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Industrial Production of Therapeutic Proteins: Cell Lines, Cell Culture, and Purification

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The biotechnology and pharmaceutical industries have seen a recent surge in the development of biological drug products manufactured from engineered mammalian cell lines. Since the hugely successful launch of human tissue plasminogen activator in 1987 and erythropoietin in 1988, the biopharmaceutical market has grown immensely. Global sales in 2003 exceeded US $30 billion.[1] Currently, a total of 108 biotherapeutics are approved and available to patients (Table 32.1). In addition, 324 medically related, biotechnology-derived medicines for nearly 150 diseases are in clinical trials or under review by the U.S. Food and Drug Administration.[2] These biopharmaceutical candidates promise to bring more and better treatments to patients. Compared to small molecule drugs, biotherapeutics show exquisite specificity with fewer off-target interactions and improved safety profiles. Protein engineering technologies have advanced to create protein drugs with improved efficacy, specificity, stability, pharmacokinetics, and solubility. Strategies that have been employed to implement these changes include mutagenesis, recombination, and other directed evolution methods, as well as rational design and structure-based computational approaches.[3–7] These advanced protein engineering technologies are creating novel drug designs and clever treatment strategies that are fuelling the biopharmaceutical market growth.

Currently, 60 to 70 percent of all biotherapeutics based on recombinant proteins are produced in cultivated mammalian cells.[8] Mammalian systems are often preferred over other hosts, such as bacteria, plants, and yeast, because of their capability for proper protein folding, assembly, and posttranslational modification. The quality and efficacy of a protein can thus be enhanced when expressed in mammalian cells. With the recent expansion in the biotherapeutics market, the demand for proteins derived from mammalian cells

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TABLE 32.1 Approved Biotechnology Products

Trade Name	*Proper Name*	*Manufacturer*	*Approval Date*	*Application*	*Cell Type*
Abbokinase	Urokinase	Abbott Laboratories	10/2002	Lysis of acute massive pulmonary emboli	Human neonatal kidney
Actimmune	Interferon gamma-1b	InterMune Pharmaceuticals	12/1990	Chronic granlomatous disease/osteopetrosis	NA
Activase	Alteplase recombinant	Genentech	11/1987	Acute myocardial infarction/acute massive pulmonary embolism/ischemic stroke	CHO
AcuTect	Tc-99m apcitide	Berlex	1998	Scintigraphic imaging of acute venous thrombosis	NA
Adagen	Injection pegademase bovine	Enzon	1990	Severe combined immunodeficiency disease	NA
Aldurazyme	Laronidase	Biomarin	4/2003	Hurler and Hurler–Scheie forms of mucopoly-saccharidosis I	CHO
Alferon N injection	Interferon alfa-n3	Hemispherx Biopharma	10/1989	Genital warts	NA
Amevive	Alefacept	BiogenIdec	1/2003	Moderate to severe chronic plaque psoriasis	CHO
Apligraf	Graftskin	Novo Novartis	5/1998	Venous leg ulcers	NA
Aranesp	Darbepoetin alfa	Amgen	9/2001	Anemia associated with chronic renal failure/ chemotherapy induced anemia	CHO
Argatroban		GlaxoSmithKline	6/2000	Heparin-induced thrombocytopenia syndrome	NA
Avastin	Bevacizumab	Genentech	2/2004	Metastatic colorectal cancer	CHO
Avonex	Interferon beta-1a	BiogenIdec	5/1996	Multiple sclerosis	CHO
BeneFIX	Human factor IX	Wyeth	2/1997	Hemophilia B	CHO
Betaseron	Interferon beta-1b	Chiron/Berlex	7/1993	Multiple sclerosis	*E. coli*
Bexxar	Tositumomab and Iodine I-131 tositumomab	Corixa	6/2003	CD20 positive, follicular, non-Hodgkin's lymphoma	Mammalian cells
BioTropin	Human growth factor	Bio-Technology General	5/1995	Growth hormone deficiency in children	NA
Botox	Botulinum toxin type A	Allergan	4/2002	Cervical dystonia/ moderate to severe glabellar lines	*C. botulinum*
Campath	Alemtuzumab	Millennium/Ilex	5/2001	B-cell chronic lymphocytic leukemia	CHO
Carticel	Autologous cultured chondrocytes	Genzyme	8/1997	Symptomatic cartilaginous defects of the femoral condyle	Human cartilage
CEA-Scan	Arcitumomab	Immunomedics	6/1996	Imaging agent for detection of colorectal carcinoma	NA
Cerezyme	Imiglucerase for injection	Genzyme	5/1994	Type 1 Gaucher disease	CHO

(*continued...*)

Trade Name	*Proper Name*	*Manufacturer*	*Approval Date*	*Application*	*Cell Type*
Comvax	Haemophilus b conjugate	Merck	10/1996	Vaccination against both invasive haemophilus influenzae b and hepatitis B	*S. cerevisiae*
DACS SC	Stem cell enrichment device	Dendreon	9/1999	Rescue therapy following high-dose chemotherapy	NA
Elitek	Rasburicase	Sanofi-Synthelabo	7/2002	Management of plasma uric acid levels in patients with leukemia, lymphoma, and solid tumor malignancies	*S. cerevisiae*
Enbrel	Etanercept	Amgen	11/1998	Moderately to severely active rheumatoid arthritis/ polyarticular course juvenile rheumatoid arthritis/ psoriasis	CHO
Engerix-B	Hepatitis B vaccine	GlaxoSmithKline	8/1989	Hepatitis B	*S. cerevisiae*; CHO
Epogen	Epoetin alfa	Amgen	6/1989	Multiple types of Anemia	CHO
Erbitux	Cetuximab	ImClone Systems	2/2004	Advanced colorectal cancer	NS0
Fabrazyme	Agalsidase beta	Genzyme	4/2003	Fabry disease	CHO
Follistim	Follicle stimulating hormone	Organon	9/1997	Infertility	CHO
Fortéo	Teriparatide	Eli Lilly	12/2002	Osteoporosis	*E. coli*
Fuzeon	Enfuvirtide	Roche	3/2003	HIV infection/AIDS	NA
Genotropin	Somatropin	Pfizer	8/1995	Short stature in children due to growth hormone deficiency	*E. coli*
Geref	Human growth hormone	Serono	12/1990	Prediatric growth hormone deficiency	NA
GlucaGen	Glucagon	Novo Nordisk	6/1998	Hypoglycemia	*S. cerevisiae*
Gonal-F	Follicle stimulating hormone	Serono	9/1997	Female infertility	CHO
Helixate FS	Antihemophilic factor	Bayer	6/2000	Hemophilia A	BHK
Herceptin	Trastuzumab	Genentech	9/1998	Metastatic breast cancer	CHO
Humalog	Insulin lispro	Eli Lilly	10/1982	Diabetes	NA
Humatrope	Somatropin	Eli Lilly	3/1987	Human growth hormone	*E. coli*
Humira	Adalimumab	Abbott Laboratories	12/2002	Moderately to severely active rheumatoid arthritis	CHO
Humulin	Human insulin	Eli Lilly	10/1982	Diabetes	*E. coli*; *S. cerevisiae*
Infergen	Interferon alfacon-1	Amgen	10/1997	Chronic hepatitis C virus (HCV) infection	*E. coli*
	Interferon alfa-2a	Hoffman-LaRoche	9/2003	Removal of Kaposi's sarcoma indication	NA
Intron A	Interferon alfa-2	Schering-Plough	6/1986	Hairy cell leukemia/genital warts/AIDS-related Kaposi's sarcoma/ hepatitis C/follicular lymphoma in conjunction with chemotherapy/ hepatitis B	*E. coli*

(*continued...*)

TABLE 32.1 Approved Biotechnology Products (*Continued*)

Trade Name	*Proper Name*	*Manufacturer*	*Approval Date*	*Application*	*Cell Type*
Kineret	Anakinra	Amgen	11/2001	Moderately to severely active rheumatoid arthritis	*E. coli*
KoGENate	Antihemophiliac factor	Bayer	2/1993	Clotting treatment of hemophilia A	BHK
KoGE-Nate-FS	rFVIII	Bayer	6/2000	Clotting treatment of hemophilia A	BHK
Leukine	Sargramostim	Berlex	3/1991	Colony autologous bone marrow transplantation	Yeast
LymErix	Lyme disease vaccine	GlaxoSmithKline	12/1998	Prevention of lyme disease	*E. coli*
Mylotarg	Gemtuzumab ozogamicin	Wyeth	5/2000	CD-33-positive acute myeloid leukemia	Micromonospora echinospora. sp. calichensis
Myobloc	Botulinum toxin type B	Elan Pharmaceuticals	12/2000	Cervical dystonia to reduce the severity of abnormal head position and neck pain	*C. botulinum*
Myoscint	Imciromab pentetate	Centocor	7/1996	Cardiac imaging agent for detection of myocardial necrosis	Mammalian cells
Natrecor	Nesiritide	Scios	8/2001	Acute decompensated congestive heart failure	*E. coli*
Neulasta	Pegfilgrastim	Amgen	1/2002	Chemotherapy-induced neutropenia	*E. coli*
Neumega	Oprelvekin	Genetics Institute	11/1997	Prevention of chemotherapy-induced thrombocytopenia	*E. coli*
Neupogen	Filgrastim	Amgen	2/1991	Chemotherapy-induced neutropenia/autologous bone marrow transplantation/chronic severe neutropenia/acute myeloid leukemia	*E. coli*
Norditropin	Somatropin	Novo Nordisk	5/1995	Growth failure in children due to inadequate growth hormone secretion	*E. coli*, CHO
Novolin 70/30	Human insulin	Novo Nordisk	6/1991	Insulin-dependent diabetes mellitus	*S. cerevisiae*; *E. coli*
Novolin L	Human insulin zinc	Novo Nordisk	6/1991	Insulin-dependent diabetes mellitus	S. cerevisiae; *E. coli*
Novolin N	Human insulin isophane	Novo Nordisk	7/1991	Insulin-dependent diabetes mellitus	S. cerevisiae; *E. coli*
Novolin R	Regular, human insulin	Novo Nordisk	6/1991	Insulin-dependent diabetes mellitus	S. cerevisiae; *E. coli*
NovoLog	Insulin aspart	Novo Nordisk	11/2001	Insulin-dependent diabetes mellitus	S. cerevisiae; *E. coli*
NovoSeven	Factor VIIa	Novo Nordisk	3/1999	Bleeding episodes in hemophilia A or B	BHK
Nutropin	Somatropin	Genentech	3/1994	Growth failure in children due to chronic renal insufficiency	*E. coli*

