a Chinese hamster (*Cricetulus griseus*) [38]. A glycine-dependent strain (CHO-K1) was derived from the original cell lines and mutagenized to generate CHO-DXB11 (also referred to as CHO-DUKX or CHO-DUK-XB11), a cell line lacking DHFR activity [49]. These cells have a deletion of one *dhfr* allele and a mis-sense mutation in the other. Subsequently, the proline-dependent CHO-pro3$^-$ strain, another derivative of the original CHO cell line, was mutagenized to yield CHO-DG44, a cell line with deletions of both *dhfr* alleles [50]. These two DHFR-minus strains require glycine, hypoxanthine, and thymidine (GHT) for growth.

Although not initially intended recombinant protein manufacture, DHFR-minus CHO cells were used for a number of pioneering experiments demonstrating stable transfection with an exogenous *dhfr* gene via selection in GHT-minus medium [39,24,40]. This genetic selection scheme remains one of the standard methods to establish stably transfected CHO cell lines for the production of recombinant therapeutic proteins. The multistep process begins with the molecular cloning of the gene of interest (GOI) and the *dhfr* gene in a single or in separate mammalian expression vectors. The plasmid DNA(s) carrying the two genes are then delivered into cells by transfection, and the cells are grown under selective conditions in GHT-minus medium. Each surviving cell will have one or more copies of the exogenous dhfr gene, usually along with the GOI, integrated in its genome [39,24,40]. The integrated plasmid copy number varies widely from one recombinant cell to another, but there is almost always only one integration site per cell even if multiple plasmids are transfected [53]. The growth rate and the level of recombinant protein production of each cell line also vary widely. To obtain a few stably transfected cell lines with the desired phenotypic characteristics, it may be necessary to evaluate several hundred candidate cell lines.

The *dhfr* gene is not the only selection marker available for generating recombinant CHO cell lines. The glutamine synthetase (*gs*) gene, initially considered for the selection of murine NS0-derived cell lines since their endogenous GS activity is low, is also used for the selection of stably transfected CHO cells even though they have a higher endogenous GS activity than NS0 cells [5,4]. After transfection with the GOI and *gs* genes, recombinant cells are selected in medium without glutamine. With both DHFR- and GS-based selection, expression of the recombinant gene can be significantly increased by exposing the cells to a drug that blocks the enzymatic activity of the selection marker. DHFR and GS are inhibited by methotrexate (MTX) and methionine sulfoximine (MSX), respectively [36,23,5]. For CHO-derived cell lines that express an exogenous *dhfr* gene, a majority of the cells die after 2–3 weeks of exposure to increasing concentrations of MTX. The rare survivors have a higher integrated plasmid copy number than the original cell line as the result of amplification of the *dhfr* gene and the neighboring DNA, including the GOI [36,23,24]. Similar observations have been made following the exposure of recombinant NS0 cell lines to MSX [4].

3. Improved recovery of high-producing cell lines

The major problem with the standard methods of cell line generation and selection is that the specific productivity of many of cell lines recovered is low. This necessitates the screening of hundreds if not thousands of individual cell lines to obtain a sufficient number that have the desired phenotype of high protein productivity and high growth rate. One way to reduce the number of cell lines screened is to increase the stringency of selection. With DHFR-minus CHO cells, for example, the GHT-minus medium may be supplemented with 30–100 nM MTX to increase the probability of selecting cell lines with high *dhfr* activity. Since the GOI is integrated at the same site as the exogenous *dhfr* gene, this selection strategy is expected to increase the number of high-producing cell lines (De Jesus, unpublished data). One advantage of this approach is that the recovered cell lines do not require further DNA amplification in the presence of increasing concentrations of MTX. Induced gene amplification is time-consuming and unpredictable, and the amplified DNA may not be stable in the absence of MTX. High-throughput methods to screen candidate cell lines are being developed to reduce the time necessary for the recovery of high producers [8]. Most of these methods are based on fluorescence-activated cell sorting (FACS). For example, the GFP gene can be co-expressed with the GOI and the cells sorted for GFP-specific fluorescence [32,29]. The GFP gene may be used as the sole selection marker or in combination with one of the selection markers described above. Alternatively, the GOI may be co-expressed with a gene encoding a cell surface protein. The recombinant cells expressing the latter are then stained with a fluorescently labeled antibody specific for this protein and then sorted by FACS [7]. Lastly, recombinant cells selected for the presence of the *dhfr* gene have been incubated with fluorescent MTX that binds to DHFR. As with the techniques described above, the DHFR-positive cells are then sorted by FACS [55]. The level of MTX-specific fluorescence is expected to correlate with the level of the recombinant protein of interest.

