



New Technologies Optimize Biotech Process Efficiency

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Product quality and biotech processing efficiency are improving, thanks to new expression systems, advanced purification materials and applications, and novel process monitoring technologies.

SARTORIUS

The pace of biotechnology advancement has never been more rapid, with the number of biotech research centers, academic programs, and proposed manufacturing sites at an all-time high. Though biotechnology products are targeting a steadily widening range of therapeutic applications, their manufacture also entails levels of complexity previously unseen in the pharmaceutical industry. As a result of this complexity, manufacturers have their eyes squarely focused on two critical considerations: time and cost. As a result, any technology that promises to reduce one or both of these factors, without compromising product quality, is sure to gain the industry's attention.

New expression systems

To begin with, therapeutic proteins must be generated either through fermentation (for microbial- and plant-based expression systems) or cell culture (for mammalian cell-based systems). This is a critical step in the process because it not only establishes the initial quality of the media required but also the maximum yield of the therapeutic being created. Any reduction of yield can translate to millions of dollars lost. As a simple example, for a \$100 million drug, a 1% yield loss would be equivalent to losing \$1 million.

Yield levels depend greatly on the type of expression system used. Mammalian cells are the most commonly used host systems for producing therapeutic proteins. Cell culture systems, however, produce media rich in impurities, thereby requiring

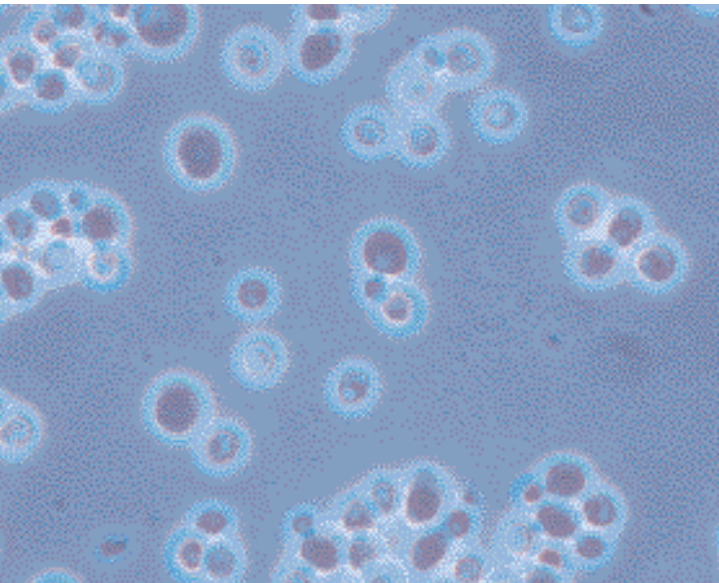
a complex purification process. Furthermore, some proteins are not easily expressed in mammalian cells.

Two well-established alternative expression systems are microbial systems and plant-based systems. One advantage of microbial expression systems (e.g., bacterial, fungi, or yeast) is that they require very few additives during fermentation, thus simplifying subsequent purification processes.

Choosing between a microbial or mammalian expression system starts with a close examination of the protein of interest. For example, Tillman Gerngross, PhD, chief scientific officer at GlycoFi (Lebanon, NH) suggests first looking at whether the protein is glycosylated. "Depending on that answer," says Gerngross, "you're going to go down one of two very different paths."

Glycosylation is the attachment of specific complex branched carbohydrate structures to the protein, facilitating therapeutic efficacy by allowing the immune system to recognize the therapeutic agent. However, while a choice of expression systems (typically *E. coli* or yeast) is possible for nonglycosylated proteins, until recently, glycoproteins had to be produced exclusively via mammalian cell culture. Other platforms were unable to carry out the necessary post-translational modifications similar to those that occur in the human glycosylation process.

Today, however, other options exist. GlycoFi, for example, has developed a way of engineering yeast (see Figure 1) such that it mimics the human glycosylation process. "From the beginning we felt that given a choice of making a protein in yeast or in mammalian cell, it would be cheaper in yeast," says Gern-



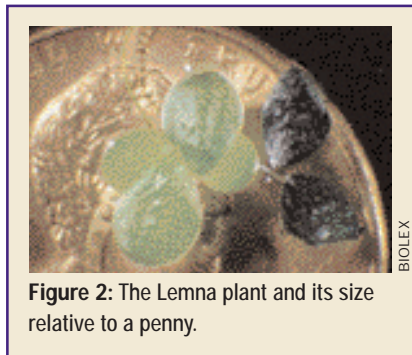
GLYCOFI INC.