(*continued...*)

Trade Name	*Proper Name*	*Manufacturer*	*Approval Date*	*Application*	*Cell Type*
Nutropin AQ	Somatropin	Genentech	12/1995	Growth failure in children due to chronic renal insufficiency	*E. coli*
Oncospar	PEG-L-asparaginase	Enzon	2/1994	Acute lymphoblastic leukemia	NA
Ontak	Denileukin diftitox	Ligand Pharmaceuticals	2/1999	Persistant or recurrent cutaneous T-cell lymphoma	*E. coli*
Orthoclone Okt3	Muromonab-CD3	Ortho Biotech	6/1991	Reversal of acute kidney transplant rejection	NA
Ovidrel	Choriogonado-tropin alfa	Serono	10/2003	Induction of final follicular maturation and early luteinization	CHO
Pegasys	Peginterferon alfa-2a	Hoffman-La Roche	10/2002	Chronic hepatitis C	*E. coli*
PEG-Intron	Peginterferon alfa-2b	Schering-Plough	1/2001	Chronic hepatitis C	*E. coli*
Procrit	Epoetin alfa	Ortho Biotech	12/1990	Anemia	CHO
Proleukin	Aldesleukin	Chiron Corporation	1/1998	Metastatic melanoma/ renal cell carcinoma	*E. coli*
ProstaScint	Capromab Pendetide	Cytogen Corp	10/1996	Labeled with Indium 111 for imaging of prostatic carcinoma	Mammalian cells
Protropin	Somatrem	Genentech	10/1985	Growth hormone deficiency in children	*E. coli*
Pulmozyme	Dornase alfa	Genentech	12/1993	Cystic fibrosis/advanced cystic fibrosis	CHO
Raptiva	Efalizumab	Genentech	10/2003	Chronic plaque psoriasis	CHO
Rebetron	Ribavirin/ interferon alfa-2b combination	Schering-Plough	6/1998	Chronic hepatitis C	*E. coli*
Rebif	Interferon beta-1a	Serono	5/2003	Relapsing forms of multiple sclerosis	CHO
Recombinat	rAHF	Baxter Healthcare	12/1992	Control of bleeding episodes in hemophilia A	NA
ReFacto	Antihemophilic factor VIII	Wyeth	3/2000	Clotting hemophilia A	CHO
Refludan	Lepirudin	Berlex Laboratories	3/1998	Heparin-induced thrombocytopenia	NA
Regranex	Becaplermin	Ortho-McNeil	12/1997	Diabetic ulcers	*S. cerevisiae*
Remicade	Infliximab	Centocor	08/1998	Moderately to severely active Crohn's disease/ rheumatoid arthritis	NS0
ReoPro	Abciximab	Centocor/Eli Lilly	12/1994	Anti-platelet prevention of blood clots/ refractory unable angina	Mammalian cells
Retavase	Reteplase	Centocor	10/1996	Acute myocardial infarction in adults	CHO
Rituxan	Rituximab	Genentech, BiogenIdec	11/1997	Relapsed or refractory low-grade or follicular, B-cell non-Hodgkin's lymphoma	CHO

(*continued...*)

TABLE 32.1 Approved Biotechnology Products (*Continued*)

Trade Name	*Proper Name*	*Manufacturer*	*Approval Date*	*Application*	*Cell Type*
Roferon-A	Interferon alfa-2a	Hoffman-LaRoche	6/1986	Hairy cell leukemia/ AIDS-related Kaposi's sarcoma/hepatitis C/chronic myelogenous leukemia	*E. coli*
Saizen	Somatropin	Serono	10/1996	Pediatric growth hormone deficiency	Mouse C127
Serostim	Somatropin	Serono	8/1996	AIDS-associated catabolism	Mouse C127
Simulect	Basiliximab	Novartis	5/1998	Prevention of renal transplant rejection	Myeloma cell
Synagis	Palivizumab	Medimmune	6/1998	Prophylaxis of serious lower respiratory tract disease caused by respiratory syncytial virus	NS0
Thymoglobulin	Thymocyte globulin	SangStat	12/1998	Prevention of kidney transplant rejection	NA
Thyrogen	Thyrotropin alfa	Genzyme	11/1998	Thyroid cancer follow-up	CHO
TNKase	Tenecteplase	Genentech	6/2000	Reduction of mortality associated with acute myocardial infarction (AMI)	CHO
Trisenox	Arsenic trioxide	Cell Therapeutics	6/2000	Acute promyelocytic leukemia	NA
Velosulin BR	Human insulin	Novo Nordisk	7/1999	Diabetes mellitus	*S. cerevisiae*
Verluma	Nofetumomab	DuPont Pharma	8/1996	Detection of small cell lung cancer	NA
Visudyne	Verteporfin	QLT	4/2000	Minimally classic age-related macular degeneration	NA
Vitravene	Fomiviren sodium	Ciba Vision	8/1998	Cytomegalovirus retinitis	NA
Xifaxan	Ritaximin	Salix Pharmaceuticals	5/2004	Traveler's diarrhea	NA
Xigris	Drotrecogin alfa	Eli Lilly	11/2001	Severe sepsis	NA
Xolair	Omalizumab	Genentech	6/2003	Moderate to severe persistent asthma	CHO
Zenapax	Daclizumab	Hoffman-La Roche	12/1997	Prophylaxis of acute renal allograft rejection	SP2/O
Zevalin	Ibritumomab Tiuxetan	BiogenIdec	2/2002	Relapsed or refractory low-grade, follicular, or transformed B-cell non-Hodgkin's lymphoma	CHO
Zorbtive	Somatropin	Serono	12/2003	Short bowel syndrome	Mouse C127

NA = not available; BHK = Baby Hamster Kidney; CHO = Chinese Hamster Ovary.

continues to grow, and the biopharmaceutical industry is faced with the challenge of efficiently producing these proteins in large quantity. To keep up with demand while driving manufacturing costs down, mammalian cell production expectations are rising every year, with product titers reaching 4 g of protein/L of culture.[8] The high yields obtained today are the result of combined efforts made in improving host cells, expression vectors,

screening methods, medium development, and process development. In this chapter, we outline the common methods applied to mammalian cell line development and describe the typical industrial processes used in cell culture and purification for the production of recombinant proteins. Recent advances in this field are also presented.

Cells Used For Industrial Production

This section focuses on the generation of engineered mammalian cell lines that stably produce therapeutic proteins. Hybridomas, transient expression systems, and insect and bacterial cell line development are outside the scope of this section. The commonly used cell lines, expression systems and vectors, as well as cell banking and stability are described.

HOST CELL LINES

Mammalian cell lines commonly used to manufacture therapeutic proteins include NS0 mouse myeloma, baby hamster kidney (BHK), and Chinese hamster ovary (CHO) derived lines such as CHO-S, CHO-K1, CHO-DUXB11, and CHO-DG44. The latter two cell lines are engineered to have single or double knockout mutations, respectively, for the dihydrofolate reductase (DHFR) gene. NS0 and CHO cell lines are also available with the glutamine synthetase gene knocked out. Stable cell lines are generated by integrating the genes that encode the desired proteins into the genome of one of the above host cell lines. The CHO-DHFR system uses a plasmid that contains a variety of promoters driving the production of the genes for the proteins and dihydrofolate reductase, which acts as a selectable marker. Amplified expression can be achieved by applying methotrexate, the selective agent for dihydrofolate reductase. The NS0-GS and CHO-GS systems use a vector containing the glutamine synthetase gene, which functions as the selection marker. This type of system can be amplified to boost expression levels by using methionine sulphoximine. In addition to being engineered for selection, host cells have also been genetically constructed by inserting growth factor genes, anti-apoptosis genes, and cell cycle control genes to generate superior production hosts.[9]

Expression Systems

The goal of cell line development is to engineer cells to express a large amount of a recombinant protein and to stably maintain this level of production for many cell doublings. The basic schema is:

1. Generate plasmid expressing recombinant protein.
2. Introduce plasmid into cell.
3. Identify cell line expressing high levels of recombinant protein.
4. Select cell line maintaining high levels of expression through scale-up and bioreactor processes.