Clonal cell line recovery has been automated with such instruments as the ClonePix system (Genetix Ltd., United Kingdom) and the CellCelector™ (Aviso GmbH, Germany). For these instruments, the putative recombinant cells are suspended in semisolid medium. Under these conditions, the secreted recombinant protein remains near the cell and can be stained with a fluorescently labeled antibody. These automated systems can then detect and transfer the cell lines to another cultivation container for further analysis.

It is also possible to increase the average specific productivity of recombinant cell lines by increasing the amount of plasmid DNA delivered to cells. We have recently shown, for example, that calcium phosphate (CaPi)-mediated transfection of CHO-DG44 cells results in both a higher plasmid copy number and a higher average specific productivity compared to PEI-mediated transfection [11]. Furthermore, the specific productivity of recombinant cell lines generated by microinjection of either BHK-21 or CHO-DG44 cells depended on the amount of plasmid DNA injected per cell [10].

Once clonal cell lines are established, they need to be characterized for the stability of recombinant protein production. This is necessary because expression of the integrated GOI is not necessarily maintained at a constant level over time due to a phenomenon termed gene silencing (the reduction or elimination of gene-specific transcription). Thus, candidate cell lines for large-scale productions must be cultivated for several months to exclude stability problems. A major determinant of gene silencing is assumed to be the structure (sequence composition and modification

of histones and other proteins) of the chromatin at the site of integration of the recombinant gene. In general, heterochromatin is condensed and transcriptionally inactive whereas euchromatin is relaxed and transcriptionally active [27]. The two chromatin states are associated with specific histone modifications including acetylation, methylation, and phosphorylation that function to control chromatin condensation and transcriptional activity [22]. We have observed two different rates for gene silencing in recombinant CHO-DG44 cell lines [11]. Rapid gene silencing occurs in about half the cell lines within days after release of the cells from selective pressure. This type of gene silencing does not appear to be correlated to the level of GOI expression. For about one-third of the cell lines, a slow and gradual reduction in GOI expression occurs within 6 months after removal of the selective pressure (data not published). Finally, about 15–25% of cell lines have a stable level of protein productivity in the absence of selection. These observations did not depend upon the method of gene delivery [11]. For cells treated with MTX to amplify the copy number of the integrated gene of interest, the stability of the recombinant protein production in the absence of MTX appeared to be due mainly to gene silencing rather than to loss of transgene copy number [12].

The choice of the promoter/enhancer used to drive GOI expression may also influence the extent of gene silencing. Furthermore, DNA elements like scaffold/matrix attachment regions (S/MARs), insulators, antirepressor elements, and ubiquitous chromatin opening elements (UCOEs) have been shown to support stable protein production in recombinant cell lines [45,56,26,52]. These DNA elements are small enough so that they can be cloned in the expression vector employed for GOI delivery. The mechanism(s) associated with their observed function, however, is not entirely clear. They may ameliorate the effects of gene silencing directly through the inhibition of heterochromatin formation or they may affect plasmid DNA integration itself. For example, they may influence the integrated plasmid copy number or the site of integration.