Figure 1: 1000× magnification of GlycoFi's *Pichia pastoris* yeast cells cultured in a bioreactor fermentor.

gross. The approach also offers advantages in production rate, potency, media impurity levels, and the number of process steps. "This manufacturing process requires little if any modification to existing infrastructures, and we felt that any technology that was going to provide improvements to manufacturing had to be compatible with the way industry is already set up," says Gerngross. Because fermentation can be conducted in a thermally sterilized environment that allows the entire medium in the tank can be autoclaved, Gerngross notes that "there's no need for a sterile filtration process, and no known retroviruses are present in yeast. We liken it to a software solution for a hardware problem."

Another alternative to mammalian cell expression is the use of plants as supporting hosts. Biolex (Pittsboro, NC), for example, has developed its "Lemna Expression System" that is based on the Lemna (duckweed) plant, a tiny aquatic plant that the company grows in aseptically sealed vessels (see Figure 2). The Lemna plant has high transformation efficiency, and the company is able to obtain a homogenous plant line, with up to several percent of the total tissue soluble protein composed of the protein of interest.

Lemna also is fast growing, doubling its biomass every 36 hours, and because the plants reproduce clonally, no seed or pollen is involved. Purification is also easier, says David G. Spencer, PhD, chief operating officer and senior vice-president of R&D for Biolex, noting the inorganic media requires only salts and water. "It gives you all the benefits of mammalian cell culture without the complications and expense." So far, the company has expressed 19 proteins and is currently working on ex-



BIOLEX

Figure 2: The Lemna plant and its size relative to a penny.

pressing its own protein products, as well as those of partners such as Bayer, Centocor, and Debiopharm.

Healthy diet

Fermentation and cell culture are delicate processes, with host cells grown to a specific density under controlled environmental conditions. Any change in these conditions, or any contaminants inadvertently introduced to the cells, may lead to cell damage or death and potentially to the loss of an entire batch.

Successful cell growth also requires precise feeding of nutrients such as basic amino acids, vitamins, salts, and sugars. Traditionally, the process of feeding mammalian cells has been accomplished by introducing these nutrients as dry-powder media (DPM) into a bioreactor. However, the use of DPM has considerable drawbacks, most notably its tendency to produce "clouds" of powder when poured into a tank.

To deal with the considerable risk of DPM cross contamination, companies typically must invest in dust-collection systems. Also, because some additives such as growth factors and cholesterol don't conform to the DPM format, these ingredients must be introduced separately. Finally, because of the hydrostatic property of the material, DPM can take as long as 60 minutes to dissolve in solution.

A more controlled method of introducing nutrients to bioreactors is now offered as a result of a collaborative effort between Invitrogen (Carlsbad, CA) and Stedim (Aubagne, France). An alternative to traditional dry powder or liquid media formats, the disposable bioprocessing system uses Invitrogen's "Advanced Granulation Technology" (AGT) media granules in Stedim's sterile, single-use plastic bags. Invitrogen applies fluid-bed granulation technology to produce complex, serum-free, protein-free, or chemically defined media in granular format (see Figure 3).

AGT granules contain all the additives needed for cell growth, and they produce specific ionic and pH conditions upon reconstitution in water. As observed by Shawn Smith, business

segment director for cell culture production at Invitrogen, "the cell culture industry has historically been a bit conservative in terms of trying new things, but because of the utility of [the AGT system] and because it enables more complex, animal-free types of media, it has been taken up quickly." Smith adds that the company's new venture with Stedim allows the granules to be supplied within a self-contained unit. Water is added to the closed system and when the media is dissolved, it is then fed through a sterile connecting device into a bioreactor or robotic filling system. "The media never have to [be exposed to] the external environment," says Smith. Future plans for the system include the incorporation of filtration devices and customized tubing configurations.

