



Antibody production[☆]

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Abstract

The clinical and commercial success of monoclonal antibodies has led to the need for very large-scale production in mammalian cell culture. This has resulted in rapid expansion of global manufacturing capacity [1], an increase in size of reactors (up to 20,000 L) and a greatly increased effort to improve process efficiency with concomitant manufacturing cost reduction. This has been particularly successful in the upstream part of the process where productivity of cell cultures has improved 100 fold in the last 15 years. This success has resulted from improvements in expression technology and from process optimisation, especially the development of fed-batch cultures. In addition to improving process/cost efficiencies, a second key area has been reducing the time taken to develop processes and produce the first material required for clinical testing and proof-of-principle. Cell line creation is often the slowest step in this stage of process development. This article will review the technologies currently used to make monoclonal antibodies with particular emphasis on mammalian cell culture. Likely future trends are also discussed.

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Keywords: Fed-batch culture; CHO; NS0; Gene expression systems; Downstream processing; Fermentation; Cell line selection

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1. Introduction

Currently, there are 18 monoclonal antibodies approved for therapeutic use [2]. The majority (15) of these antibodies are produced by recombinant DNA technology although three are murine antibodies made in hybridomas. The recombinant antibodies are produced in mammalian cell expression systems using Chinese hamster ovary (CHO) or murine lymphoid cell lines (e.g., NS0, Sp2/0-Ag14). Most products in clinical trial are whole antibodies made in mammalian cell systems but some are antibody fragments, which can be made in microorganisms such as *E. coli*. For example, CIMZIA™ [2] is a pegylated Fab' fragment made in a microbial system and is currently in phase III trials. Reichert et al. [2] report that, of the 15 antibodies they identified in phase III trials, six were single chain or Fab fragments. In this article, we review the technologies used to manufacture antibodies focusing particularly on the current status of mammalian cell culture and approaches taken in process development. There are two crucial issues, which have to be faced in process development. The first is to minimise the time taken to provide material for clinical studies and the second is to develop a process which can deliver sufficient drug substance to meet market demands at an acceptable price per dose.

The industry continues to look at new technologies and process development strategies that will reduce

timelines. The resulting processes must be easily scaleable, robust and meet quality and safety criteria. One approach to shortening the timelines is the use of platform technologies for cell culture processes, for example using standard media, feeds and growth conditions. Cell line construction and selection is often a critical path activity and needs to be completed rapidly without compromising quality criteria. Ideally, one would like to rapidly create a highly productive cell line that could be used for long-term manufacture obviating the need to create an improved second generation cell line at a later stage of development.

Productivity of mammalian cell processes has improved dramatically in recent years [3] and modern cell culture processes can achieve antibody concentrations exceeding 5 g/L [4,5]. This has resulted from improvements in expression technology and from process optimisation, particularly of the upstream, cell culture stage. Most current processes are based on fed-batch culture and the development of feeds in particular has made a significant contribution to increased antibody yields. Highly productive cell lines result from using a host cell line that has the desired characteristics, an appropriate expression system, and a good transfection and selection protocol. A number of expression systems with the potential to produce cell lines with high specific production rates (Q_p) are available. The challenge is to create cell lines that not only have high Q_p but also

have the growth characteristics that will lead to high productivity in the manufacturing process. This can be achieved by selecting cell lines with the combination of a high Q_p , an ability to achieve high space–time yields of viable biomass and consequent high volumetric production rate in a screen designed to mimic the final production process. The resulting cell lines are also selected for stability, growth in suspension culture using a chemically defined, animal component-free (CDACF) medium and the ability to make the desired post-translational modifications.

Purification strategies are based on chromatographic procedures usually including a protein A affinity step. With increasing upstream concentrations, significant attention is now being paid to improving downstream efficiency.

2. Expression systems

The ability of a cell line to achieve high volumetric productivities results from a combination of characteristics. Efficient transcription of the antibody genes is achieved by using an appropriately designed expression vector. Secondly, one requires a cell line capable of efficiently translating antibody mRNAs, assembling and modifying the antibody at high rates with minimal accumulation of incorrectly processed polypeptides, and having sufficient secretory capacity for secreting the resulting assembled antibody. It is probable that with the current generation of cell lines, productivity is not limited by transcription. It has been reported that, for a panel of antibody-producing GS-NS0 cell lines with Q_p values varying between 0.05 and 0.95 pg/(100 pg cell protein h), there was no correlation between Q_p and mRNA levels [6]. These data suggest that events downstream of transcription are the limiting factors. Thirdly, the cell line must be capable of achieving high viable cell concentration within an acceptable time. An additional criterion is that the cell line must produce antibody with the desired product quality characteristics, such as glycosylation.

2.1. Expression vectors

The expression vector systems most frequently used for the production of therapeutic monoclonal antibodies are the Glutamine Synthetase (GS) Gene

Expression System (Lonza Biologics; [5]) and those based on dihydrofolate reductase (DHFR) genes. A selection of expression vectors developed for the expression of immunoglobulin genes is shown in Table 1. To achieve high levels of gene expression, GS and DHFR vectors usually have strong promoters to drive expression of the antibody genes. The promoters are typically of viral origin (e.g., human cytomegalovirus) or they are derived from genes that are highly expressed in a mammalian cell [10,11]. Historically, the vectors have also included favourable RNA processing signals such as polyA tail, 5' and 3' untranslated region, presence of an intron to encourage export from the nucleus and a splice site to remove this intron. Coding sequences may also be optimised to remove, for example, cryptic splice sites or cryptic polyA tails, or sequences that lead to unfavourable folding of the mRNA. To increase mRNA processing and improve secretion, codon usage can be optimised for the target cell type, GC content increased and signal sequences used to target the heavy and light chain polypeptides to the correct part of the secretory pathway.