Plasmids. The plasmid contains all genetic elements necessary for the expression of recombinant protein and for the selection of the cells generating the desired product. For stable cell lines, the plasmids must integrate into transcriptionally active chromosomal regions. The vector promoter elements drive the expression of recombinant protein. The strong cytomegalo virus (CMV) promoter is in general use, although the weaker simian virus 40 (SV40) and rouse sarcoma virus (RSV) promoters are also used. Recently, the strong promoter, CHO-EF1α (CHEF1)[10] has been generating CHO cell lines with high production levels. The inclusion of a chimeric intron in the primary transcript leads to higher stable expression of recombinant protein through enhanced transport and processing of the mRNA from the nucleus into the cytoplasm where it is translated.[11,12]

To allow the selection of cells expressing the desired protein, the plasmid also contains selectable markers such as the *neo* gene generating aminoglycoside 3′-phosphotransferase (APH 3′ II) for G418 geneticin resistance, the *hph* phosphotransferase gene for hygromycin resistance, the *Sh ble* gene for zeocin resistance, or puromycin N-acetyl-transferase

encoded by the *pac* gene for puromycin resistance. Selection can also occur using a complementation gene such as DHFR or GS, which can be included in plasmids when using DHFR-negative or GS-negative cell lines, such as CHO-DG44 and NS0-GS, respectively. The use of an internal ribosomal entry site (IRES) element can facilitate the co-expression of selectable markers and protein product when integrated into the genome.[13] This system generates a single transcript accessible to ribosomes at two locations just prior to the start site of each gene. The fact that the selectable marker and the product gene are under the control of a single promoter, which generates one transcript, is likely to improve cell line stability. Elements such as matrix attached regions (MARs)[14,15] and ubiquitous chromatin opening elements (UCOEs)[16] can also be included in plasmids, as they are known to generate transcriptionally active genomic environments once integrated into the cell genome.

DNA Delivery. Several methods are in use for introducing plasmid DNA into cells. $Ca_2(PO_4)_2$ precipitation is the earliest method, but has been surpassed by the more convenient and consistent lipid-basereagents (Lipofectamine, Fugene, Transfectin) and electroporation.[17] Polyethyleneimine (PEI)[18] is a low-cost alternative DNA delivery method. Retroviral transduction has a long history for inserting DNA into cells, but use for manufacturing is only now emerging (Gala Biotech, Middleton, Wisconsin). Transfection efficiencies can vary from 5 to 100 percent depending on cell line and DNA delivery method. CHO cells can achieve 5 to 40 percent with $Ca_2(PO_4)_2$, 20 to 60 percent with lipids, and ~100 percent with retroviral transduction.

Amplification Systems. The DHFR and GS amplification systems have successfully generated manufacturing cell lines with high protein titers (Lonza, Basel, Switzerland).[19,20] These systems employ a $DHFR^-$ or GS^- cell line that is transfected with the plasmid encoding product of interest along with DHFR or GS, respectively. The use of methotrexate and methionine sulphoximine results in chromosomal amplification events that increase the DHFR or GS gene copy number, respectively, to overcome the drug toxicity. The gene encoding the product of interest is usually co-amplified with the DHFR or GS genes as they are inserted into the genome in the same locations. Tenfold or greater improvements in expression can be achieved with this amplification system. The DHFR amplification system has the potential to experience the loss of transgene copy number,[21–23] so stability studies are especially important to characterize cell lines derived from drug-induced genomic amplification approaches.

Sequential Transfection. A simple approach to boosting expression is to repeat the transfection on previously transfected cells but with selectable markers not used in the first transfection. Xoma (Berkeley, CA), Sunol Molecular (Miramar, FL), and ICOS (Bothell, WA) have used this approach successfully. Fivefold or greater improvements in expression can be achieved in a single sequential transfection. ICOS reports the added advantage of balancing heavy and light chain ratios to improve the secretion and expression of recombinant monoclonal antibodies.

Table 32.2 summarizes expression systems that are currently used in the biotechnology industry for generating stable cell lines. Before one makes a decision as to which expression system is preferred, it is important to evaluate all aspects including productivity and time needed for cell line development, as well as potential license fee and cost.

Identifying High-Expressing Cells

For industrial cell line development, moderate to high-throughput methods are needed to identify the cells expressing the highest levels of the protein pharmaceutical. Typically, the cells are grown in selective or amplifying environments in several 96-well plates. For secreted proteins, enzyme-linked immunosorbant assays (ELISA) on conditioned media can identify the cells expressing the highest

TABLE 32.2 Expression Systems for Establishing Stable Cell Lines

Expression System	*Cell Line*	*Specific Productivity Prescale-Up (pg/Cell/Day)*	*Length of Development (Months)*	*Industrial References*
Unamplified	CHO	0.5–4	4	Various
Unamplified	NS0	2–14	6	Various
Amplified (GS or DHFR)	CHO	5–15	6 (GS) 9 (DHFR)	Lonza (GS) Various (DHFR)
CHEF1	CHO	6–15	3	ICOS
MARs	CHO	15	2–3	Selexis
UCOE	CHO	4–9	NA	Cobra
Sequential transfection	CHO	10–15	6–8	Xoma, Sunol, ICOS
Retroviral transduction	CHO	17–35*	3	Gala Biotech

NA = not available.
Data are from Gala Biotech, a wholly owned subsidiary of Cardinal Health, Middleton, WI.

protein levels. AlphaScreen™ (Perkin-Elmer, Boston, MA) is a recent homogeneous assay that is well suited for high-throughput quantification of protein production. The Guava Inc. (Hayward, CA) personal cell analyzer (PCA) economically and conveniently generates fluorescence-activated cell sortinglike (FACS) expression profiles of cells with moderate throughput in 96-well microtiter plates. Using Guava PCA, clonal populations and cells with the highest average productivity can be identified. A critical element of generating stable cell lines is identifying clonal populations of expressing cells. Pools of expressing cells tend to express lower levels of desired protein; they can drift to lower expression levels, and are more difficult to adapt to serum-free suspension. Limited dilution methods have been used for years whereas FACS sorting of live cells has also proven successful. FACS can be used to simultaneously clone and enrich for the highest expressing cells.[24] Recent automated approaches for identifying clones include picking high-expressing colonies in semisolid media (Genetix, New Milton, UK) and enriching for high-expressing cells by laser ablation of low and nonproducing clones (Cyntellect, San Diego, CA).

Cell Banking

A stock of cells must be preserved to ensure continuity for research, development, and manufacturing production programs. For a small research program, only a small number of frozen vials may be needed. However, to continue to supply a cell line for the manufacture of therapeutic proteins, it is usually best to prepare two-tiered cell banks: a master cell bank (MCB) and working cell banks (WCB). A single cell line demonstrating suitable expression levels and stability is used to generate an MCB, and a WCB is derived from one vial of the MCB. Each MCB and WCB usually includes 100–300 vials. As a WCB is depleted during manufacturing runs, another frozen vial of MCB is used to generate a new WCB.

Making cell banks involves the process of cryopreserving cells. During cryopreservation, cells can be damaged by the formation of intracellular ice crystals or by osmotic effects that occur with decreased water content. To minimize cell damage, the rate of freezing must be controlled and cryoprotectants must be used. Dimethylsulfoxide (DMSO) at 7.5–10 percent is routinely used as a cryoprotectant. In some cases, a low percentage of fetal calf serum or serum albumin is added to the freezing medium. However, when freezing cell lines for the production of therapeutic proteins, it is generally preferable to eliminate any animal-derived material to minimize the risk of disease transmission from animal to human. To control the rate of freezing, Nalgene Cryo freezing containers (Nalge Company, Rochester, NY) are routinely used

in making small cell banks, whereas programmable controlled-rate cell freezers are needed for large cell banks.

Cell Stability

The properties of a cell line are likely to change during a long period of continuous passage. For example, cell lines can lose their expression and can generate undesired proteins clipped from the product. Therefore, it is critical to characterize the cells to ensure consistency for large-scale production and to guarantee that the properties of the protein derived from the cells are maintained. For a production cell line, an acceptable level of stability of the desired characteristics must be established and a maximum passage number must be defined so that comparison of the cells' characteristics can be made after low and extensive passages. Tests such as peptide mapping, amino acid sequencing, DNA fingerprinting, and determination of gene copy number and phenotype markers must be conducted to ensure the cells' genetic stability. In addition, productivity and product identity must be examined to assess the stability of the cell line. A good production cell line should be able to maintain its productivity and product quality through the many generations required to reach the end of large-scale production. In most instances, stability retained for 50 generations will satisfy the rigors of large-scale manufacturing.

MEDIA

Mammalian cell culture is the most important source of therapeutic proteins and monoclonal antibodies. Just as mammalian cells are more complicated than most other microorganisms, the media required for their growth is also more complex. The extracellular medium must provide the same nutrients and growth factors that mammalian cells are exposed to in vivo in order for them to survive, proliferate, and differentiate. Serum contains many important components that support the growth of mammalian cells including growth factors, hormones, transport and binding proteins, attachment factors, protease inhibitors, and lipids. Serum was therefore commonly supplemented in the media in early mammalian cell culture and for large-scale production of therapeutic proteins and monoclonal antibodies in the 1980s. However, the use of serum in mammalian cell culture has many disadvantages: (1) it is a potential source of bacterial, mycoplasmal, and viral contamination; (2) it is the most expensive additive to cell culture media; (3) it has a high degree of batch variability, making production consistency difficult; (4) it contains a high concentration of proteins that can interfere with product recovery. In the early 1990s, these drawbacks, especially the serious concern about the risk of transferring diseases from animal to human, led to an important regulatory-driven trend to eliminate serum and animal-derived components from mammalian cell growth media. This trend sparked an industrywide interest in developing serum-free media.

Commercial Serum-Free Media

Many new medium companies formed in the early 1990s. Today, more than a decade later, the medium development industry has matured. Currently, a variety of serum-free (SF) media are available commercially. Table 32.3 lists some SF media produced in the United States. Many of these media are also made in powder form to facilitate use in large-scale production. As listed in Table 32.3, there are different types of SF media, which can be categorized into protein-free (PF), chemical-defined (CD), and animal-component-free (ACF) media. The relationships among these different types of media are illustrated in Figure 32.1.

