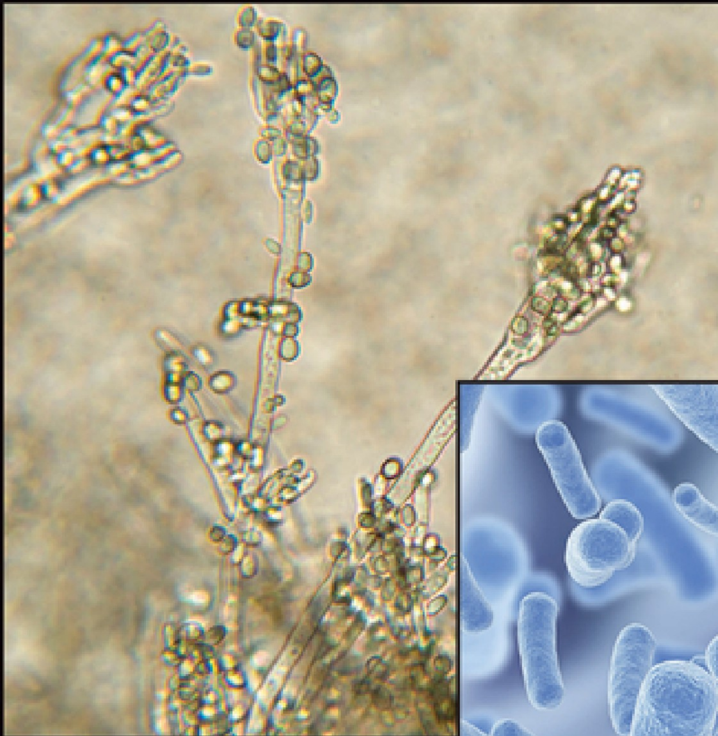

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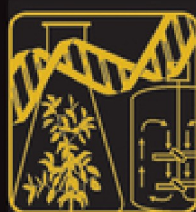

UPSTREAM INDUSTRIAL BIOTECHNOLOGY

Expression Systems and Process Development

MICHAEL C. FLICKINGER, EDITOR

UPSTREAM INDUSTRIAL BIOTECHNOLOGY

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Edited By

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PREFACE

Upstream Industrial Biotechnology is a compilation of essential in depth articles, organized topically and listed in alphabetical format, for biopharmaceutical, bioprocess and biologics process scientists, engineers and regulatory professionals from the comprehensive seven volumes of the *Encyclopedia of Industrial Biotechnology*. Process development for the manufacture of complex biomolecules involves solving many scientific, compliance and technical problems quickly in order to support pilot, preclinical and clinical development, technology transfer and manufacturing start-up. Every organization develops new processes from accumulated process knowledge. Accumulated process knowledge has a very significant impact on accelerating the time to market (and reducing the financial resources required) of products manufactured using recombinant DNA and living microbes, cells, transgenic plants or transgenic mammals. However, when an entirely new upstream platform is needed, there are few books that will quickly provide the depth of industry-relevant background. *Upstream Industrial Biotechnology* can fill this void as a 2 volume advanced desk reference. These volumes include relevant biology, protein purification and engineering literature with abundant process examples provide by industry subject matter experts (SMEs) and academic scholars. This desk reference will also be useful for advanced biomanufacturing students and professionals to quickly gain in depth knowledge on how to design processes (and facilities) capable of being licensed to manufacture enzymes, biopharmaceutical intermediates, human and veterinary biopharmaceuticals or vaccines. The opportunity is yours to leverage the combined knowledge from scores of industry professionals from around the world who have contributed to *Upstream Industrial Biotechnology* to reduce the time and cost to deliver engineered proteins, biomolecules and cost-effective biologics to the market and especially to millions of patients worldwide.