2.1.1. DHFR expression systems

DHFR expression systems use the folate analogue methotrexate (MTX) to inhibit the function of DHFR, an essential metabolic enzyme. Transfection with an expression vector containing a DHFR gene prevents MTX poisoning of transfected cells. The antibiotic resistance gene frequently used in DHFR expression vectors acts as the selectable marker: the primary function of the DHFR gene is then to facilitate vector amplification. The DHFR gene is usually under the control of a weak promoter, such as one from SV40. The use of a weak promoter to regulate DHFR

Table 1
Expression vector systems for use with expression of immunoglobulin genes

Expression vectors and selectable markers	References
GS vectors	www.lonza.com , [5]
DHFR intron vector/hygromycin	[7]
Neomycin	[8]
DHFR bicistronic vector	[9]
CHEF/DHFR	[10,11]
DHFR/neomycin	[12]

Where resistance to an antibiotic is used to select transfectants, the target antibiotic is shown.

gene expression should reduce promoter interference (due to read-through from the upstream promoter inhibiting expression from the downstream promoter) thus increasing expression of the immunoglobulin genes.

2.1.2. *Glutamine Synthetase (GS) Expression System*

GS synthesises glutamine from glutamate and ammonium. Since glutamine is an essential amino acid, transfection of cells that lack endogenous GS with the GS vector confers the ability to grow in glutamine-free media. GS expression vectors contain the GS gene downstream of a SV40 promoter, which offers similar benefits to those seen when a weak promoter is used to drive DHFR gene expression.

2.2. *Increasing specific production rate by improving transcription*

Transcription is probably not limiting antibody secretion in the current generation of cell lines. This is because they were constructed using expression vector systems developed to give high mRNA levels. Several options exist to increase transcription. In early expression systems, this was generally by gene amplification. Gene amplification is usually achieved by constructing the expression vector so that the genes of interest are linked to an amplifiable gene (e.g., thymidine kinase, adenosine deaminase, GS or DHFR). Transfected cells are then exposed to increasing levels of a specific enzyme inhibitor at concentrations substantially higher than those used for selection of transfectants. If the drug inhibits an enzyme (e.g., GS or DHFR) essential for the survival of the cell, only cells that overproduce this enzyme will survive. The overproduction of the enzyme commonly results from increased levels of its particular mRNA, resulting from either an increase in gene copy number (i.e., amplification), or from more efficient transcription [13]. Often more DNA (up to 1000 kb) than just the target gene is amplified. Therefore, when the transfected genes are amplified, other tightly linked sequences, including the immunoglobulin genes, on the vector are co-amplified. The high copy numbers of the expression vector seen upon amplification, especially with the DHFR expression system, may increase Q_p but it can also have a detrimental effect on other cellular properties. Ampli-

fication of the transgenes will frequently result in poor growth performance of the resulting cell population and may alter cellular metabolism. Amplification and the resulting variation in copy number can also alter the inherent stability of expression and often requires the continued presence of the selective agent. If the selective agent is required in the production bioreactor, it will be necessary to demonstrate that the purification process removes this compound from the bulk drug substance.

The GS system [5] and some variants of the DHFR one [12] do not rely upon amplification to achieve high productivities. Instead, these systems rely upon insertion of the antibody construct into a transcriptionally active region to achieve high productivities, selecting against insertion into the heterochromatin.

One approach is to use site specific recombination of the gene(s) of interest into a known transcriptionally active locus. Expression vectors can be constructed that contain a specific targeting sequence that will direct the vector to integrate by homologous recombination into a particular active site. Such a sequence has been identified in the immunoglobulin locus of the murine myeloma cell line NS0 [14]. Vectors containing this sequence are targeted to the immunoglobulin locus in more than 75% of high producing NS0 cell lines.

A corollary of this approach is to take the sequences flanking the transcriptionally active locus and incorporate them into the expression vector. Thus, the vector should create a favourable environment for expression independent of its integration site into the genome. Vectors incorporating ubiquitous chromatin opening elements [15], matrix attachment regions and anti-repressor sequences [16,17] or the flanking sequences of the Chinese hamster elongation factor-1 α gene [10,11] have been shown to increase and maintain transgene expression and are being actively evaluated with immunoglobulin genes.

An alternative approach is to transfect the cells with a conventional expression vector (i.e., randomly integrate the expression vector into the genome) but then bias the selection method so that only transfectants where the vector integrated into a transcriptionally active site are selected. This can be done by using a selection system that only allows transfectants producing sufficient levels of the selectable marker

gene product to proliferate. Expression systems using a selectable marker gene with either the weak SV40 promoter [5] or an impaired Kozak sequence upstream of the marker gene [12] are included in this class of selection system. Linkage of the antibody construct to the selectable marker gene results in the overproduction of antibody as both genes are integrated into a transcriptionally active locus. The choice of selection conditions is extremely important for the success of this approach [5].

3. Cell lines

The key issues affecting the choice of a cell line for use in a manufacturing process are: the capability to produce high antibody concentrations in the chosen production system, the ability to consistently produce a product of uniform characteristics, and the speed with which a high yielding cell line can be obtained. The availability of a suitable expression system and the importance of post-translational modifications of the recombinant antibodies may also affect this choice.