4. High-throughput bioprocess development

In the 1980s, small-scale process development studies for recombinant protein production have mainly been performed in instrumented or non-instrumented spinner flasks. However, they are not easily adapted to high-throughput applications since the minimal culture volume for these containers is about 50 ml, more frequently even bottles with working volumes of 250 or 500 ml were used. In these vessels, volumes smaller than 50 ml were not possible. Also, in these containers, cell growth is limited since the volumetric mass transfer coefficient (k_La) is about 1–3 h^{-1}, limiting the maximally achievable cell densities to 2–3 $\times$ [35], while high-performance media allow densities of 10 $\times$ 10^6 cells/ml when the culture is performed in a fully controlled and properly oxygenated bioreactor. Therefore, the cell cultivation conditions in spinner flasks have been an inaccurate predictor of those in large-scale stirred-tank bioreactors.

As an alternative and in an attempt to address the urgent needs for simpler, high-performance cultivation systems, we developed orbitally shaken 50-ml ventilated tubes (TubeSpin® bioreactor 50, now marketed by TPP, Trasadingen Switzerland, short "TubeSpins"). These have been found to be efficient scale-down bioreactors for mammalian cell cultivation in suspension [14]. Cell densities >10^7 cells/ml have been achieved in volumes of 5–20 ml with CHO cells [42]. At agitation speeds appropriate for mammalian cell cultivation, k_La values of 10–20 h^{-1} have been measured [57]. Due to the excellent mass transfer in these tubes, oxygen limitation has not been observed at cell densities up to 3 $\times$ 10^7 cells/ml. We and others have used these TubeSpins for high-throughput screening campaigns to optimize bioprocesses for recombinant CHO cell lines. The low cost and ease of operation of orbitally shaken TubeSpins allows 100s of small-scale cultures to be run simultaneously [43]. TubeSpins are expected to significantly reduce the time necessary for medium design and the development of feeding strategies for fed-batch cultures. Other scale-down cell culture systems have also been described recently. Examples include the SimCell microfluidics technology (Invitrogen, USA) and microbioreactors in 24- and 96-deepwell plates [16,9].

5. Disposable bioreactors

Today, large-scale mammalian cell culture is almost exclusively performed in stainless steel stirred-tank bioreactors. However, there is a trend in the protein manufacturing industry toward the use of disposable equipment. The most successful of the disposable bioreactors is the wave-type bioreactor that was introduced about 10 years ago [41]. The cells are cultivated in disposable plastic bags of volumes of up to 500 l mounted on a rocking table. However, the k_La values in wave bioreactors are less than 4 h^{-1}, meaning possible oxygen limitation at densities >5 $\times$ 10^6 cells/ml when only air is used as an oxygen-providing gas [41]. Disposable stirred-tank bioreactors at volumetric scales of up to 1000 l have also become available very recently from a number of suppliers.

Erlenmeyer flasks have been used in the past for both microbial and cell culture operations by applying orbital shaking as the mixing principle. However, the scale-up in these flasks is limited since the working volume is only 10–20% of the nominal volume. More recently, both cylindrical and square-shaped vessels with working volumes in the range of 100 ml to 30 l have been used, mostly in our laboratories, for the suspension cultivation of mammalian cells by orbital shaking [28,33,42,44,57,48]. Mammalian cell cultivation has also been performed in disposable plastic bags of 200 l and 2000 l that were mounted within cylindrical containers on orbital shakers custom-made for this purpose [42].