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Volume 1

Expression Systems & Process Development

PART I

INTRODUCTION

INTRODUCTION

Volume 1: *Expression systems & Process Development*
Volume 2: *Equipment, Process Design, Sensing, Control and cGMP Operations*

Upstream biomanufacturing processes are designated on the basis of volume or surface area (liters, gallons, cubic meters, square meters etc.). As the process is developed and optimized, it is scaled up in volume or area and optimized for biocatalyst specific reactivity to match the market demand for the product. Therefore, close integration of the characteristics of the biological system that produces the product with the engineering and optimal performance of the manufacturing scale bioreactors is essential. This means that engineers, life scientists, and manufacturing operations staff with broad expertise all need to work and communicate effectively as a team to design an upstream process that can be scaled from the laboratory bench and transferred to the manufacturing scale.

Designing the upstream process requires focusing on the biochemistry of the final product (peptide, protein, hormone, and low-molecular-weight metabolic intermediate) *and working backwards* to design living cells or enzymes that will generate the product in the precise form for optimal biological activity or clinical efficacy. Many peptides and proteins are post-translationally modified or are synthesized on a multienzyme complex and the biological catalysts that carry out these modifications differ from cell line to cell line. This results in different degrees of modification or mixtures of partially modified products depending on the choice of cell line used and the growth conditions. The enzymes in some cell lines or microorganisms also inactivate or degrade the product as it is being produced. Fortunately, the range of these modifications can now be minimized or precisely altered by strategic host cell line engineering. These same concepts also apply when the product is more complex such as in the manufacture of biologics (cells, virus particles, virus-like particles, and complex antigens) used as vaccines or artificial tissues. In some cases the products are isolated from living tissues (eggs, blood, whole organs, milk, and fluids from individual patients) and this isolation step is considered as a component of the upstream manufacturing process. Fortunately, the genes encoding many complex biologics can now be cloned from their tissue of origin and expressed in microorganisms, fungi, mammalian or insect cell lines thereby diminishing or eliminating the need for direct tissue isolation. Regardless of their biological source, the precise biochemical characteristics of all of these products must be carefully defined at the beginning of the process design and these characteristics are often expressed as target product profiles (TPPs).

The first section of Volume I of *Upstream* provides indepth information on industrial cell gene expression systems and methods to quantify cell growth in order to design processes that are highly reproducible. Choice of the gene expression platform (host cell line, vector, promoters, the site of protein accumulation, and optimal expression conditions) has a major impact on the overall process design and final product yield. This is often determined by in-house gene expression expertise, existing process equipment as well as intellectual property restraints (composition of matter or process patents, licensing agreements, and freedom to operate).

How cells grow and the extent to which they grow are affected by media composition, growth conditions, and upstream process design (batch, fed-batch, continuous, cell recycle, immobilized biocatalysts, illumination, and heat transfer), which are included in Section III. For some cells

that require attachment to a surface, the chemistry of the surface and the available surface area are critical to optimizing growth. Upstream process design and development, even at the laboratory bench scale, must also consider eventual scale up to manufacturing scale. Scale-up approaches are included in Volume 1 Section III, and in Volume 2 Section IV.

Volume 2 includes important engineering information on the materials and design of specific types of bioreactors that have been found by the industry to be optimal for the growth of specific types of microorganisms and cells. Also included are reactors engineered for immobilized biocatalysts (whole cells, enzymes, and photo reactive cells). Each type of bioreactor must be designed to grow cells at optimal rates to a desired concentration and, therefore, methods for calibrating bioreactors for oxygen transfer, cell illumination, mixing, shear, foam formation, design of aseptic sampling systems, culture fluid rheology and effective sterilization/decontamination are included in Sections IV and VI.

As stated above, the eventual goal of bioreactor design is to scale up to the total volume needed on the manufacturing scale to meet market demand. However, there are critical regulatory considerations that need to be included in upstream process design and process operations for products manufactured under current Good Manufacturing Practice (cGMP) mandated by USFDA federal regulations (CFRs) and guidelines. These include monitoring of the process to obtain detailed process knowledge used to determine multivariant design space for optimal performance of each unit operation. Methods are included for these Process Analytical Technologies (PAT) as well as upstream cGMP operations in Volume 2, Sections V and VI. While these cGMP regulations may vary from country to country, a significant international harmonization effort has resulted in common global guidance documents (ICH, International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use) referred to in these sections.