There are 18 therapeutic antibodies currently licensed for use of which 10 are manufactured in Chinese hamster ovary (CHO) cell lines and 8 are made in murine lymphoid cells (including NS0 and Sp2/0-Ag 14). These parental cell lines are also the ones most commonly used for antibodies currently in clinical trials. In addition, murine hybridomas and other cell lines such as the human cell line PER.C6 are used.

3.1. CHO

CHO cells are widely used to produce recombinant antibodies using both the DHFR and GS expression systems. The most commonly used CHO strains with DHFR expression vectors are DUKX-B11 and DG44, which both lack dhfr. The GS system uses the CHO-K1 strain, or a derivative of the CHO-K1, CHOK1SV. Although both CHO-K1 and CHOK1SV [18] express functional GS enzyme, inclusion of the GS inhibitor methionine sulphoximine (MSX) in the medium allows use of the GS expression vectors. Endogenous GS in CHO cells is inhibited by 3 μ M MSX, which is a cytotoxic concentration. By selecting GS-CHO transfectants in the presence of 50 μ M MSX, only

those cell lines that have stably incorporated the expression vector into a transcriptionally active locus will form transfectant colonies. These cells produce enough GS enzyme to titrate out the MSX whilst leaving sufficient functional enzyme to meet the cellular demand for glutamine. Recombinant CHO cell lines show efficient post-translational processing of complex proteins, while the glycosylation patterns of native and CHO-derived recombinant proteins are similar.

The preferred culture format for large-scale (substantially greater than 10 L) is single cell suspension, ideally using chemically defined, animal component-free (CDACF) media. Wild type CHO strains have adherent cell morphology and require serum supplementation for growth. Adaptation of recombinant CHO cell lines from adherent to suspension culture formats and adaptation to CDACF media can take up to 9 months, which is not compatible with short development timelines. The industry trend has been to pre-adapt the parental CHO cell line to suspension culture in CDACF media, reducing timelines by about 6 months [18,19].

3.2. Murine lymphoid cell lines

The host cell lines NS0 and Sp2/0-Ag14 are widely used for antibody production. Both cell lines were derived from a plasmacytoma cell line originating from a BALB/c mouse. The starting cell line underwent numerous rounds of cloning, and in the case of Sp2/0-Ag14, fusion with spleen cells from another BALB/c mouse, to generate these two parental cell lines [20,21]. Both the cell lines lack the ability to synthesise and secrete immunoglobulin proteins. The parental cell type of the two cell lines is a differentiated B cell, which is inherently capable of high levels of immunoglobulin production. These two characteristics favour their use for manufacturing antibodies. The genotype of NS0 cells makes them particularly suited for use with the GS expression system. Unlike other cell types, NS0 cells are obligate glutamine auxotrophs: glutamine independence can be conferred upon NS0 cells following transfection with a functional GS gene. In the case of Sp2/0-Ag14, mutants that no longer require glutamine occur spontaneously with relatively high frequency. This is not observed with NS0 cells [22].

3.3. Hybridomas

In addition to murine antibodies, it is now possible to make human antibodies using murine hybridoma technology. However, unlike the original hybridomas [23], the spleen cells are taken from a transgenic mouse which has the murine immunoglobulin locus replaced by the human genes.

3.4. Other cell lines

There are reports of other types of cell line being used to produce monoclonal antibodies including the hamster line BHK21 [24] and the human PER.C6 cell line [8]. The PER.C6 cell line is derived from human embryonic retinal cells by transfection with the adenovirus E1 region followed by selection for transfectants with an immortal phenotype. Analysis of the glycosylation profiles of IgG₁ antibody revealed no high mannose or hybrid structures; all were biantennary with core fucose. Galactosylation was similar to human serum IgG₁.

4. Screening of cell lines

The function of the expression vectors described in the previous sections is to generate cell lines with high Q_p values. Transfectants with high Q_p are rare events and this is the reason to use expression technologies that provide stringent selection and/or an increased frequency of high producers. However, a transfectant with a high Q_p does not necessarily result in a cell line that performs well in the production process. Hence, a sufficient number of cell lines need to be generated to allow for the attrition in numbers when screening for other desired characteristics.

The issue is, therefore, how can the hit rate for finding highly productive cell lines be increased? The simplest approach is to screen more transfectants, but how many? Simulations run by one of us (AJR, unpublished) suggest that several thousand should be screened, even after being enriched with a stringent selection system, to be confident of getting multiple transfectants with the desired productivity characteristics [5].

Conventional methods for the screening of cell lines are labour intensive, which limits the number of cell lines that can be screened. Increasingly robotics is being used to automate the liquid handling and cell transfer stages. This does not address the need to screen large numbers of transfectants to identify sufficient high producers to screen against the additional growth criteria that contribute to high productivity in a manufacturing process. The number can be reduced by using a fluorescence-activated cell sorting (FACS) technique to identify cells secreting high levels of antibody and sort them away from the lower producers. One such method, based on the capture of the secreted antibody by a capture matrix and its detection by a labelled probe, has been described by Holmes and Al-Rubeai [25]. The cells can be sorted into large populations (“bulk sorting”), from which cell lines can be isolated by conventional cloning methods, or by single cell sorting using FACS. Strictly, these approaches enrich for cells with a high Q_p since the secreted monoclonal antibody is captured close to the cell surface and secretion occurs over a short time period. Q_p is not the only phenotypic characteristic contributing to productivity and cell lines need to be screened for other characteristics. Typically, several criteria are used to select the production cell line including a high Q_p , growth characteristics such as the magnitude of the time integral of the viable cell concentration and maximum cell concentration, antibody concentration at harvest, cell line stability and product quality. An important feature of any screening scheme is that it incorporates a technique that is a predictive model of the manufacturing process. This screening step is often carried out in shake flask cultures, which may include the feeding techniques used in the final process.