Stirred-tank bioreactors are aerated by the sparging of oxygen or oxygen-enriched air into the culture from a position near the bottom of the vessel. In contrast, surface aeration is the preferred operational mode for shaken bioreactors, highlighting the importance of the surface (area) on oxygen transfer in this type of cultivation system. The flow structure at or near the liquid–air interface is the dominant factor determining the rate of mass transfer into and out of the cell culture medium. For cylindrical shaken vessels of up to 100 l working volume, shaking at moderate shaking speeds, k_La values of 3–7 h^{-1} were obtained. For the orbitally shaken 2000 l reactor mentioned above, a k_La value of 2–3 h^{-1} was observed when using cell culture compatible conditions in shaking [57]. This somewhat lower k_La value is still surprisingly high when compared with similar scale stirred-tanks for cell culture applications. It is known that limiting oxygen transfer rates in bioreactors (due to low k_La values) can be overcome by aeration with oxygen-enriched air or pure oxygen, since such gas mixtures or pure oxygen has a higher driving force into the liquid. Thus, we firmly believe that suspension cell culture using orbitally shaken bioreactors will become an attractive, possibly even superior option to stirred stainless steel tanks at scales of up to 2000 l because of increased flexibility, better mixing and gas transfer rates and, most importantly, due to the reduced costs associated with disposable cultivation systems [42,51]. Although operating conditions in large-scale orbitally shaken bioreactors require further study and in-depth analysis, the results to date demonstrate the potential of this simple bioreactor for applications in high-density mammalian cell cultivation.

6. Transient gene expression (TGE)

Large-scale TGE is a relatively new technology that was only recently considered for recombinant therapeutic protein production

[37,3]. TGE is defined as the production of a recombinant protein over a short period (1–14 days) following DNA transfer into single-cell suspension cultures. The recombinant gene(s) is usually cloned in a non-viral expression vector and transfected into cells with a chemical delivery agent like calcium phosphate (CaPi) or polyethylenimine (PEI). In contrast to stable gene expression from recombinant cell lines, genetic selection is not applied to the transfected cells during the protein production phase. The process has been developed mainly with CHO and HEK-293 cells since they are easily transfected, grown in single-cell suspension, and have been used for the production of therapeutic proteins which have gained regulatory approval [31,17,15,47]. TGE is typically performed in stirred-tank bioreactors or in agitated containers including shake flasks, wave-type bioreactors, and plastic or glass bottles [18]. The main advantage of TGE over stable protein production is time savings. In the past, the specific and volumetric productivities achieved by TGE were significantly lower than those seen in stable cell lines. Recently, with HEK-293 cells, however, volumetric productivities of 1 g/l have been achieved in a bioprocess lasting 14 days [2]. Thus, significant quantities of recombinant protein can be obtained within a few days of transfection. While 1–10 l scale TGE with yields of hundreds of mg/l has been developed as a reliable method at ExcellGene (founded by the authors of this paper), the routine use of this approach for volumetric scales in the 100–1000 l range is still not yet feasible. To date, the largest volumes for TGE have been 100 l [19,34].

Up to now, there has not been a therapeutic protein produced by TGE that has gained regulatory approval. This must be accomplished before large-scale TGE becomes a standard method of therapeutic recombinant protein manufacturing. This is likely to be first attempted with a low-dose protein such as a vaccine that does not require a large amount of recombinant protein to cover the market needs. There is also a perception in the industry that large-scale TGE is not reproducible. However, as an emerging technology, it needs to be addressed from all angles of science and engineering which is a matter of time and effort. The application of high-throughput culture systems as described above will help to alleviate existing problems with TGE.

7. Conclusions

Much has been accomplished in the last 25 years of recombinant therapeutic protein manufacturing with typical volumetric yields from bioprocesses having increased approximately 20-fold during this period. With gram per liter yields in highly optimized fed-batch processes, the production of kilograms and even hundreds of kilogram of a desired protein in large-scale bioreactors has become a routine. Continued improvements in the generation of high-producing cell lines, the composition of media, and the strategies for feeding are expected to result in further yield increases, probably also from shorter run times than those that are now in place. With this, the overall annual capacity of manufacturing plants can be increased dramatically. It remains to be seen whether the required bioreactor volumes may actually decrease over time, due to the improved yields. Surely, the time required to establish high-yielding processes will decrease to a few months (3–6), offering more flexibility and choices. Overall, the improved speed for the development, the higher yields, the use of disposable reactors, and the application of generic processes will have a beneficial economic impact on protein manufacturing. Transient gene expression is likely to become an attractive manufacturing technology for highly urgent needs, such as in the case of an emergency vaccine when a new viral or bacterial agent threatens public health.

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