SF, PF, ACF, and CD media tend to be highly specific to one cell type and sometimes even to one particular cell line. It is not uncommon that a different optimal medium is required for a particular cell line. Developing SF, PF, ACF, or CD media requires considerable experience and expertise and can be very time-consuming. One approach is to start with a commercially available SF medium and add necessary nutrients to optimize growth and production for a particular cell type. This approach can shorten the timeline needed for in-house medium development.

TABLE 32.3 Examples of Commercial Serum-Free Media

Media	*Type of Medium*	*Cell Type Applied*	*Vendor*
CD CHO	PF and CD	CHO	Invitrogen
CHO-S-SFM II	SF with low-protein	CHO	Invitrogen
HyQ® CDM4CHO™	CD and ACF	CHO	Hyclone
HyQ SFM4CHO	PF	CHO	Hyclone
HyQ PF CHO	PF	CHO	Hyclone
EX-CELL™ 302 SF	SF with low-protein	CHO	JRH Biosciences
EX-CELL™ 325 PF CHO	PF	CHO	JRH Biosciences
EX-CELL™ CD CHO	CD and ACF	CHO	JRH Biosciences
CHO Animal-Component Free	ACF	CHO	Sigma-Aldrich
CHO DHFR- Medium	ACF	$DHFR^-$ CHO	Sigma-Aldrich
CHO CD-3 Chemically Defined	CD and ACF	CHO	Sigma-Aldrich
BD Cell™ MAb Medium (220509)	SF	Sp2/0, CHO and myelomas	BD Biosciences
BD Cell™ MAb Medium (220513)	ACF	Sp2/0, CHO and myelomas	BD Biosciences
IS CHO™ Medium	SF	CHO	Irvine Scientific
IS CHO-CD™	CD and ACF	CHO	Irvine Scientific
CD Hybridoma	PF and CD	Hybridomas and myelomas	Invitrogen
Hybridoma-SFM	SF with low-protein	Hybridomas and myelomas	Invitrogen
HyQ® CDM4NS0™	CD and ACF	Hybridomas and myelomas	Hyclone
HyQ® SFM4MAb™	SF with low-protein	Hybridomas and myelomas	Hyclone
HyQ® ADCF-MAb™	ACF	Hybridomas and myelomas	Hyclone
EX-CELL™ 620-HSF	SF with low-protein	NS0 hybridomas	JRH Biosciences
EX-CELL™ NS0	PF and CD and ACF	NS0 hybridomas	JRH Biosciences
Hybridoma Medium	CD and ACF	Hybridomas and myelomas	Sigma-Aldrich
Hybridoma Medium	SF and PF	Hybridomas and myelomas	Sigma-Aldrich
IS MAB-V™	ACF	Hybridomas and myelomas	Irvine Scientific
IS MAB-CD™	CD and ACF	Hybridomas and myelomas	Irvine Scientific

Information is from the vendors' Web sites.

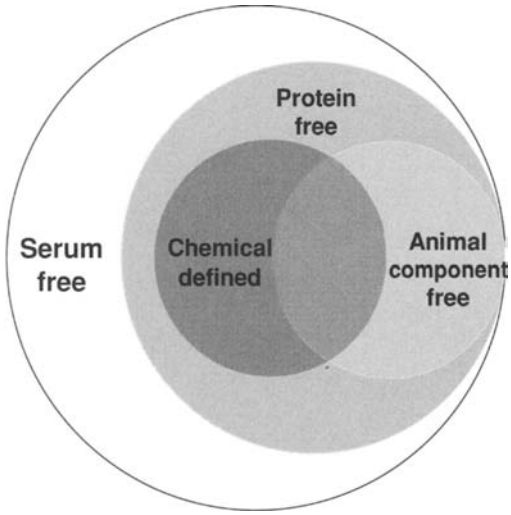

Fig. 32.1. Relationships among serum-free, protein-free, chemical defined and animal-component-free media.

However, using commercial media has two major drawbacks: commercial SF media are expensive, which can lead to a high cost of goods for large-scale production; the composition of a commercial SF medium is proprietary to the medium company and the buyer will not know its formulation. This makes it difficult to gain full knowledge about the growth and production characteristics of your cell line. To fully understand the metabolism and characteristics of your cell line, there is no substitute for developing your own media.

Approaches for Serum-Free Medium Development

Medium development is part of the process of cell culture optimization. It can be very complicated and time consuming, and will require

expertise and resources. In this section, we outline the common and novel methods used for SF medium development. Details of each strategy can be found in the referenced literature.

In the early 1980s, efforts were initiated to eliminate serum or animal-derived components from the culture media used to produce human therapeutics. At that time, different strategies were recommended for the development of SF media:

1. Limiting factor method:[25] Starting with an existing formulation, the serum concentration is lowered until cell growth becomes limited; then the concentration of each component of the medium is optimized until cell growth recovers.
2. Synthetic method:[26] A variety of growth factors is added to the existing basal media to replace the serum's functions.

The last decade saw the development of many new genomic and automated screening tools. These advancements, as well as an improved understanding of mammalian cell culture, allowed novel concepts and approaches to be applied to the development of SF and CD media. Some representative strategies are briefly summarized below.

1. Rational design:[27] This method uses four complementary methods, including component titration (CT), media blending (MB), spent media analysis (SMA), and automated screening (AS), to achieve the best SF culture medium in the shortest timeline. An example of how to use this strategy is outlined in Figure 32.2.
2. Factorial design:[28–30] This method identifies key growth factors in a lean basal medium by performing experiments

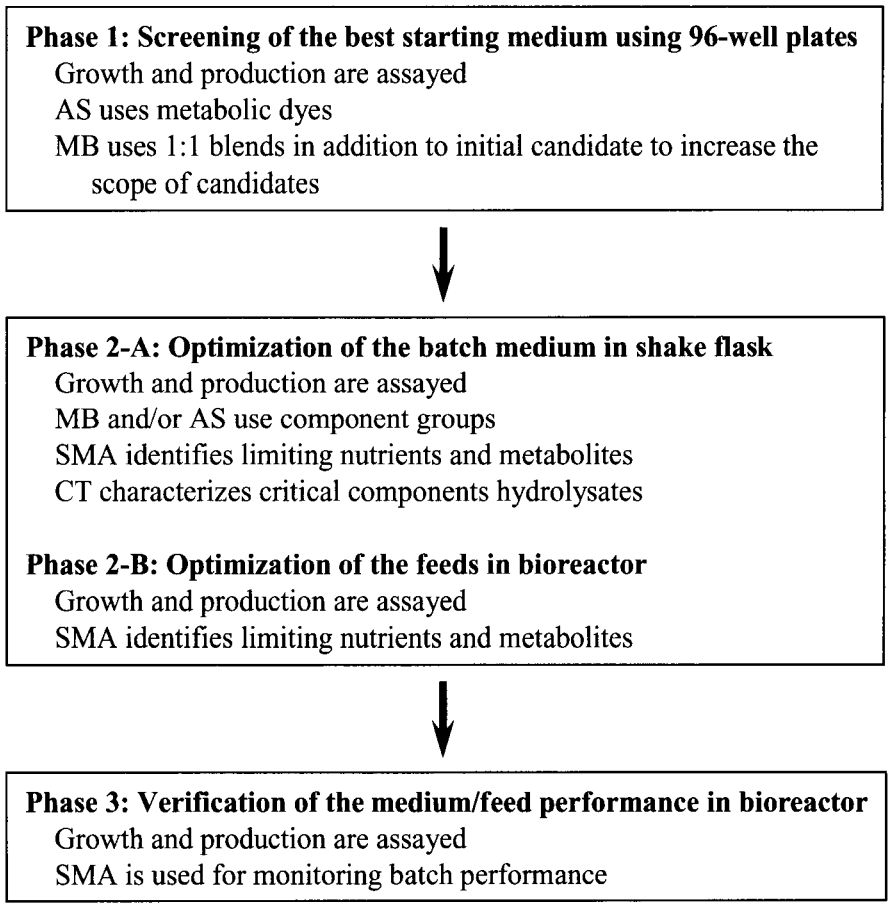

Fig. 32.2. Procedure of using rational design.

using a full factorial matrix. It allows calculating the maximum number of interaction effects and gives insights into growth factor biology.

3. Genomic tools:[31] This method takes advantage of select genomic (microarray- or PCR-based approaches) and proteomic (antibody array analysis) tools to identify the receptors for growth factors, hormones, cytokines, and other components of cell signaling pathways expressed by a culture of interest.

Serum-Free Adaptation

In most cases, cells are grown in a serum-containing environment during the early stages of cell line development, such as transfection and selection. Once a SF medium with a good nutrient balance is chosen, the next step is to adapt cells to SF growth. Two adaptation strategies, sequential adaptation and starve/save adaptation, are described.

Sequential Adaptation. This method weans cells off serum gradually (e.g., from 10% to 5% to 2.5% to 1.25% to 0.5% to 0.1%) until the serum is completely removed. This strategy is conservative and easy to follow, and therefore commonly used in the biotechnology industry. A drawback is that it takes a relatively long time (up to six months) before achieving a full SF adaptation. A typical protocol includes these steps:

1. Grow the cells in a targeted SF medium containing 10 percent serum and split the cells until the same doubling time is achieved as in the old serum-containing medium.
2. Reduce the serum concentration to 5 percent and continue to split the cells until a relatively consistent doubling time is achieved. Reduce the serum concentration to 2.5 percent and repeat the process.
3. Transfer the cells to the medium containing 1.25 percent serum. At this level of serum, it will be more difficult for the cells to adapt. The cells may become stressed and show diminished survival. It is therefore wise to maintain the cells at the previous serum concentration while spliting them to a lower serum concentration.
4. Continue to reduce the serum to 0.5, 0.25, 0.1, and 0 percent by repeating step 3. At the low serum concentrations, seeding the cells at a high cell density ($\sim 5 \times 10^5$ cells/mL) will facilitate adaptation. This can be achieved by centrifuging the cells out from the medium containing the current serum concentration and resuspending them into a smaller volume of medium containing a lower serum concentration.
5. Once the cells are able to survive and grow in a SF medium with a high seeding density, split them several times at a lower density ($\sim 2 \times 10^5$ cells/mL) to ensure that the cells are truly adapted.