5. Transient and other expression systems for production of development material

In order to produce quantities of material for process development studies, rapid expression technologies are frequently used that allow the generation of milligrams to grams of material in advance of a stable manufacturing cell line becoming available. These methods include the use of uncloned transfectant pools and the application of transient expres-

sion technology. Large-scale (up to 100 L) transient expression systems are being developed to meet this demand [26].

6. Cell engineering to increase productivity or modify product characteristics

High monoclonal antibody concentrations are the result of high Q_p values and space–time yield of viable cells. As discussed above, Q_p is probably limited by events downstream of transcription, only some of which can be addressed by vector engineering. An alternative approach is to modify the translational or secretory pathways where antibody production is considered limited at folding and assembly reactions. Dinnis and James [27] recently reviewed ways of increasing Q_p through cell line engineering. These authors proposed that, since foldases and chaperones exist as large multi-protein complexes, global expansion of all components of the secretory pathway is required for generic improvement of antibody secretion rather than over-expression of selected proteins. This would be similar to the events occurring during the differentiation of B cells into plasma cells, where the unfolded protein response (UPR), an important intracellular signalling pathway, is induced. Protein expression in differentiating B cells is coordinated by components of the UPR to achieve maximum antibody production. It has been proposed that there may be benefit in over-expression of proteins known or suspected of having a key role in modulating signalling pathways, e.g., BLIMP-1, or initiation of ER expansion (XBP-1, ATF6) [27]. An alternative approach to over-expression of specific genes is to use randomised zinc finger protein-transcription factor (ZFP-TF) libraries [28]. Theoretically, the ZFP-TF libraries can modulate the expression of any gene, so that a specific phenotype can potentially be created without a detailed knowledge of the molecular basis of the phenotype.

High space–time yields of viable biomass are achieved by using a cell line capable of growing to a very high viable cell concentration and then maintaining it for extended periods. The maintenance of high viability for such cultures requires minimisation of the death rate. The major cause of cell death in animal cell cultures is by apoptosis pathways. Since apoptosis can be induced by various chronic insults and is mediated

by a number of pathways, numerous strategies have been developed to limit cell death [5,29]. Nutrient limitation can induce apoptosis, so one strategy for limiting apoptosis is to prevent nutrient limitation. Although operating the culture in fed-batch mode can delay the onset and reduce the extent of apoptosis, the cells will still eventually die by apoptosis. Alternatively, resistance to apoptosis can be engineered into the cell lines. As activation of the apoptotic pathways is lethal to the cell, the pathways must be tightly regulated. The best understood regulatory mechanism involves the Bcl-2 family of proteins. The anti-apoptotic properties of Bcl-2 family members have been used to protect industrially important cell lines from insults typically experienced during cell culture operations. However, the results are contradictory with respect to productivity and there are few reports describing the behaviour of cell lines engineered to have increased apoptosis resistance in modern antibody manufacturing processes.

In addition to changing characteristics related to productivity, there are also examples where it has been advantageous to alter the cell's ability to carry out particular post-translational steps such as glycosylation. This has been driven by increasing awareness of the role of glycosylation in effector functions such as antibody-dependent cellular cytotoxicity (ADCC). ADCC is believed to play a role in the function of some therapeutic monoclonal antibodies, with various studies showing that oligosaccharide engineering may optimise ADCC. The degree of galactosylation and fucosylation and the proportion of bisecting GlcNAc residues have all been implicated in modulating effector functions. Oligosaccharide engineering has thus become an important research area for increasing antibody potency. Yamane-Ohnuki et al. [30] created a FUT8 double knockout of the CHO DG44 host that lacks α -1, 6-fucosyltransferase activity and cannot synthesise fucosylated antibodies. The ADCC of the resulting antibody was increased 100-fold compared to the fucosylated form. In a different approach, over-expression of *N*-acetylglucosaminyltransferase III in CHO increased the proportion of bisecting GlcNAc residues, increasing the ADCC substantially compared to the parental molecule [31,32].

Increasingly, our ability to isolate useful variant cells or to engineer them will come from a better understanding of the biology which defines the

desired phenotype. The “omics” tools which have become available in recent years will play a major part in providing this knowledge base and we are already seeing examples of the power of these methods. Recently, Smales et al. [6] compared the proteomes of GS-NS0 cell lines with varying monoclonal antibody production rates and were able to demonstrate changes in abundance of several proteins associated with changes in productivity.

7. Reactor systems used for large-scale antibody production

A consequence of the rapidly growing demand for monoclonal antibodies has been a dramatic increase in capacity in the industry [1] and an increase in the scale of reactors used for production. Two types of culture system are used for large-scale manufacture, fed-batch and continuous perfusion culture [33]. The principles of these types of reactor are shown in Figs. 1 and 2. Fed-batch processes are by far the most common and are now operated at scales up to 20000 L working volume. Several authors [33,34] have reviewed the types of cell culture reactor systems and processes in industrial use.

