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Protein Production from Mammalian Expression Systems for Research and Early Development at Contract Facilities — A Primer

By TRISH BENTON

Photo courtesy of Millipore

In the late research/early development stage of a biologic, it is often necessary to produce a larger quantity of a protein than a company is capable of producing internally. At this stage, the availability of the purified protein may govern its continued development. The protein needs to be produced with a minimal amount of waste; i.e., with a minimal amount of personnel and expense in the shortest amount of time possible.

Often, at this stage, the expression system is in its infancy. Expression levels are often quite low and procedures for production and purification are not robust. Small amounts of the protein may have been produced for antibody production and assays to analyze and quantitate the protein. These assays facilitate the scale-up and production of larger quantities of the protein.

The reasons vary why an organization may not be able to scale up protein production. In some cases, it is a lack of available equipment and skilled personnel. In others, the time frame in which the protein is needed necessitates a change of the production philosophy from one of small batches that will fit in an incubator to large batches that require spinner flasks or bioreactors.

In general, a "batch" run will take five to ten days of residence time in the incubator or bioreactor. Therefore, the number of batches that is necessary to produce the desired amount of the protein can have a significant effect on the delivery time. Chances are, if the capacity for upstream (fermentation) is not available, the capacity for downstream (purification) operation will also be lacking. However, downstream purification can be split into discrete batches more easily than fermentations, and will have less of an effect on the timeline than running multiple fermentations.

Often the answer to protein production at this scale and at this point in development is in developing a relationship with a skilled contract manufacturing organization (CMO) that has the equipment and experience necessary to produce the material. Before beginning a search for a CMO, gather detailed information about the capabilities of the expression system in order to estimate the scale of the project. This, in turn, will allow assessment of potential CMOs. Providing more accurate information will increase the chances of receiving an estimated cost and a realistic project plan.

A general description of the cell line (Fig. 1) will help define the capabilities of a CMO that is likely to produce a realistic project plan and timeline, and meet your needs cost effectively. It may also point out areas that need to be addressed before the scale-up can take place.

A culture that is adapted to suspension is easier to scale up than one that requires a plastic substrate or other solid support for cell growth. Therefore, choosing an adherent cell line may limit the number of CMOs that have the

Trish Benton *(trish_benton@pacbell.net) is an independent consultant specializing in mammalian media development, gene expression, and production of recombinant proteins.*

facilities and skills to complete the project. In addition, the cost of the plastic substrate is a potentially significant additional expense. The culture of cells on microcarrier beads often requires so much development work that adaptation of the cells to suspension may be more economical and time efficient.

In the research and early development stages, there are few, if any, regulatory concerns about the composition of the media used in producing a protein, including the addition of large amounts of serum. Theoretically, any media formulation can be scaled up, although the use of high-protein media formulations in scale-up may be impractical. For example, when formulations that contain large amounts of serum or other proteins are used in an air-lift or well sparged bioreactor, the foaming that occurs can result in the need to add large amounts of anti-foam, potentially affecting both protein quantity and quality. As a result, a media formulation that is rich in basic nutrients may give better results than a traditional formulation supplemented with serum, even though there is little difference in the cost of the media. In many research laboratories, it is a common practice to grow cells to peak density in a high-serum media, then switch the cells into a low-serum media for protein production. In a bioreactor system, this can be very stressful to the cells and is technically difficult. It also disregards the protein that may have been produced during the growth phase, necessitating additional scale-up.

The care and feeding instructions, average doubling time, and the maximum cell density are necessary to estimate the timing and scale of the seed for the production culture. Figure 2 contains an example calculation for a cell line from the freezer to a 100-L bioreactor.

A growth and viability profile will allow the determination of the cumulative viable cell days (CVCD) in a typical batch culture. The CVCD is the cumulative number of live cells per day of culture and can be thought of as the "area under the growth curve." Knowing the CVCD and the average specific productivity (pg/cell/day) or Q*p* allows determination of the expected volumetric production of the culture. For example, if the CVCD of a culture is 4×10^7 cell/ml and the average specific productivity is .1 pg/cell/day, the estimated protein production for the run is 4×10^6 pg/ml (4 mg/L).