Starve and Save Adaptation. This method uses a SF medium to starve the cells of components contained in serum, thereby selecting for adaptable cells. Switching to a serum-containing medium then saves the cells that became stressed during starvation. This process is repeated allowing selection and adaptation to continue until full SF adaptation is achieved. The advantage of this method is its effectiveness in selection, so it usually only takes four to six weeks to achieve the goal.[32] The following steps are used.

1. Define a SF medium and a serum-containing medium that have exactly the same formulation except for the serum.
2. Grow cells in the serum-containing medium to reach a high cell density.
3. Split and grow the cells in the SF medium, monitoring changes in cell density and viability.
4. When cell viability drops to about 50 percent, save the cells by transferring them back to the serum-containing medium.
5. Switch back to the SF medium to further select the cells and repeat steps 3–5 until the cells grow successfully in the SF medium.

BIOREACTOR SYSTEMS

Many therapeutic proteins are produced using genetically modified mammalian cells, as described in the preceding sections. This section describes the basic design and function of bioreactors used for suspended mammalian cell culture. Bioreactors should provide a sterile environment, adequate mixing, ease of operation, and control of temperature, pH, and dissolved oxygen. Traditionally, these requirements were met using glass or stainless steel stirred-tank systems. At production scale, therapeutic proteins are primarily produced in stirred-tank bioreactors. However, single-use, disposable systems such as the Wave bioreactor are gaining acceptance for certain applications.

Stirred-Tank Systems

Stirred-tank bioreactors are generally glass or stainless steel tanks with an impeller to provide mixing. Air or oxygen is usually bubbled through the media to supply oxygen to the cells. An example of a stirred-tank bioreactor is depicted in Figure 32.3. Sterility is obviously an important issue in mammalian cell culture, and therefore stirred-tank systems

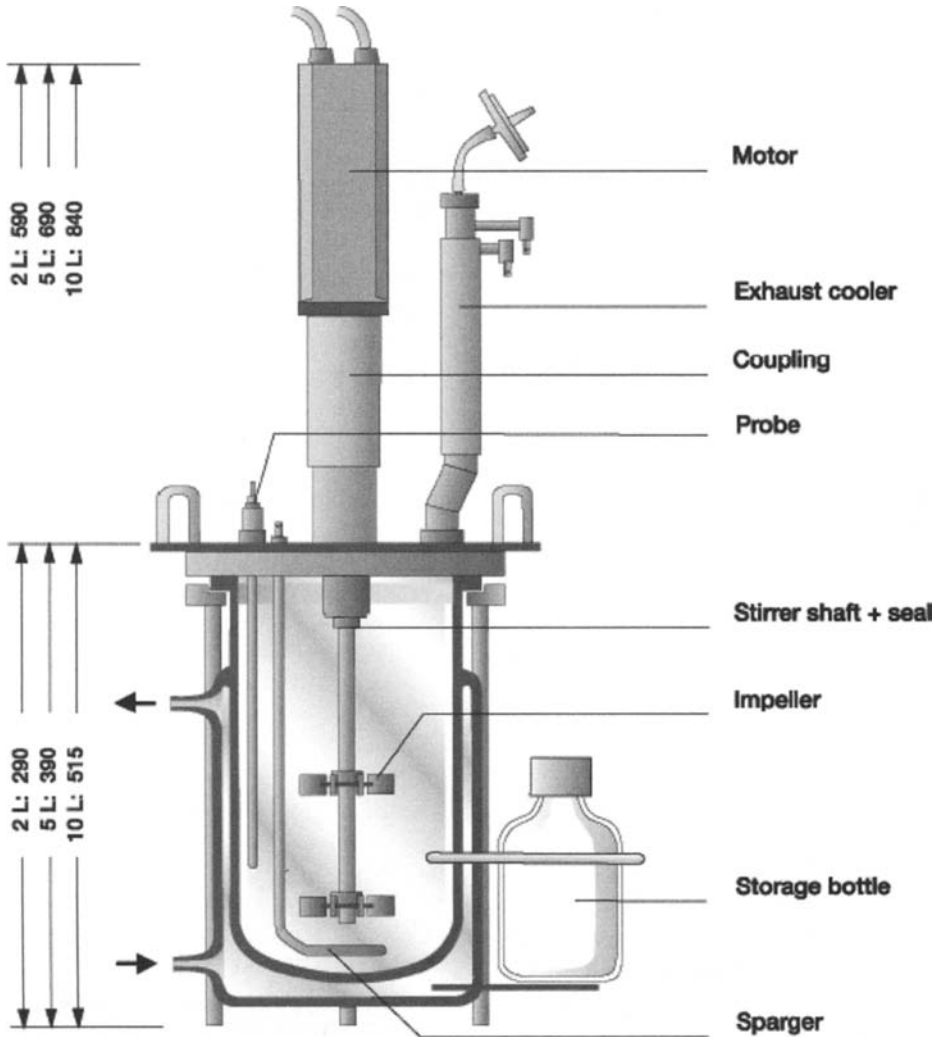

Fig. 32.3. Standard bench-scale, water-jacketed stirred tank bioreactor (permission of Sartorius BBI Systems Inc.).

require extensive cleaning and autoclaving. A significant portion of the labor involved with operating these systems is related to cleaning and sterilizing.

Mixing in Stirred-Tank Bioreactors. Adequate mixing is essential to suspend the cells and to facilitate heat and mass transfer. Historically, however, due to concerns regarding mammalian cells' sensitivity to hydrodynamic stress, most stirred-tank bioreactors were agitated just enough to keep the cells in suspension.[33] This low level of mixing can result in large concentration gradients of pH, oxygen, and other nutrients. Ozturk demonstrated this experimentally by adding base to a poorly mixed bioreactor.[34] The base was added to the top of the bioreactor, and because of poor mixing, a high pH region was created at the top. The cells in this region ruptured, forming a "snowball" of cellular debris in the vicinity of the base inlet.

Proper impeller selection and sizing will improve mixing. Generally, the diameter of the impeller should be approximately one-third to one-half the tank diameter. The lowest impeller should produce a radial flow pattern to aid in gas dispersion, and the upper impeller(s) should produce axial flow to eliminate "zones of mixing." Paddle or Rushton impellers produce radial flow, and hydrofoils and pitched blade impellers produce axial flow. Retrofitting existing bioreactors with different impellers, however, may prove difficult because the motor driving the impellers may not produce enough torque to turn them. Adequate baffling will also improve mixing; baffles prevent solid body rotation and vortex formation.[35]

Aeration. Oxygen can be introduced to the culture in many different ways. Membrane aeration provides efficient oxygen transfer with minimum shear damage to the mammalian cells and minimal foaming. However, due to the design complexity and the difficulty involved in cleaning and sterilizing membrane reactors, membrane aeration has limited utility in large-scale bioreactor systems. Sparger aeration offers high oxygen transfer rates and is widely used in both bench and production-scale bioreactors. Because oxygen is only sparingly soluble in water, a large surface area is needed to maximize diffusion of oxygen into the cell culture media. Frit spargers with micropores provide a large surface area for diffusion, but this type of sparger can cause foaming problems at large scale. Traditional large-hole-ring spargers reduce foaming and are therefore often used in production-scale stirred-tank bioreactors.[36]

Biosensors. Sensors are required to adequately monitor bioreactor performance. Ideally, one would like to have online sensors to minimize the number of samples to be taken from the bioreactor and to automate the bioreactor process. Most bioreactors have autoclavable pH and dissolved oxygen (D.O.) electrodes as online sensors, and use offline detectors to measure other critical parameters such as glucose and glutamine concentration, cell density, and carbon dioxide partial pressure (pCO_2). An online fiber-optic-based pCO_2 sensor is commercially available and appears to be robust.[37] Probes are also commercially available that determine viable cell density by measuring the capacitance of a cell suspension. Data from perfusion and batch cultures indicate that these probes are reasonably accurate at cell concentrations greater than 0.5×10^6 cells/mL.[38,39]

All of the aforementioned biosensors are designed for a standard stirred-tank bioreactor; they will not work with smaller-scale vessels such as shake flasks. Small noninvasive sensors have been developed to measure D.O. and pCO_2 inside shake and T-flasks.[40,41] A D.O. or pCO_2 detecting "patch" is placed in the flask, and this patch contains an oxygen or carbon dioxide luminescent dye. The color shifts of these dyes are detected using an external fluorometer.

Disposable Bioreactors

One of the major drawbacks of the stirred-tank bioreactors is the difficulty of cleaning and sterilizing the vessels. Disposable bioreactors are now commercially available that

are based on the use of presterile plastic bags. The sterile bag is partially filled with cell culture media and is then pressurized with a mixture of carbon dioxide and air. The bag is placed on a platform that rocks back and forth creating waves inside the bag. The wave motion provides sufficient mixing and gas transfer to easily support cell growth of over 20×10^6 cells/mL.[42] Currently, wave bags are available in sizes up to 500 L culture volume and have been proven for the Good Manufacturing Practice (GMP) production of human therapeutics. Wave bioreactors have been primarily used as batch culture for inoculum preparation and transient production; however, internal perfusion filters can be applied, allowing the option of use for high-density perfusion culture.