In fed-batch culture, small volumes (in our case less than 10% of the reactor volume) of key nutrients are fed to the culture during the fermentation process to

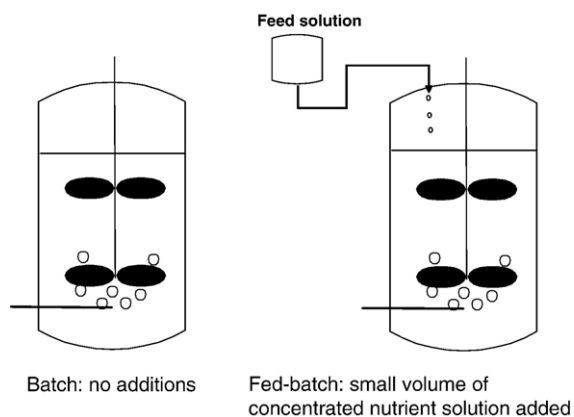


Fig. 1. Schematic representation of batch and fed-batch culture systems. The fed-batch system is supplied with a concentrated nutrient solution: no spent culture medium is removed. In a batch system, no additions of nutrient solutions are made.

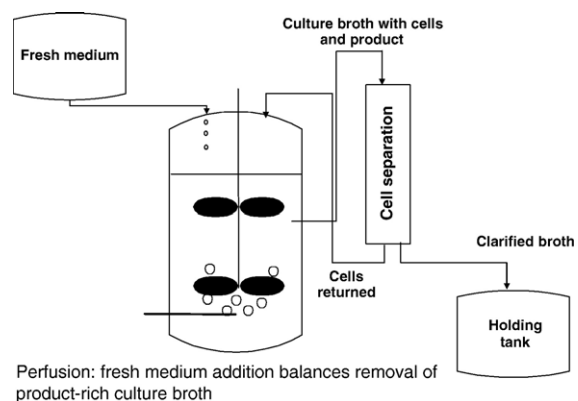


Fig. 2. Schematic representation of a perfusion culture system. Fresh nutrient solution is supplied to the vessel at the same rate that it is withdrawn. Before the spent culture is removed, however, the biomass is removed and returned to the culture.

maintain nutrient sufficiency, and the culture is harvested at the end of the batch cycle. Optimisation of feeding strategies has been a major factor contributing to improvements in growth and productivity in recent years.

In perfusion culture systems fresh medium is added continuously to the reactor and spent medium, containing product, is continuously removed. In these systems, cells are retained in the reactor and a variety of retention devices have been described which may be internal or external to the reactor [35]. An example of a 500 L industrial perfusion system for monoclonal antibody production is described by Deo et al. [36]. They were able to operate this type of system for 15 to 35 days for the production of multikilogram quantities of antibody. The authors claim that the system has a throughput of antibody, which is approximately 10 times higher than can be achieved in a batch or fed-batch system. A disadvantage of the perfusion system is the additional time and complexity involved in developing the process.

At small scale, increasing use is being made of disposable bag systems including reactors and holding tanks, particularly to meet early development needs. One such reactor system which uses wave induced agitation and is commercially available is described by Singh [37]. The use of disposables reduces the need for cleaning and sterilisation with significant economic benefits [38]. Such systems are also finding application in, for example, the inoculum stages of large-scale culture.