Optimal biopharmaceutical product yield is *the mathematical product* of the number of cells used to generate the product, the amount of product produced per cell, *multiplied* (most importantly) by the yield of recovered product in the appropriate final biologically active form and purity. Each upstream process decision impacts downstream product recovery and purification. Therefore, the companion volume *Downstream Industrial Biotechnology* should also be consulted when designing an upstream process.

PART II

INDUSTRIAL CELL GROWTH AND GENE EXPRESSION SYSTEMS

1

ANIMAL CELLS, SUSPENSION CULTURE

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1.1 INTRODUCTION

Mammalian cells can be distinguished by their requirement to grow when attached to a surface (anchorage dependence) or in free suspension. The ability to grow in suspension is frequently associated with cell lines that demonstrate an "immortal" or infinite lifespan phenotype. Suspension culture systems are preferred for most large-scale manufacturing processes because scale-up is more straightforward. Relatively homogeneous conditions can be achieved in a suspension bioreactor, allowing efficient monitoring and control of key process parameters.

Suspension culture technology for animal cell culture started in the 1950s, with the demonstration that several types of cells could be grown in simple agitated systems such as tumbling tubes and shaken flasks (1,2). By the end of the decade, methods had been developed for growing cells in magnetically stirred spinner vessels (3,4) and in bioreactors similar to those used for microorganisms (5,6). By the 1960s, pilot plant reactors at scales of hundreds of liters were in operation (7).

The initial drive to develop an industrial process based on mammalian cell suspension culture came from the need to produce very large volumes of vaccines against foot-and-mouth disease (FMD) virus. Processes were developed using baby hamster kidney (BHK) cells growing in stirred-tank reactors up to 3000 L (8–10). Subsequently, stirred-tank reactors of up to 8000 L were used for the production of interferon α from human Namalwa cells (9). The industrial application of animal cell culture has increased significantly over the last 20 years, driven by the need to produce monoclonal antibodies and recombinant proteins in addition to vaccines. It is the demonstration that these products can be made safely in immortal cell lines that has made possible their large-scale manufacture. In the case of monoclonal antibodies, requirements can be as high as hundreds of kilograms or tons per year. To meet this demand, bioreactors with working volumes up to 20,000 L are now used (11) and global reactor capacity is expected to increase from ∼2.3 million L in 2004 to ∼3.7 million L in 2011 (12).

1.2 TYPES USED FOR LARGE-SCALE PRODUCTION IN SUSPENSION CULTURE

Cell types used for large-scale production in suspension culture and those that are most commonly used industrially are described below.

1.2.1 Cell Types Used for Protein Production

1.2.1.1 CHO. The CHO line is the most commonly used cell type for recombinant protein production. Approximately 70% of all licensed biotherapeutic proteins are produced in this cell line (13). CHO cells have been used to produce a wide range of therapeutic proteins (hormones, growth factors, thrombolytics, blood clotting factors, and immunoglobulins). The choice of the CHO cell is based on several factors: compatibility with efficient gene expression systems leading to good productivity, ability to carry out important posttranslational modifications of proteins, and freedom from detectable pathogenic agents. In addition, the cell type can be grown in large-scale suspension

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bioreactors. CHO cells can also grow as attached cultures, and in fact, growth in suspension traditionally required a period of adaptation after the production cell line had been created. This requirement for adaptation, which can take several weeks or months, can be circumvented by using host cells for gene transfection that have been preadapted to grow in suspension (14). Kurano *et al*. (15) isolated several anchorage-independent sublines of CHO, one of which grew in suspension even in static flasks. A CHO variant, CHOK1SV, has been described, which grows spontaneously in suspension in chemically defined medium (11). A different approach was taken by Renner *et al*. (16), who demonstrated that expression of recombinant cyclin E (a cell cycle regulator) in CHO cells prevented surface attachment and additionally permitted growth in protein-free medium.