CVCD x Q*p* = volumetric productivity

Culture conditions affect the CVCD and the specific productivity. The ability to keep the conditions as constant as possible in the scale-up will improve the accuracy of the estimation.

Often in the early stage of development, and especially with proteins other than antibodies, the expression levels are very low. There are two manipulations that may increase expression levels that warrant small-scale testing before beginning work with a CMO. The addition of a histone deacetylase inhibitor, such as sodium butyrate, to a production culture that has reached its maximum cell density has been reported to increase protein production levels.[1–5] Another manipulation that is easily translated to large-scale culture that has demonstrated the ability to increase protein expression is to reduce the culture temperature from 37° C to 30–32° C at maximum cell density.[6] Variations of each of these manipulations are covered by pending and approved patents. As a result, you should consult with your legal department concerning the use of these techniques.

Using the example above of 4 mg/L volumetric productivity to produce 50 mg of purified protein (assuming the purification protocols allow for 100 percent recovery of the protein), 12.5 liters of conditioned supernatant would be required to obtain the protein. Often, volume is lost in bioreactor sampling and harvest, therefore, assuming a 10 – 20% loss is prudent.

In reality, purification protocols with 100% recovery are rare. At this stage of development, robust protocols are also rare. Recovery rates for antibodies can often be in the 90–95% range, but recovery rates for non-antibody proteins can be exceptionally low. In the absence of any data that indicate a recovery rate, it should be assumed that 0 to 5 percent of the protein will be recovered, and the required fermentation volume should be adjusted accordingly. In the perfect world of the above example, approximately 32 liters would need to be delivered from the upstream to the downstream processing groups:

(amount needed)/(volumetric productivity) = ideal volume required
(50 mg)/(4 mg/L) = 12.5L

[(ideal volume required) + (10 % ideal volume)](% recovery loss) = realistic volume required
[(12.5L) + (10%)](100%) = 31.25L

1. Is the culture format suspension or monolayer?
2. What media formulation has been used for cell growth?
3. What media formulation has been used for protein production?
4. What are the general "care and feeding" instructions for the cells line?
5. What is the average doubling time during the log phase of the culture?
6. What is the maximum cell density?
7. What does a typical growth and viability profile in a batch culture look like?
8. What is the average specific productivity? What culture conditions were used to make that determination?
9. How stable is the expression level? What culture conditions were used to make that determination?

Figure 1.

It should also be noted that there are two different methods to describe the size of a bioreactor: the working volume and the total volume. The working volume is the amount of liquid that can be introduced into the vessel and still have the bioreactor components (such as the impeller) and enough headspace to allow for optimal operation of the bioreactor. The total volume is the amount of liquid that can be introduced into the vessel without the bioreactor components being installed. The configuration of the bioreactor can have an influence on the working volume, but the total volume is a constant. The bioreactor needed to produce this working volume should be at least 15–20% larger in total volume. For the example above, a total volume of 36L to 40L would be appropiate.

Another factor that should be considered in determining the methods used to scale up and the volumes required to produce the desired amount of protein is the stability of the cell line. In an example that scales up as in Figure 2, if the average specific productivity measured on the freezer vial is reduced by 50 percent in 10 generations, an additional bioreactor run would be required to produce the necessary quantity of protein. In severe cases, the cell line may stop producing protein before it reaches the production bioreactor. When this is the case, the best strategy for producing the proteins is to perform many small-scale runs initiating a new culture from frozen stock for each run.

Most CMOs will require two to five frozen vials from the cell bank that is being used for production, depending on the number of viable cells per vial and the scope of the project. The frozen cell bank should be tested for mycoplasma contamination. If the cell line (e.g., a hybridoma) was recently derived from a mouse, it may be necessary to have a mouse antibody production (MAP) test performed before the CMO will begin to work with the cell line. This will similarly be true for hybridomas derived from rat or hamster cell lines. These tests are used to detect several common viruses found in these animals.