Quality improvement

Filtration serves two basic purposes in both fermentation and cell culture processes: the removal of impurities and particulates from media and the prevention of contamination. The two

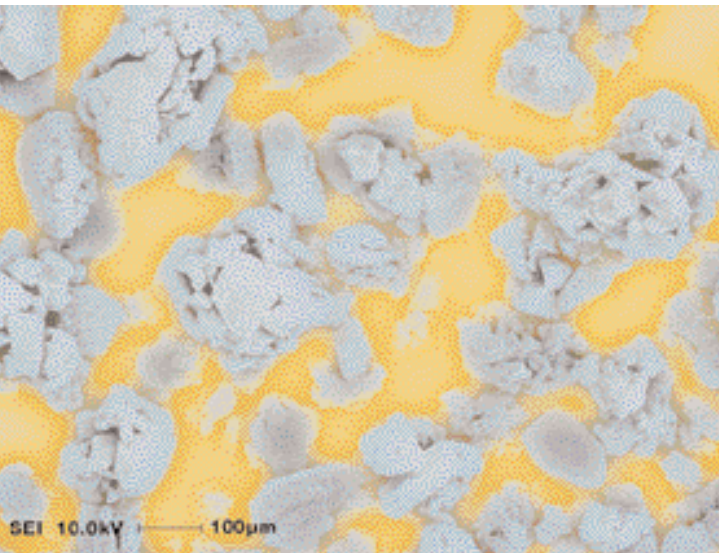


Figure 3: Invitrogen applies fluid-bed granulation technology to produce ingredient media in a granular format.

principle technologies used to remove contaminants and to separate the therapeutic protein from media are filtration and chromatography. After fermentation or cell culture, the media (often referred to as conditioned media or harvest fluid) is clarified, usually by centrifugal separation and/or filtration (either cross-flow membrane filtration or direct-flow depth filtration). The media then undergo downstream processing, which involves a series of operations resulting in a “purified” protein.

Filtration during the purification process is quite complex, with both filtration and chromatography operating to remove impurities from the media, acting as prefilters, chromatography column protectors, micro-, nano-, and ultrafiltration filters serving to affect buffer exchange and/or concentration via tangential flow filtration, and so forth. “The most critical step is the purification step,” notes Maik Jornitz, group vice-president of global product management bioprocesses at Sartorius Corporation (Edgewood, NY). “Although the expression system and media are important, if you don’t have the purification step in hand, that’s where you’re going to see most of your losses. If your purification step is not optimized, you might get protein degradation and activity losses.”

Microporous membranes are used in both chromatography columns and filter cartridges. Although membrane technology isn’t new, it’s now being used in novel configurations. For example, Meissner, which manufactures microporous membranes, has recently developed its “Protec RM0.5” two-level filter. This filter combines two types of filter media in one unit, with borosilicate glass fiber as the upstream layer and a microporous PVDF membrane as the downstream layer. Barry Bardo, Meissner’s director of Business Development, explains that the filters can be used as effective prefilters after the fermentation step because the borosilicate glass microfiber has a particularly high “dirt-holding” capacity or weight-per-surface-area (see Figure 4). “The idea is that between the two media types, the filter will remove a large amount of ‘plugging material’ such as colloids, proteins and fat, lipids, and other solids that might be

in a bioprocessing fluid,” says Bardo. Companies perform this prefiltration before proceeding to the purification steps—often after centrifugation separation and before the use of a downstream membrane—because the filter is able to remove particles down to approximately 0.5 μm , thus extending the life of sterilizing filters that are located downstream.

Optimizing the process—sometimes referred to as “system compression”—by having more than one technology within one unit, is quickly becoming a common practice in processing. “It makes sense to do it wherever you can,” says Bardo. “It cuts down on labor and time and uses fewer housings and filters. We’re trying to streamline the process and cut costs wherever we can.”

The use of membrane adsorbers to separate recombinant proteins from whole cells is also becoming an increasingly popular practice. According to Herb Lutz, strategic marketing manager at Millipore Corporation (Bedford, MA), “Doing it on a membrane format instead of a bead format is a different way of doing a column.” One application is the removal of DNA, such as for monoclonal antibodies, by running the process at high pH. In such cases, the antibody goes straight through the column, or straight through the membrane adsorber, while the DNA becomes negatively charged and binds to a positively charged resin (or positively charged membrane). “Running it through the flow-through mode is an application of a membrane type format” says Lutz, “and we’re starting to see that being used selectively in some biotech processes.”