Modes of Bioreactor Operation

Industrial mammalian cell culture can be divided into three primary modes of operation: batch, fed-batch, and perfusion. Each mode of operation has its attributes and drawbacks, which are described in detail below.

Batch. Batch culture is the simplest of the three modes to operate. Cells are inoculated into media, and with the exception of agitation, temperature, pH, and D.O. control, essentially left alone for three to five days; no additional nutrients or media are added. Batch processes are easy to operate and require the least optimization effort of the three modes. However, cell densities and protein titer are significantly lower for batch processes, typically less than 5×10^6 cells/mL and 0.5 g/L, respectively.[43] Also, some protein products are degraded in the media during the batch process.

Fed-Batch. Fed-batch processes start out as batch cultures; after a few days of growth—when a crucial nutrient is depleted—a concentrated solution of nutrients is added to the media. Fed-batch cultures persist for one to two weeks and may produce high cell density and product titers, typically greater than 10×10^6 cells/mL and 1.5 g/L, respectively.[43] However, one needs to optimize the contents of the feed solution as well as the feeding strategy. Similar to batch cultures, fragile proteins may be degraded during the course of the culture.

Perfusion. Perfusion cultures can be considered continuous cultures. Broth is continually removed from the bioreactor, the cells are separated using a cell retention device, the cells are returned to the bioreactor, and the supernatant is collected for later protein purification. There is a variety of cell retention devices available, including spin filters, acoustic separators, continuous centrifuges, and gravity settlers. Spin filters and, to a lesser extent, acoustic settlers are the two most commonly used cell retention devices for large-scale perfusion cultures.[44] Perfusion cultures usually last many weeks, but require a longer time for process optimization, and more effort in bioreactor operation than either batch or fed-batch cultures. The increased effort is primarily due to continuous feeding/harvesting and fouling of the cell retention devices. The cell densities attained in perfusion cultures are usually on the order of 50×10^6 cells/mL, and the product titer, in most cases, is lower than that from fed-batch culture. However, the volumetric productivity is typically ten times that of fed-batch cultures. Because broth is continually removed from the bioreactor, fragile proteins can be separated immediately from cell proteases and other components that can cause degradation of the product.

CELL CULTURE PROCESS AND CONTROL

Mammalian cell culture processes must be tightly controlled to attain acceptable cell density and maximize product titer. Slight deviations in pH, temperature, nutrient, or catabolite concentrations can cause irreparable damage to the cells. This section covers the effects of pH, shear stress, catabolite, and carbon dioxide accumulation on cell growth and product formation, and discusses the importance of controlling glucose and glutamine concentrations

TABLE 32.4 General Control Parameter Setpoints for Mammalian Cell Culture

Control Parameter	*Optimal Range*
Temperature	37°C
pH	7.0–7.4
Osmolality	280–320 mOsm/kg
O_2 demand	0.5–2 mM/hr

in fed-batch and perfusion cultures. A brief discussion of scale-up heuristics in mammalian cell culture is also included.

Process Parameters

General Parameter Values. The optimal pH range for mammalian cell growth is 7.0 to 7.4, which is typically maintained using carbon dioxide and sodium bicarbonate. The optimal osmolality of the media is between 280 and 320 mOsm/kg. In general, mammalian cells achieve high cell densities most quickly with the temperature set to 37°C; however, a lower temperature may be advantageous in some cases for extending cell life, thereby increasing product titer. The oxygen demand for mammalian cells is 0.5–2 mM/hr and is one of the more critical control parameters in cell culture. The optimal ranges of common control parameters in cell culture are summarized in Table 32.4.

Mitigating Effects of Physical and Chemical Stress

Shear Stress. Because mammalian cells lack a cell wall and are larger than bacteria, they are more susceptible to hydrodynamic forces, or shear stress. Several studies have investigated the effects of shear stress on mammalian cells.[45–48] Many indicate that the action of the impeller alone does not decrease the viability of suspension-adapted mammalian cells.[46,48,49] Some bioprocess engineers in industry have seen a few cell lines that appear to be less robust, and anecdotally might have been damaged by the impeller. However, bubble rupture does cause sufficient hydrodynamic force to kill all the cells attached to the bubble.[48] The effects of bubble rupture can be greatly reduced by the addition of surfactants, such as Pluronic F-68. Pluronic F-68 renders cell-bubble adhesion thermodynamically unfavorable, so the cells do not adhere to bubbles.[50] However, Pluronic F-68 offers very little shear protection; cells rupture at the same level of hydrodynamic force regardless of the Pluronic F-68 concentration.[51] Cells attached to microcarriers, however, are very susceptible to shear stress and can quite easily be removed and killed by the action of the impeller.[47]

pH Perturbations. As mentioned earlier, significant pH gradients within the bioreactor are common, due to inadequate mixing. Cell lysis occurs at extreme pH; however, even moderate deviations from the optimal pH may be detrimental. Osman and co-workers found that pH values greater than 8.0 or less than 7.0 cause a considerable decrease in cell viability and a reduced culture time.[52] Antibody titers increased when the pH setpoint was reduced from 7.2–7.3 to 7.0.[52,53] The best way to eliminate deviations from optimal pH is to improve mixing (reduce the mixing time); this can be achieved by increasing the agitation or aeration rate, adding baffles, or optimizing the impeller design or placement.

Catabolite Accumulation. Several byproducts of cellular metabolism accumulate during the course of a bioreactor run. Many of these catabolites, such as lactate, ammonia, and carbon dioxide are detrimental to cell growth and protein production. Lactate is a product of glycolysis and lactate accumulation greater than 2 g/L tends to inhibit cell growth and increase glucose and glutamine consumption.[54,55] Lao and Toth pointed out the difficulty in completely decoupling the effects of lactate accumulation from the effects of increased osmolality (osmolality increases with increasing lactate concentration).[54] The effects of lactate accumulation can be mitigated by keeping glucose levels low, which can be achieved by optimizing media composition and feeding strategies.

In cell culture, ammonia is produced as a cellular metabolite and is converted from

glutamine in the media through deamination. Less than 20 mM ammonia inhibits cell growth, induces apoptosis, and alters glycosylation in certain cell lines.[56–58] The primary method of reducing ammonia concentrations in fed-batch and perfusion cultures is to optimize feeding strategies. In addition, cell lines containing the glutamine synthesis gene (GS systems) can be used to eliminate ammonia production; in GS systems, the cells are able to synthesize glutamine based on need. A recent report shows that substituting glutamine with pyruvate can greatly reduce ammonia production.[57]

Carbon dioxide is a product of cellular respiration. In mammalian cell culture, carbon dioxide and sodium bicarbonate are normally used to control bioreactor pH. Elevated partial pressure of carbon dioxide (pCO_2) hinders cell growth and protein production.[59–61] As with lactate accumulation, the effect of elevated pCO_2 is difficult to completely decouple from the effect of elevated osmolality.[60,61] Generally, one can reduce pCO_2 by increasing the volumetric mass-transfer coefficient (k_La), typically by increasing the sparge rate and/or the agitation rate. Mostafa and Gu were able to reduce pCO_2 in a 1000 L culture and nearly double the titer by increasing the sparge rate and using an open pipe instead of a sparger.[62] Table 32.5 summarizes the effects of shear force, pH deviation, and accumulation of lactate, ammonia, and carbon dioxide on cell culture and lists methods to minimize these effects.

Temperature Shifts. Culture temperature is one of the primary control parameters in mammalian cell culture. As stated previously, most mammalian cells grow optimally at 37°C. However, reducing the temperature setpoint slows cellular growth rate and metabolism, extends the period of high cell viability, arrests cells in the G_0/G_1 phase of the cycle, and possibly reduces intracellular protease activity.[63–66] By decreasing the temperature to 31–33°C, the period with high cell viability is extended for several days, leading to higher product titers. Fox et al.[65] and Bollati-Fogolin et al.[66] reaped the benefits of a cooler temperature without excessively extending culture time by shifting from 37°C to 32°C or 33°C, respectively. The temperature shift occurred toward the end of the cells' exponential growth phase, approximately 3–4 days after inoculation in a batch culture. Different cell lines and culture conditions may have different optimal time points for the temperature shift. However, finding the optimal time point is important and worth investing the time required. Operating cell cultures at a reduced temperature will most likely increase production of the target protein, but to avoid substantially increasing the culture time, the culture is generally started at 37°C, then decreased to 31–33°C at a predetermined time point.

TABLE 32.5 Effects of Shear Force, pH Deviation, and Accumulation of Lactate, Ammonia, and Carbon Dioxide on Cell Culture

Stress	*Cause*	*Effects*	*Possible Solution*
Shear force	Bubble rupture, impeller	Decreased viability	Add surfactants such as Pluronic F-68
pH deviations	Inadequate mixing	Decreased growth rate, viability	Increase agitation/aeration rate; optimize vessel configuration
Lactate accumulation	High glucose concentration	Decreased growth rate, increased glucose/ glutamine consumption	Optimize feed strategy/media to reduce (glucose)
Ammonia accumulation	Metabolism/deamination of glutamine	Apoptosis, decreased growth rate, altered glycosylation	Optimize feed strategy/media to reduce (glutamine); use cell line with GS system
CO_2 accumulation	Cellular respiration, sparged gas component	Decreased growth and protein production	Increase agitation/aeration rate; optimize vessel configuration

Fed-Batch Process Control and Optimization

In general, fed-batch cultures are initially operated in batch mode; when a key nutrient(s) is exhausted, a solution containing the nutrient(s) is added to the media. As mentioned earlier, the feed solution and feeding strategy should be optimized. A common approach is to use partial concentrates (i.e., 10X) of the basal media as the feed solution; typically most of the salts found in the basal media are not included in the feed solution. The bioreactor is sampled at regular intervals and when one or more of the key nutrients (usually glucose and/or glutamine) is below a certain concentration, a precise amount of feed solution is added to raise the nutrient concentration up to its setpoint. A useful rule for choosing the nutrient setpoint concentrations is to provide enough nutrients to support cell growth and production while avoiding formation of toxic levels of catabolites due to excess feeding. Another method is to determine which of the media's several nutrients become depleted during the culture and add those particular nutrients to the bioreactor independently.[67] Wong et al. selected glutamine and glucose as key control nutrients, and used two feed solutions (glucose and glutamine solutions) to maintain them at specified concentrations.[68] Using this strategy, they maximized cell viability and density while decreasing accumulation of lactate and other catabolites.