Traditional MAP, HAP, or RAP tests require one to two frozen vials from the cell bank and six to eight weeks to complete. A PCR-based alternative is available from the Research Animal Diagnostic Laboratory at the University of Missouri.[7] It requires 2 x 10^7 frozen cells and one to two weeks for results. The type of testing required varies among CMOs and should be one of the earliest questions asked in initial inquiries about capabilities and availability.

Frozen Amp: 2 x 10^7 viable cells
Doubling time: 18 hours
Seeding density: 2 x 10^5 viable cells/ml
Maximum cell density: 8 x 10^6 viable cell/ml

(2 x 10^5 viable cells/ml)(100L)= **2 x 10^{10} viable cells to seed 100L bioreactor**
2 x 10^{10} viable cells/ 8 x 10^6 viable cells/ml = **2500 mls for seed into 100L bioreactor**

(2 x 10^5 viable cells/ml)(3L)= **6 x 10^8 viable cells to seed 3L bioreactor**
6 x 10^8 viable cells/ 8 x 10^6 viable cells/ml = **75 mls to seed 3L bioreactor**

(2 x 10^5 viable cells/ml)(75 mls)= **1.5 x 10^7 viable cells to seed 75 ml flask**

1.5 x 10^7 ⇒ 6 x 10^8 = 5.25 generations x 18 hours doubling time = 94.5 hours
6 x 10^8 ⇒ 2 x 10^{10} = 5.1 generations x 18 hours doubling time = 91.8 hours

Conclusion: ~4 days in 75 ml flask, ~4 days in 3L bioreactor, ~10 day in production

Total of 18 days for protein production

Figure 2.

Locating a CMO that can deliver what you need may prove to be a bit more difficult than it would appear on the surface. A single 100-L bioreactor run and subsequent purification may seem like "small potatoes" to some of the larger contract organizations, and they may not understand the need or urgency of the project to your organization. As a result, it can be difficult to locate a CMO that meets your needs for capacity, delivery schedule, and cost. Helpful resources include the *Directory of Toll Fermentation and Cell Culture Facilities*, 5th Edition, as well as the Internet and professional contacts.[8] For projects of this scope which do not require the protein to be produced under cGMP conditions, core facilities at universities can be cost-effective CMOs. However, many small "for profit" CMOs can compete with the university facilities in cost and availability.

Early in the initial conversations with any CMO, confidentiality agreements (CDAs) will need to be executed. Once a CDA is in place, project details can be provided to the CMO. One method for providing the information is in a written "request for quotation" or RFQ. The RFQ should include a detailed description of the requested services. It should also include all of the information about the cell line and the purification process that is available, as well as the information used to determine the scope of the project. Additional considerations include the formulation and quality of the final product and the method of delivery to the client.

Carefully writing an RFQ that fully describes the scope of the project will result in a fair estimate for delivery of a quality product within the necessary time frame. The same RFQ can be provided to several different CMOs to get competitive responses. In return, a CMO should provide you with a detailed written response to the RFQ.

After evaluating the responses and determining which CMO can meet your needs, you will need a formal contract that describes the scope of the project. This document should include any time estimates and any remedies for not delivering the product on time. Including a "sliding incentive" for early

delivery can motivate CMOs to provide on-time or better performance. With a sliding incentive, a 5% bonus is paid for delivery that is more than two weeks early, the contract price is paid for on-time delivery, and a 5% penalty is paid by the CRO if the project is more than two weeks late. In addition to the final contract, materials transfer agreements may also need to be signed and executed.

While the contract negotiation is ongoing, references need to be investigated. The CMO should be able to provide references. However, they have no incentive to provide the names of unhappy customers, so turning to professional contacts can be valuable.

There are many examples of bad experiences with CMOs…and there are many examples of CMOs providing excellent service. Unfortunately, it is often the bad experiences that are remembered and talked about. To increase the possibility that the CMO and the client are both happy with the association, both parties need a clear and complete understanding of the tasks and resources required to complete the project, and clearly defined responsibilities. In traditional project management terms, this means understanding the scope of the project. To prevent miscommunication, it's best to designate an individual at both the contracting organization and the CMO with the authority to provide direction and technical support for the project. Most importantly, both the contracting organization and the CMO need to have a strong commitment to "getting the job done."

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