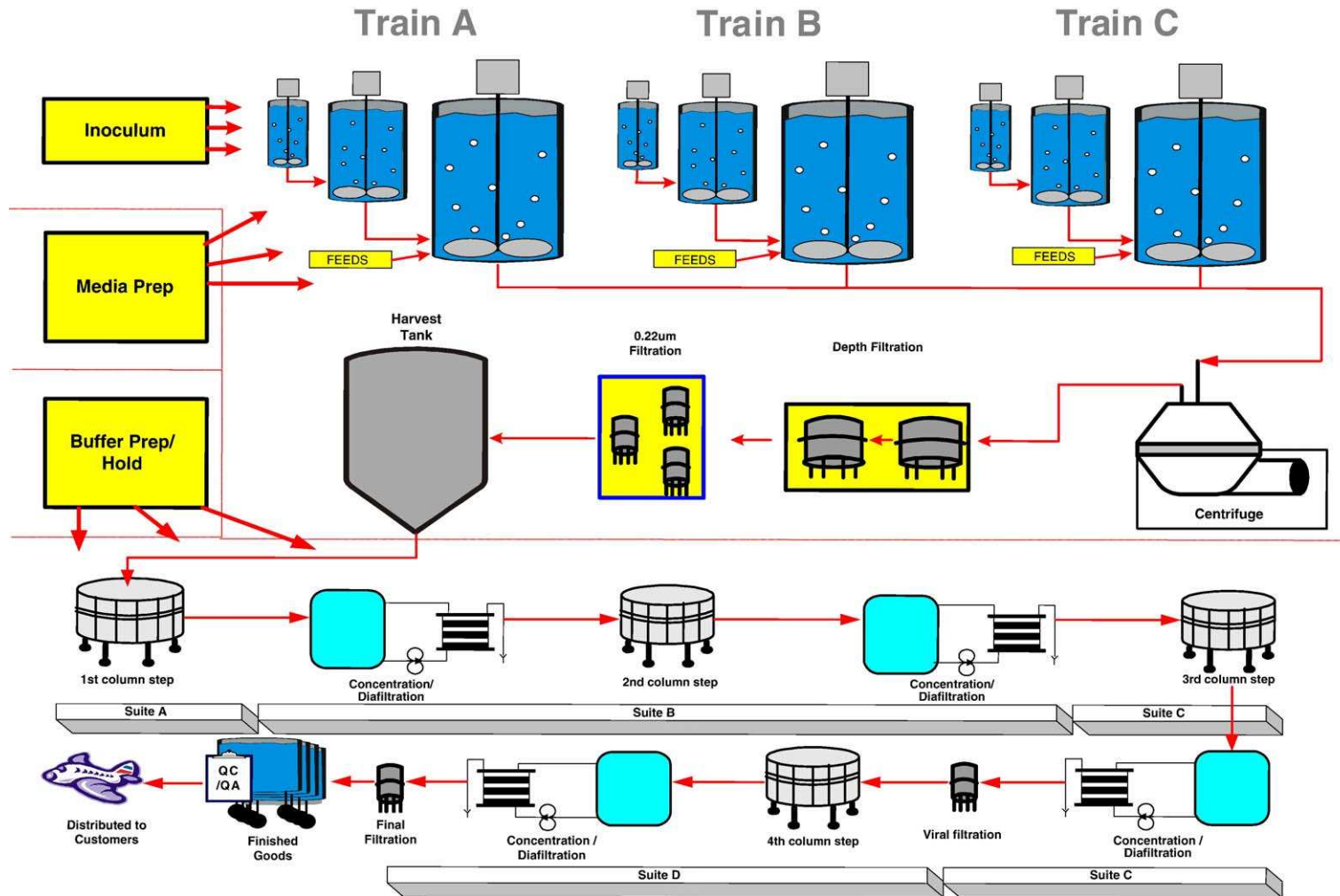


Fig. 3. Process flow for a large-scale facility for manufacturing proteins (reproduced courtesy of Lonza Biologics Inc.).

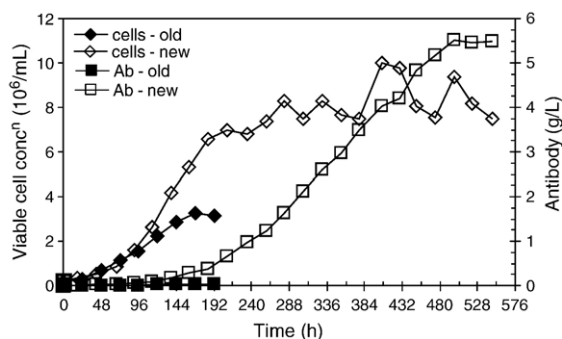


Fig. 4. Growth and productivity of two GS-CHO cell lines making the same chimeric antibody: comparison of a process from 1990 ('old') and from 2005 ('new').

7.1. Steps in a fed-batch process

Cell culture is typically done in stirred stainless steel tanks. Airlift reactors up to 5000 L scale have also been used but are much less common [39]. Detailed aspects of the issues taken into account in reactor design are summarised by Varley and Birch [40]. It is normal to control pH, dissolved oxygen concentration and temperature in reactors and careful consideration has to be given to the design of vessels to ensure adequate mixing and mass transfer of oxygen and CO₂. By way of example, addition of pH control agents to the surface of the culture can lead to high local departures from the setpoint pH value [41]. This can be avoided by adding the agent in a well mixed zone of the reactor. It is important to have laboratory and pilot reactors that can be used to predict large-scale reactor performance. Dissolved oxygen concentration is regulated by the controlled sparging of air into the fermenter and pH is controlled by the addition of CO₂ gas or an alkaline solution (e.g., NaOH) as relevant.

Table 2

The evolution of a fed-batch process for antibody producing GS-CHO cell lines showing the improvement in productivity

Process	Antibody (mg/L)	Antibody (fold increase)	Volumetric productivity (mg/L/day)
Original cell line (CHO-K1), original process	139	–	13
Iteration 1	334	2	33
Iteration 2	585	4	42
New cell line (CHOK1SV), iteration 2	1917	14	128
Iteration 3	2829	20	189
Iteration 4	3560	26	237
Iteration 5	4301	31	215
New clone, iteration 5	5520	40	240

Fig. 3 shows the steps in a production process based on Lonza's facility in New Hampshire, USA which has three 20,000 L reactors. Cells from a frozen working cell bank are expanded through small reactors into inoculum vessels in the production train. The 20,000 L vessels are operated in fed-batch mode and, at the end of the batch cycle, the contents of the reactor are clarified through a centrifuge and through filters prior to purification in a series of chromatography steps.

7.2. Culture media

There has been rapid progress in recent years in the development of serum-free media, driven principally by concerns about the possible introduction of adventitious agents in animal derived ingredients. Initially, serum was replaced by serum proteins such as albumin, transferrin and insulin, and latterly it has been possible, in many cases, to develop chemically defined, protein-free media which contain no animal derived materials. Frequently, the serum protein was acting as a carrier of an essential nutrient such as a lipid, which could be used to substitute for the protein. Defined media are inherently less expensive and make downstream processing more straightforward as there are fewer contaminants to monitor and remove. Process optimisation is also easier in a defined environment. Protein-free culture media for the commonly used industrial cell lines such as CHO and NS0 are commercially available and there are descriptions of such media in the literature (e.g., [33,42,43]). For suspension culture, it is common to add a synthetic polymer to the culture medium to act as a protectant against the damage to cells that would otherwise be caused by sparging gases into the reactor [44]. Antifoam is used to prevent foaming caused by sparging.