Membrane technology also is used to remove viruses from mammalian cell-based media. “Some earlier processes used chromatography for this step, but now virus filters are really becoming the standard,” says Lutz (see Figure 5). One reason for this trend is that filters are a more robust method of performing viral clearance. Because some types of chromatography are very sensitive to small changes, such as in buffer pH, if the amount of protein varies by only a small degree, then the ability of the chromatography step to remove viruses may be affected. Another reason is that filters offer the benefits of disposable technology, including simplified validation and ease of use.

To address the industry’s needs for effective viral clearance, Millipore recently developed its Opticap XL and XLT disposable capsule filters with its “Viresolve” normal flow parvovirus (NFP) membrane. With a diameter of ~ 20 nm, the parvovirus is one of the smallest viruses to be removed. According to the company, its Viresolve NFP membrane uses size-exclusion technology to clear the parvovirus by a log value of 4.

Membrane adsorbers also are used to remove viruses and other contaminants but through a different mechanism. For example, Sartorius’s “Sartobind” line of ligand-spiked membrane adsorbers are constructed of reinforced cellulose with a microporous structure and pore sizes larger than conventional chromatographic gel matrices (see Figure 6). Rather than trapping contaminants, the system captures the target molecules by transporting them by convective flow to the ligands where they are retained. Optimization of the process depends on what is being run through the chromatography system and its interaction with the ligand-spiked membranes. According to Jornitz, “To optimize the capture of the target protein, given that



Figure 4: Meissner UltraCap(r) disposable capsule filter with Protec RM0.5 microfiber glass and PVDF membrane media inside. Photo courtesy of Meissner Filtration Products, Inc. and Anderson Instrument Co., Inc.



Figure 5: Millipore's Viresolve NFP family of products provides rapid viral clearance from recombinant or human plasma sources with its size-exclusion technology.



Figure 6: Sartorius's Sartobind SingleSep 10-in. capsule incorporates the company's membrane adsorber technology for the rapid purification of proteins.

the protein and the chromatography ligands have specific charges (isoelectric point), salt concentrations and pH will have to be well balanced."

Once the protein is captured on the ligand, the column is washed to remove any contaminants. Then salt gradients are run through the column, with the salts becoming "competitive" with, and releasing, the target proteins bound to the ligands. "That's how you get your concentrated protein," says Jornitz, "And in development, you need to find out what is the best packing, the best loading, and the best elution situation. And that's when expertise and experience come into play."

One recent application of membrane technology incorporates the advantages of both chromatography and filter configurations. Pall Corporation's (East Hills, NY) system incorporates two membrane-based methods; namely, its "Mustang Q" technology, an ion-exchange membrane technology, and its "Ultipor VF DV50" virus filter technology. Both membranes are used sequentially for DNA and viral clearance (see Figure 7).

The approach is currently being implemented by BioMarin Pharmaceutical for the development of Aldurazyme, a drug developed through a joint venture with Genzyme Corporation. It marks the first time the two-sequential-membrane technique has been applied, recognized, and approved by both FDA and EMEA for the removal of contaminant DNA and contaminating viruses. "No one step can be proven to inactivate or remove all potential viruses," says Jerold Martin, senior vice-president

and global technical director of Pall Life Sciences. "However, you can take an approach to use multiple steps that are each capable of either inactivating or removing viruses."

Combining chromatography and filtration delivers a cumulative effect. "Removing or inactivating viruses by different mechanisms increases the total process clearance by adding up the logarithmic clearances that you get in each step," says Martin. He explains that BioMarin's application was unique because the company chose to incorporate additional dedicated viral clearance steps downstream from the purification steps. They accomplish this by passing the fluid through a wall so that the viral clearance steps are conducted in a dedicated area.