1.2.1.2 Hybridomas and Myeloma Cell Lines. Rodent monoclonal antibodies are typically produced in hybridoma cells, which can be readily grown in suspension culture (see, e.g. Ref. 17). In general, rodent antibodies are used in diagnostic and research applications and are required only in modest quantities. For the vast majority of therapeutic applications, antibodies are now genetically engineered and produced in CHO cells or in mouse lymphoid cell types (particularly NS0 and SP2/0), which, like CHO, can be grown in large-scale suspension culture (13).

1.2.1.3 Other. BHK cells are used for the production of recombinant blood clotting factors VIIa and VIII (18,19). Immortalized human cell lines such as PER.C6® are also being developed for the production of recombinant proteins (20).

1.2.2 Cell Types Used for Vaccine Production

Many vaccines are produced in anchorage-dependent cell systems, but suspension culture is also used, particularly where the scale of production is large. BHK cells have been used for large-scale production of FMD vaccine because of their ability to propagate the virus and their capacity to grow in large-scale suspension culture (10). Rabies vaccine for veterinary use is also manufactured in BHK cells (21).The availability of cell lines that can be grown in large-scale suspension culture is leading to a shift in technology in some key areas of human vaccine manufacture. In the case of influenza vaccine in particular, the new cell culture processes may be an attractive alternative to the traditional egg-based processes (22,23). Examples of cell lines that have been developed for the production of large-scale human vaccine include the PER.C6 cell line derived from human retinal cells by immortalization with adenovirus E1 genes (20), the canine cell line MDCK (23), and EBx™ diploid cell lines derived from avian embryonic stem cells (24). There are also examples of suspension cultures of insect cells being used in the development of virus vaccines (25).

1.2.3 Cell Lines for Transient Production of Proteins

Transient expression technologies are frequently used for the rapid production of research quantities (milligrams to grams) of protein. The human HEK293 cell line has been very widely used for this purpose and suspension culture processes up to 100-L scale have been described (26). A process at similar scale for transiently transfected CHO cells has been described (27). Insect cells are also used for the rapid production of research materials using baculovirus expression technology (e.g. see Ref. 28).

1.3 SUSPENSION CULTURE REACTORS

Bioreactors up to 20,000-L scale are now used in the manufacture of recombinant therapeutic products from mammalian cells (11); the most commonly used systems being based on stirred tanks. Airlift reactors are also used, but much less frequently. The principles underpinning the design and scale-up of animal cell bioreactors have been reviewed by several authors (29–31). In addition to stainless steel systems, a variety of simpler technologies are used at smaller scales (<100 L) including spinner flasks and, more recently, disposable bag reactors.

1.3.1 Stirred Reactors

The majority of products made using large-scale mammalian cell culture are produced in stirred-tank reactors (see Ref. 32 for description of reactors used for a range of licensed products). Stirred bioreactors up to 20,000-L scale are now in use particularly for the production of monoclonal antibodies, which can be required in very large quantities. Birch and Racher (11) provide details of the process flow for 20,000-L scale reactors. Typically, reactors are stainless steel with height-to-diameter ratios in the range 1:1–3:1. In some cases, mammalian cell bioreactors have been developed by retrofitting microbial vessels, usually by changing the agitators and aeration system. Backer *et al*. (17) described the retrofitting of 150- and 1300-L reactors. Rushton impellers were replaced with marine propellers, and the agitator drives were altered to allow operation between 25 and 250 rpm. Air was sparged through a sintered stainless steel sparger with 10-μm pore size. Garnier *et al*. (28) describe the retrofitting of a 150-L microbial bioreactor (height-to-diameter ratio 3). They used two large pitched-blade impellers (45°), three surface baffles, and a polypropylene porous sparger (80-μm pore size). Agitation was controlled at 60–120 rpm. Nienow *et al*. (33) found