7.3. Optimisation of culture conditions

Improvements in recombinant protein production in cell culture have been dramatic. In a review of progress in this area, Wurm [3] noted that concentrations have increased 100-fold since 1980. Some of this progress has resulted from improvements in expression technology and strain selection as noted earlier, but an equally significant contribution has come from optimisation of the fermentation processes. In Fig. 4, we show current growth and productivity profiles for a GS-CHO cell line making a chimeric antibody compared with data for a process we were using in 1990. Productivity is over 100-fold higher in the current process. It can be seen that there has been a dramatic increase in cell concentration in the reactor and the cells can be maintained in a viable state for a much longer period. This is crucial because productivity is a function of the specific production rate of the cells and the integral of the viable cell count with respect to time (IVC). Cell concentrations exceeding 10^7 /mL are now commonly seen in reactors.

Much of this improvement has come from the optimisation of media and feeds based on a better

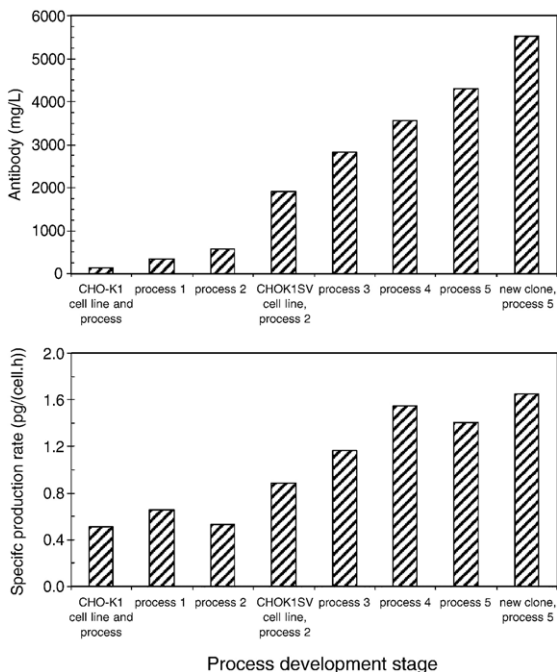


Fig. 5. Improvement in Q_p and antibody concentration during the development of a fed-batch process for GS-CHO cell lines.

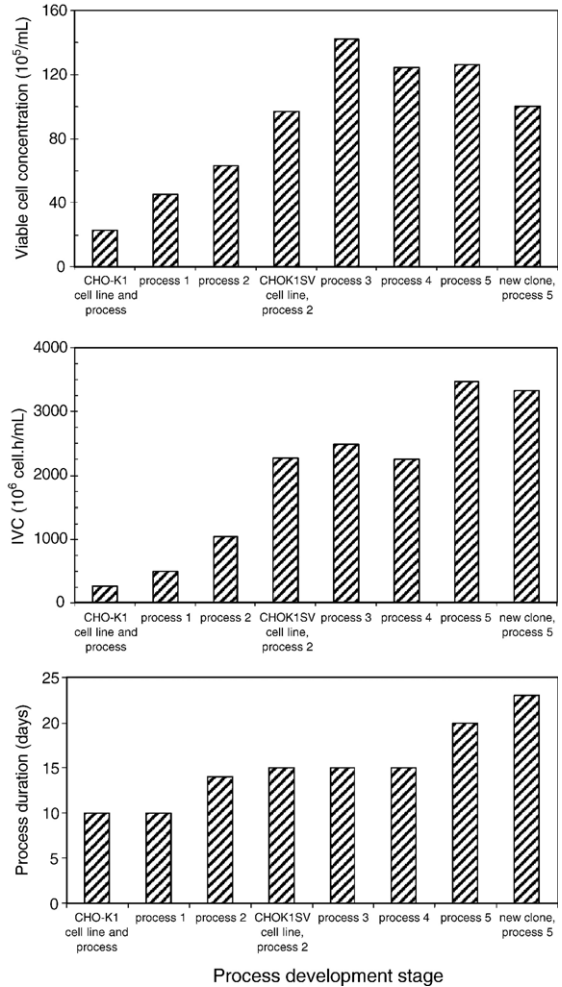


Fig. 6. Improvement in growth kinetics for an antibody-producing GS-CHO process using a fed-batch culture system.

quantitative understanding of cells' nutritional requirements. This understanding is often based on iterative analysis of nutrient depletion followed by supplementation of the relevant nutrient in the medium or feed. Strategies for medium and feed development have been reviewed [5,45]. The overall aim of the fed-batch approach is to increase maximum cell concentrations in the reactor and to prolong the production phase. Dempsey et al. [46] and Sauer et al. [47] give examples of NS0 and Sp2/0 processes in which they improved productivity by approximately an order of magnitude.

Another factor to be taken into consideration in designing media and feeds is the potentially toxic effect

of accumulated catabolites such as ammonium and lactate. Various strategies have been adopted to minimise accumulation of these growth inhibitors. It is possible to use stoichiometric feeding of key energy substrates (glutamine and glucose) to reduce accumulation of catabolites [47,48,49]. In the case of ammonium, the problem can be substantially reduced by eliminating the main source (glutamine) from the culture medium. This is one of the advantages of using cell lines, which have glutamine synthetase and do not require glutamine. It has also been found that CO₂ concentrations can reach inhibitory levels in cell culture (e.g., [50]). It is important to ensure that gas exchange systems in reactors can remove CO₂ adequately. It has also been found that osmolality can reach inhibitory levels for example because of addition of feeds and pH control agents. The situation is made more complex by the fact that many parameters interact. For example elevated osmolality can lead to an increase in rates of production of lactate and ammonium [50].