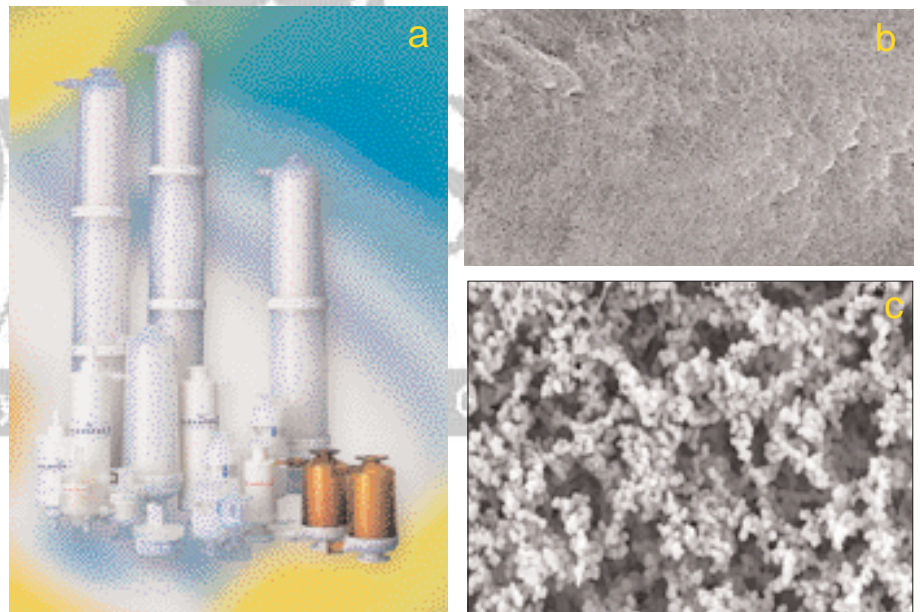


Figure 7: (a) Pall Ultipor VF DV50 membrane and filters; (b) Cross section of Pall's Ultipor VF DV50 membrane (0.05 μm rating); (c) Cross section of Mustang Q membrane used for viral clearance (0.8 μm rating). Photos courtesy of Pall Corporation.

Quality maintenance

In addition to ridding a product of impurities, manufacturers also must detect the presence of contaminants that may have been introduced into the material during processing. Recently introduced tools have made this task easier to accomplish by moving process analysis and monitoring procedures out of the laboratory and onto the manufacturing floor.

For example, a routine part of bioprocess monitoring is endotoxin detection. Endotoxins are ubiquitous pyrogenic agents that are very hard to remove once in the process. For example, a highly pyrogenic culture of bacteria can be put into an autoclave and, though the autoclave will kill the bacterium, the endotoxin pyrogens will still be present in the sample. In general, it is not possible to remove endotoxins by filtration on the basis of size because their molecular weights are fairly large and similar to the molecular weights of many proteins. Once in the system, they are there to stay.

To address this concern, Charles River Laboratories (Charleston, SC) has produced a portable, handheld Limulus Amebocyte Lysate (LAL) pyrogen test unit that provides results in about 15 minutes and uses disposable cartridges, thereby eliminating the need for tubes and other laboratory products. The "Endosafe PTS" LAL system is specifically designed for point-of-use testing, and according to John Dubaczak, operations manager at Charles River Laboratories, the process lends itself well to in-process monitoring. "Manufacturing personnel can now pull their own sample, run their own tests, get a quantitative real-time test report, and move on," he says. Although the method has not yet been FDA approved, the formulation used in the cartridge is an FDA-licensed reagent. Current users implement it as a "go-no go" tool (see Figure 8).

BioPAT progress. FDA and industry both hope to take at-line monitoring of product quality one step further. One currently debated topic is the application of FDA's process analytical technology (PAT) initiative to the manufacture of biological and biotech therapeutics. Under consideration is the feasibility of the rapid microbiology method (for bioburden testing) for use in bioprocesses as well as other technologies such as membrane introduction mass spectrometry (MIMS), temperature monitoring and sensing, oxygen monitoring, and pH sensor technology.

One PAT candidate under development is NIR spectroscopy. For example, Foss NIRSystems (Silver Spring, MD) recently designed an on-line system for monitoring the process of mammalian-cell cultivation at the pilot scale. At a presentation before FDA's Advisory Committee for Pharmaceutical Science in April, Robert A. Mattes, laboratory instrumentation scientist at FOSS NIRSystems, presented the company's findings on the use of NIR spectroscopy in biotech process applica-

tions. The NIR system was used to study chinese hamster ovary (CHO) cell cultivation. Mattes points out that many companies have been using NIR spectroscopy to analyze the feedstocks in the "broth" used for cell culturing. Possible measurement parameters include the amount of glucose, glutamate, glutamine, lactate, and ammonia (which builds up over time).