Perfusion Process Control and Optimization

In perfusion bioreactors, supernatant is removed from the bioreactor at certain times, the cells are separated from the supernatant, the supernatant is harvested, and the cells are returned to the bioreactor. Perfusion bioreactors can be operated in a variety of modes. The simplest mode is to consistently remove a certain amount of broth each day (i.e., one bioreactor volume/day) and replace with fresh media. This mode is relatively easy to control. However, as the cell density increases, the required nutrient level may not be met. Also, the protein product tends to become diluted in the collected supernatant.[34,38] Another mode of perfusion operation is to remove relatively small volumes of media at a time and replace the volume with a concentrated solution of nutrients. This mode is similar to fed-batch bioreactors. One might think of this as a perfusion/fed-batch hybrid, and as with fed-batch bioreactors, considerable effort goes into determining the optimal nutrient setpoint concentrations and the feeding strategy. Sophisticated analysis and control schemes have been developed for this type of perfusion bioreactor.[69,70] A third mode of perfusion bioreactor operation attempts to maintain a pseudo-steady-state cell concentration, after an initial growth period. Dowd and coworkers employed an online cell density meter and were able to maintain a relatively constant cell concentration by altering the perfusion rate, the amount of media removed from the bioreactor.[38] This mode of perfusion reduces the frequency of sampling and analysis required to uphold a set nutrient concentration, but requires a well-characterized online cell density probe and a well-calibrated pump control scheme.

Scale-Up of Mammalian Cell Bioreactors

When scaling up a process to large-scale, it is important to maintain the same physical and chemical conditions as in small-scale. The chemical conditions include pH, oxygen level, concentration of medium components, and concentrations of toxic metabolites; these must be monitored and controlled to keep the cells in the proper physiological environment. The physical conditions include the bioreactor configuration and the power provided to the bioreactor. In scale-up, it is critical to preserve a similar geometrical configuration of the bioreactor in order to facilitate duplication of mixing patterns.

Impellers are an important physical component in a stirred bioreactor; they convert electronic energy to hydrodynamic motion and generate the turbulence required to keep the cells in suspension and achieve good

mass transfer. At large-scale, efficient oxygen delivery and carbon dioxide stripping become increasingly difficult due to poor mixing. At the same time, the energy generated at the tip of the impeller blades must be limited, as certain cell lines can be damaged by the elevated shear force. Therefore, to achieve good mixing and minimize possible damage of the cells from high shear force, one must determine the proper impeller shape, ratio of impeller to vessel diameter, and impeller tip speed.

The mixing time as well as the oxygen and carbon dioxide mass transfer rates can be correlated to the power per unit volume of the reactor, also known as the average or overall energy dissipation rate.[71] Maintaining constant power per unit volume is a commonly used scale-up strategy because of its simplicity. Other strategies include keeping the average shear force experienced by the cells constant, or keeping the maximal shear force constant (the shear force experienced by the cells when passing the impeller tip).

In addition to scale-up models, scale-down models are widely used to establish the operating ranges of critical large-scale process variables.[72,73] Conducting the many experiments required to define a validated range for each parameter is not feasible at large scale. A scale-down model based on an existing large-scale process therefore becomes an efficient and economic tool in reaching this goal.

PURIFICATION PROCESS

The process of purification, also called "downstream processing," depends on the product and the degree of purification required.[74] Current strategies used for purification of therapeutic proteins generally involve these steps: (1) sample preparation (clarification or extraction), (2) product capture (product concentration), (3) intermediate purification (removal of bulk impurities), and (4) polishing (removal of trace impurities) as shown in Figure 32.4.

Microbial cells such as *E. coli* and yeast are limited in their ability to make glycoproteins or correctly structured glycoproteins. Therefore, therapeutic proteins (including monoclonal antibodies) that require glycosylation

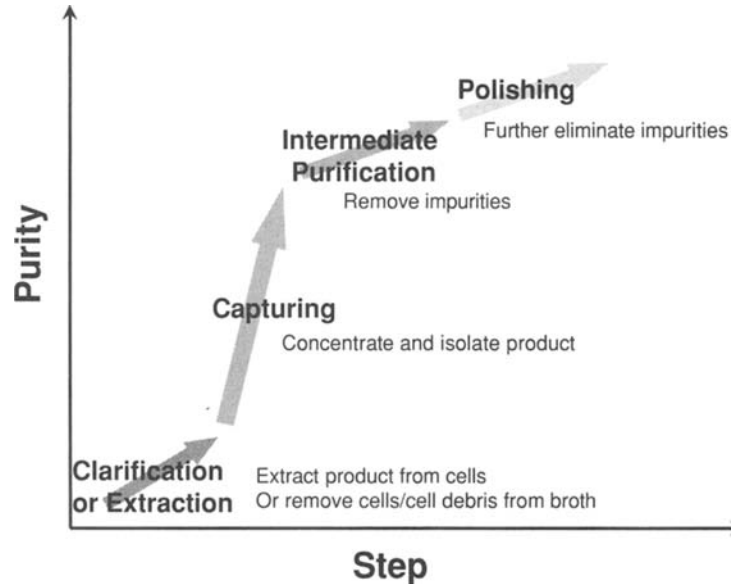

Fig. 32.4. General strategies for protein purification (permission of Amersham Biosciences, GE Healthcare).

for their biological activity are often produced via cultivation of mammalian cells. Compared to microbial fermentation, mammalian cell culture generates complex broths with a variety of impurities. Typical impurities found in culture broths include:

1. Host cells and cell debris
2. Host cell protein (HCP) and DNA released by the cells
3. Aggregated proteins or cleaved proteins produced by the cells
4. Medium additives (such as serum and protein used to support cell growth)

These impurities pose risks for the safety of proteins used as therapeutics and must be removed to a final concentration below their target limit. In addition, the product stream contacts materials such as filters and resins. Extractables, such as leachates from protein A resins, can pose an immunogenic risk to the patient and must be eliminated.[75] Finally, adventitious agents such as viruses and bacterial pathogens or related contaminants such as endotoxins can lead to serious problems with the safety of the protein preparation and therefore must be minimized. Table 32.6 lists concentrations for the above impurities that are generally considered acceptable in a final protein product.[76]

This section describes some general processes used for protein purification, including methods and tools currently employed by the bioseparation industry to achieve clarification, capture, and removal of impurities.

TABLE 32.6 Concentrations of Impurities Generally Acceptable in Final Protein Product

Impurity	*Concentration in Final Product*
Cell debris	0
Host cell protein and serum	<0.1–10 mg/L
Aggregated/cleaved proteins	Various
DNA	10 ng/dose
Leachates	0.1–10 mg/L
Virus pathogens	$<10^{-6}$/dose
Endotoxins	0.25 EU/mL

Generic Processes

As shown in Figure 32.3, each purification step has different goals. The purification problems associated with a particular step will greatly depend on the properties of the starting material. Thus, the goal of a purification step will vary according to its position in the process.

Chromatography is one of the most important tools in protein purification. Chromatographic purification techniques include affinity chromatography (AC), ion exchange chromatography (IEX), hydrophobic interaction chromatography (HIC), and gel filtration (GF). These techniques separate proteins according to differences in specific protein properties. The protein property used for separation, the attributes of each technique, and its suitability for different purification steps are summarized in Table 32.7.

Selection of the purification strategy will depend on the specific properties of the sample and the required level of purification. Due to the widely differing properties of proteins, a final purification strategy that is most suitable for one protein may be unsuitable for another. A logical combination of chromatographic steps can usually achieve the final goal of protein purification. Figure 32.5 shows two flow diagrams commonly used for the purification of proteins expressed by mammalian cells.

Clarification of Broth

For proteins secreted into broth, the first objectives after cell culture are to remove cell mass and debris, to reduce processing volume, and to bring the product to a stable holding point for further purification steps.

At bench-scale purification, a two-step operation of depth filtration followed by sterile filtration (or centrifugation followed by sterile filtration) can efficiently remove large particles, colloidal particles, and bacteria. At industrial scale, however, the clarification step is usually completed by three stages in series as shown in Figure 32.6.

Centrifuges can function in batch-mode and continuous mode. Batch-mode works well for laboratory-scale processes, whereas continuous-mode can handle larger volumes and is thus

TABLE 32.7 Attributes and Purification Suitability of Chromatographic Techniques

Technique	*Protein Property*	*Main Attributes*	*Capture*	*Intermediate Purification*	*Polishing*
Affinity chromatography (AC)	Ligand specificity	High capacity High resolution High speed	+ + +	+ + +	+ +
Ion exchange chromatography (IEX)	Charge	High capacity High resolution High speed	+ + +	+ + +	+ + +
Hydrophobic interaction chromatography (HIC)	Hydrophobicity	High capacity High resolution High speed	+ +	+ + +	+
Gel filtration (GF)	Size and shape	Low capacity High resolution	−	+	+ + +

Permission of Amersham Biosciences, GE Healthcare.
(+ + +) = highly suitable; (+ +) = very suitable; (+) = suitable; (−) = not suitable.

conducive to large-scale processes. Both depth and tangential flow filtration are commonly used in the clarification. For large-scale purifications, depth filters may have volume and throughput limitations. Tangential flow filtration becomes a better choice when processing large harvest volumes.