Table 2 shows the effect of iterative process improvement for a GS-CHO process making a chimeric IgG₄ antibody. A 40-fold improvement in productivity was achieved in part as a result of optimising the culture process and in part due to the introduction of a more productive cell line based on a suspension variant of CHO.

Figs. 5 and 6 show the effect of optimisation on various aspects of growth and productivity. It can be seen that improvements were made to antibody concentration, Q_p , maximum viable cell concentration and IVC. The improvements were achieved without a dramatic increase in process duration. The overall objective is to increase throughput in the plant and hence attention is focused on those parameters that increase yield without increasing culture duration, e.g., Q_p and maximum cell population density.

7.4. Impact of process improvements on product

Any process improvement programme must take account of possible product changes, such as aggregation, altered glycosylation or degradation resulting from modifications to the process. There are several examples in the literature of such changes. Patel et al. [51] for example showed that the glycosylation pattern of an IgG made from a murine hybridoma varied depending on the culture system which was

used. Muthing et al. [52] discuss the effect of pH on glycosylation of a murine antibody. Galactosylation of antibody glycans varied with pH.

8. Alternative production systems

All licensed antibodies and the majority of those in clinical development are currently made in mammalian cell culture. There is however continuing interest in alternative processes that might provide less complex and less expensive alternatives in the longer term. Good progress has been made in the production of both whole antibodies and antibody fragments in bacteria and in fungi. Humphreys [53] and Bowering [54] describe methods for producing antibody fragments in *E. coli* including procedures for subsequent pegylation of the fragments to decrease rate of clearance in vivo. Antibody fragments are more readily produced in microbes than are whole antibodies. As stated above, Reichert et al. [2] note that of the 15 antibodies they identified in Phase III trial, three were single chain fragments and three were Fabs. The production of whole antibodies, albeit non-glycosylated, in *E. coli* has been described by Simmons et al. [55]. Antibody concentrations exceeding 100 mg/L were reported in a process in which the antibody was secreted into the periplasm of the bacterium. This approach may be useful for indications where glycosylation is not needed for biological activity. Production of antibodies has also been studied in yeasts and in filamentous fungi such as *Aspergillus* [56]. There is already experience in using these organisms for protein production, including very large-scale manufacture of industrial enzymes in the case of *Aspergillus*. Although fungi can glycosylate proteins, the resultant glycans are not the structures one would normally find on human proteins. However, good progress is being made in the resolution of this problem and Choi et al. [57] have described the use of a genetic engineering approach to humanize the glycosylation of the yeast *Pichia pastoris*. Apart from microbial systems considerable effort has gone into the evaluation of transgenic production systems particularly those based on plants [58] and transgenic milk from animals [59]. Most recently, eggs have been discussed as a possible production route. Given the rapid

progress made in cell culture it remains to be seen what impact the transgenic route will make, particularly given the fact that downstream costs make up a significant proportion of total manufacturing costs and are likely to be similar regardless of the production method. Chadd and Chamow [60] discuss the economics of different production routes.

9. Downstream processing of antibodies

Large-scale purification of monoclonal antibodies is based on chromatography. Protein A affinity purification is used in the majority of cases in combination with at least one ion exchange step. It is common for there to be at least one and sometimes more polishing steps using ion exchange, hydrophobic interaction and/or size exclusion chromatography. The steps are designed to remove contaminant proteins from cells or media to ppm levels and DNA to ppb levels. Depending on the process there may be additional specific contaminants (e.g., leached protein A) to be removed. In addition to contaminants, it may also be necessary to remove undesirable derivatives of the product itself such as degradation products and aggregates. For mammalian cell processes, one also has to take account of potential virus risks and establish the ability of the purification steps to remove a range of virus types. In addition, at least two virus removal/inactivation steps are included, typically based on filtration, low pH treatment and, sometimes, use of solvent/detergent. Typical yields from a purification process for antibodies are in the range 60–80% depending on the number of steps. For a general discussion of purification process steps and their relevance to removal of different contaminants, see Birch et al. [39] and Berthold and Walter [61].

With increasing upstream concentrations increasing attention is being paid to downstream recovery because this becomes a significant proportion of total cost and it can also limit overall plant throughput. If we consider just the issue of buffers for chromatography, the volumes used can be an order of magnitude higher than those upstream. Hence, we are seeing improvements to reduce volumetric handling as well as, for example, the introduction of improved chromatography matrices, which allow increased throughput and the use of rapid membrane separation steps.

10. Conclusions

The demand for monoclonal antibodies seems set to increase for the foreseeable future. Pavlou and Belsey [62] project that the market will grow by 20.9% per year to reach \$16.7 bn in 2008. This is likely to be matched by increased global capacity for mammalian cell culture on the one hand and by technological progress on the other. It is likely that mammalian cell culture will be dominant for the immediate future and that improvements will continue to be made in process efficiency. This will result from improvements to the inherent productivity of cell lines which in the future will be driven to a greater extent by a better understanding of the fundamental biology of the cell, informed by the systems biology that is evolving from the various ‘omics’ approaches. It is generally believed that such improvements combined with further progress in media and feed development will lead to antibody concentrations of at least 10 g/L. As concentrations increase, downstream processing will become a much more significant component of cost and this will be a driver to develop more efficient processes, potentially using radically different approaches to those used now. It also seems likely that other expression technologies, especially those based on bacteria and/or fungi will become more dominant not just for fragments but potentially for whole antibodies as well. A final factor that will impact on process volumes and cost of goods is product potency. Some of the recent progress in improving effector functions by glycosylation engineering demonstrates the potential in this area.

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