Mattes is currently working with researchers on measuring amino acids. Here, however, the difficulty is the number and variety of amino acids with similar absorption spectra, requiring a large number of samples. "We hope to be able to differentiate them, but we haven't completely finished the job yet," he says. Mattes admits that one limitation of NIR is that it doesn't measure inorganics well, such as those present in salts, and that there's a lower limit to what it can detect (i.e., it is not suitable for trace analysis). Also, Mattes notes that when there's a high cell density in the reactor, as is sometimes the case with *E. coli*-based systems, it becomes impossible to make transmission measurements, so reflectance measurements must be used. "This isn't a problem with mammalian cells because they never reach such a high cell density that they become opaque," says Mattes. Future plans include determining the number of constituents the technology can analyze, building models for them, and scaling up the process to production scale (see Figure 9).

Elizabeth Fowler, vice-president of quality and regulatory affairs at Xcellerex (Marlborough, MA), a contract services provider for biotherapeutics process development and manufacturing, says the industry currently performs a considerable amount of process monitoring.

For example, during the fermentation step, real-time off-line process monitoring typically includes the evaluation of cell growth and cell viability as well as metabolic parameters such as pH and glucose. Likewise, during the purification process,

parameters such as flow rate, conductivity, and pH are continually monitored on-line. These parameters serve as surrogates to verify process consistency, which in turn helps to ensure biosafety, product quality, and (in the case of the purification process) the removal of impurities. "There is presently no way to assess many of the important product quality attributes in real time," says Fowler. "There are real opportunities for technology development to meet this need."

Fowler points out that key drivers for monitoring during production of biologics are biological variation in material (including animal to animal variation and cell culture variation), biological safety related to unknown pathogens, and unrelated impurities with unknown activities.

Future goals for PAT principles in bioprocessing include the evaluation of the characteristics of the active pharmaceutical ingredient such as quality,

Figure 8: A portable, handheld LAL test system incorporates disposable cartridges to allow for testing at the point of use during processing. Results are available in approximately 15 minutes and as many as 100 quantitative results can be stored or downloaded.



CHARLES RIVER LABORATORIES



Figure 9: Foss NIRSystems has recently configured a pilot-scale NIR spectroscopy sensor for use in the analysis of CHO cells during cultivation.

content, and concentration as well as the assessment of bioburden and presence of viruses during fermentation and purification to ensure that product quality remains consistent throughout development. "PAT is not an all-or-nothing phenomenon," says Fowler. "Companies will need to evaluate where PAT fits best." She suggests that once a company determines the key process factors that should be monitored, it will have to examine the data it already has, the best ways of obtaining those data, and whether the benefits justify the cost.

Biotech growth: gains and pains

When even a 1% difference in product yield can be equivalent to millions of dollars, process efficiency is vital. While FDA encourages adoption of its risk-based approach to the manufacture of conventional pharmaceuticals, those in biotech manufacturing point out that this has always been their strategy because of the realities of the industry.

Recognizing the tremendous gains provided by running their processes at optimum levels, some companies are, in fact, willing to go through extra regulatory documentation, filings, and scrutiny just for the chance of maximizing their yield and reducing their costs. Such strategies also may circumvent the manufacturing capacity shortages that have been predicted. Says Sartorius's Jornitz, "There are two camps: One says there's a capacity crunch, while the other says that because of new expression systems and process optimization, no such crunch will materialize." Pointing to the full-order books of fermentation businesses, Jornitz admits a capacity need may exist but suggests that it's a need that can be, at least in part, covered by optimizing manufacturing processes. "We're seeing a high demand to conduct surveys of existing processes to optimize their fermentation and purification," he says. "In fact, demand is so high that even if there exists a possibility of meeting the 'barrier' of filing postapproval changes, companies aren't afraid, because they see the value behind it. And the value is tremendous." **PT**