Capture of Product

The goal in the capture step is to concentrate and isolate a protein product. This step is usually accomplished with ion exchange or affinity chromatography. Ion exchange separates protein molecules based on interactions between charged side chains on the protein surface and oppositely charged groups, such as ammonium or sulfate, covalently linked to the chromatography matrix. The charge characteristics of a protein can be altered by changing the pH of the separation. Based on the charge differences of a targeted protein and other impurities, conditions at the capture step are selected to maximally bind the targeted protein

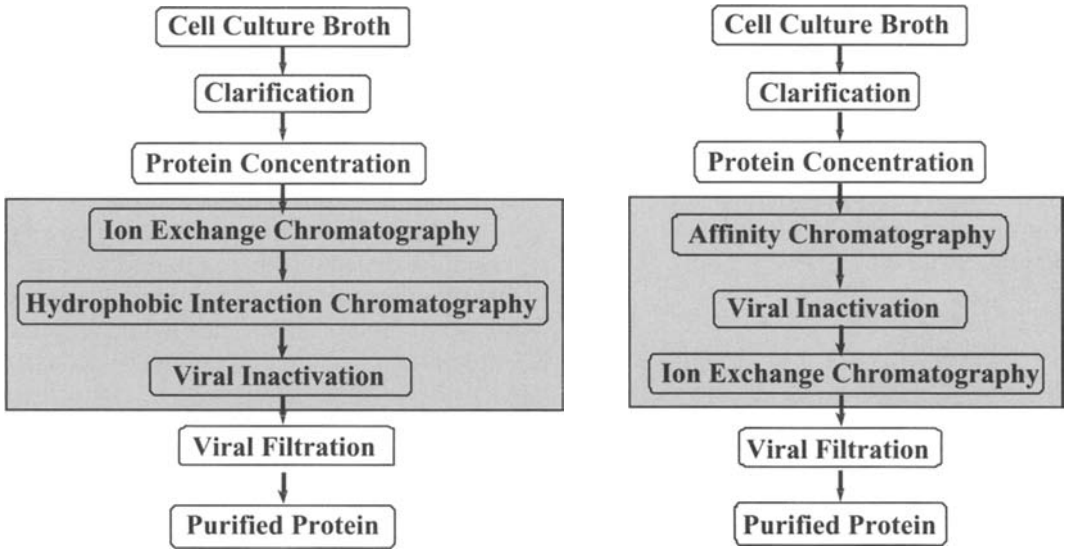

Fig. 32.5. Flow diagrams for protein purification.

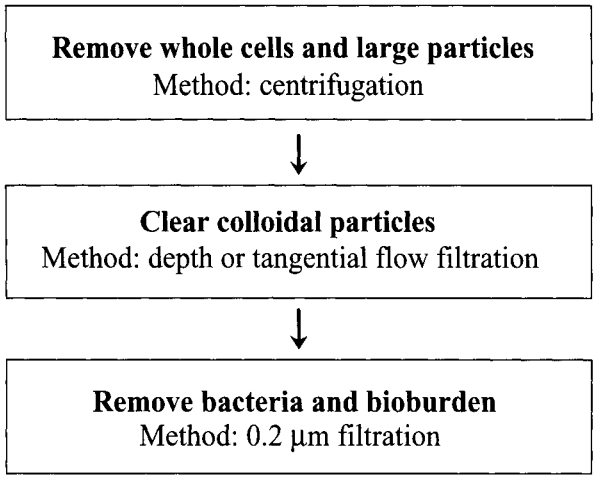

Fig. 32.6. Procedures for broth clarification at large-scale.

and minimally bind the impurities to achieve isolation of the product. Ion exchange is probably the most frequently used chromatographic technique for the separation and purification of proteins. It offers many advantages such as high capacity, long resin lifetime, and low cost. Its operation is relatively simple and easy to control. A major disadvantage is that it usually only leads to moderate purity.[77]

Affinity chromatography separates proteins based on a reversible interaction between a protein and a specific ligand coupled to a chromatography matrix. One of the most important attributes of this method is its excellent selectivity. As a result, affinity purification offers immense time savings over less selective multistep procedures. A single-step operation with affinity chromatography can achieve purification levels on the order of several thousandfold with high yield. The most successful example of affinity chromatography in protein purification is the use of Protein A to capture monoclonal antibodies. One-step purification with Protein A not only results in high yields of targeted antibodies, but also removes bulk contaminants such as host cell protein, DNA, and virus, leading to purity greater than 95 percent. Another advantage of using Protein A is that it works for multiple antibodies. Major disadvantages are: (1) high cost, (2) cleaning and sanitization difficulties, and (3) ligand leakage.

There are many different types of Protein A affinity media: MabSelect™ (GE Healthcare, Chalfont St. Giles, UK) and PROSEP Ultra (Millipore, Billerica, MA) are the two most commonly used at production scale. Recently, GE Healthcare released a new type of Protein A called MabSelect SuRe™. This latest MabSelect resin is resistant to deterioration from cleaning and sanitization-in-place with 0.1–0.5 NaOH.

Removal of Impurities

Although the capture step dramatically enriches a targeted protein and removes some impurities, bulk impurities such as host cell protein, DNA, endotoxin, virus, and leaching ligand remain in the eluted pool. Additional procedures are needed to eliminate these impurities.

Depending on the required level of purity, the step used for removing impurities can be a single or multiple-step chromatography operation. Table 32.8 summarizes chromatographic methods that can be used to remove impurities following the capture step.[76,78–80]

TABLE 32.8 Chromatography Methods Used to Remove Impurities

Impurity	*Methods Used to Remove Impurity*
Host cell protein	AEX, CEX, HIC, AC
Aggregated/cleaved proteins	CEX, HIC
DNA	AEX, CEX, HIC, AC
Leachates	AEX, CEX, HIC
Virus pathogens	AEX, CEX, HIC, AC
Endotoxins	AEX, CXE, AC

For monoclonal antibody purification in which Protein A is primarily used for protein capture, cation and anion exchange chromatography are employed to reduce the remaining impurities.[81] Because the majority of monoclonal antibodies have a pI greater than 7 and predominating impurities (HCP and protein A leachates) have pIs lower than 7, cation exchange can retain the product and allow the impurities to flow through. Anion exchange can be employed as a noproduct-binding step to remove residual DNA and endotoxin. In this mode, the trace impurities will be retained on the column and the antibody will flow through. This step typically clears 2–5 logs endotoxin and 3–5 logs DNA.[76]

Separation by hydrophobic interaction chromatography (HIC) is based on the reversible interaction between a protein and the hydrophobic surface of chromatographic medium. This interaction is enhanced by high ionic strength buffers. Thus, HIC usually follows an ion exchange step where a high salt buffer is used for elution. It can be used to remove impurities such as host cell protein, DNA, and virus. However, at production scale, it is not as widely used as ion exchange chromatography.

Gel filtration separates proteins based on size and shape and can also be applied to remove the trace impurities. However, because this method is usually very slow, its application is often limited to bench or pilot scale.

In addition to chromatography, membrane filters have also proven to be effective in removing virus and endotoxin. For example, application of Sartobind™ membrane adsorbers made by Sartorius (Goettingen, Germany) results in up to 5 logs reduction of endotoxin. The Ultipor® VR grade DV50 membrane by Pall Corporation (East Hills, NY) demonstrated a greater than 7 log clearance in removal of retroviruses.[82]

Strategies for Scaling Up Purification Processes

The great demand for high-quality therapeutic proteins requires efficient manufacturing processes, both upstream and downstream. When bioreactors are scaled up from bench to pilot to production scale (10,000–20,000 L), downstream processes must be scaled up accordingly. Equally complicated as upstream processes, scale-up of downstream processes requires more than simply increasing the size and volume of the laboratory equipment. The use of larger piping dimensions, larger filters, different types of pumps, and larger dead volumes can introduce variation to the large-scale process. We've limited our description here to common strategies used by the industry to scale up chromatographic columns.

Scale-Up of Affinity Chromatography.[78]

1. Prior to scale-up, different protein A resins should be screened. To achieve high production rate, defined as the amount of protein purified per unit time and per unit column volume, a resin must have a high dynamic binding capacity and be able to operate at low backpressures.
2. Before scale-up, the column volume based on the required binding capacity must be calculated and the optimum bed height that allows high flow rates and high dynamic capacities must be determined.
3. During scale-up, the residence time, which is equal to the bed height divided by the linear velocity of the fluid, must be kept constant.
4. The operation flow rate should not exceed 70 percent of the maximum flow rate specified by the resin's vendor.

Scale-Up of Ion Exchange Column.[78]

1. Prior to scale-up, screen resins to obtain those that offer a long lifetime, lot-to-lot consistency, and long-term availability.
2. During scale-up, the column volume should be increased by keeping the height of the resin bed constant and increasing the column diameter.
3. During scale-up, the total load of protein per unit of resin should be the same and the linear flow rate should be identical.

4. Scale-up usually involves two steps: the first step results in an increase of 50- to 100-fold, from laboratory to pilot scale; the second step results in an increase of 10- to 50-fold, from pilot scale to full production scale.[83]

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