Microbial Process Development for Biopharmaceuticals

From Science and Engineering to Regulatory Requirements

Xiaoming Yang and Longze Cui

Microbial expression systems play an essential role in today's biopharmaceutical processes. Recombinant *E. coli* and yeast systems are dominant tools in early discovery of effective medical targets. These systems are also routinely used to produce biological reagents for analytical methods in research and quality controls of products. In the biopharmaceutical industry, microbial systems have delivered major medicines such as blood proteins (factors VIII and IX), G-CSF, tPA, recombinant hepatitis B vaccine, interferons, interleukins, and human growth hormone (1). Microbial systems continue to improve to meet the challenges in expressing new and more complex biomolecules (2, 3). The advantages of microbial systems over mammalian systems are clearly recognized for their short development cycles and substantially lower manufacturing costs.

Microbial process development is an integrated activity involving science, engineering, and regulatory requirements. Process development professionals must master microbial science and technology and thoroughly understand key quality issues and regulatory drivers.

PRODUCT FOCUS: RECOMBINANT PROTEINS

PROCESS FOCUS: PROCESS DEVELOPMENT

WHO SHOULD READ: PROCESS ENGINEERING AND MANUFACTURING PERSONNEL, QA/QC, REGULATORY AFFAIRS, PROJECT MANAGEMENT

KEYWORDS: *E. COLI*, *PICHIA*, PROCESS DEVELOPMENT

LEVEL: INTERMEDIATE

THE BIG PICTURE: KEY KNOWLEDGE AND DEVELOPMENT DRIVERS

Biopharmaceutical development competes on the basis of effectiveness and efficiency. The winning criterion of process development is to deliver a predictably stable and reliable process over a short development time. The following areas contain key knowledge and drivers for success.

The biology of microbial expression systems includes the microbiology of the host cell line, especially its physiology relating to cell growth dynamics, nutrient use, and metabolism. It is important to understand the plasmid vector system, its control and stability. Understanding process parameters for controlling protein expression kinetics in soluble (or secreted) and insoluble (inclusion body) phases is critical (4–6).

Process Engineering and Manufacturing Ability: This area includes process design, control, and monitoring as well as process scale-up, equipment specifications, and considerations of manufacturing-practice issues. It is important to follow the advances of new process technologies that may significantly affect the direction of process development.

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Methodologies for Design, Development, and Project Management: Process development efficiency can be improved significantly by the use of smart experiment-optimization methods. Effective methods include statistical design of experiments (DOE) for screening and optimization of critical process parameters, control charts and standard deviation analysis for evaluating process

reliability, and failure mode evaluation and assessment (FMEA) for evaluating development risks and characterization priorities. Overall project management skill is essential.

The Biologics Quality System: In the biopharmaceutical industry, understanding the quality control system and detailed regulatory requirements of a process is crucial. Biological product quality and consistency are usually derived from process quality and consistency. This is because analytical tools today are as yet insufficient for detecting functional and even structural changes of large biological molecules during process changes. This concept is very different compared with that for small synthetic drugs.

Biologics process specifications need to strictly adhere to FDA and ICH guidelines (International Conference on Harmonisation of Technical Requirements for the Registration of Pharmaceuticals for Human Use), especially the CMC (Chemistry, Manufacturing, and Controls) and cGMP (current good manufacturing practice) sections.

Quality control considerations must be given to raw materials and reagents used throughout a process because material quality significantly affects process consistency and even product quality. Documentation is paramount and includes laboratory notebooks, batch records, and analytical methods and results. Timely completion of development reports and raw data archiving are necessary. We can never overstate the importance of documentation in biotechnology.

Emerging Regulatory Challenges: The dymanic field of biological development is experiencing rapid advances in science and risk assessment. These changes are having a significant impact on process development. The discovery of "mad cow" disease prompted serious concern over potential contamination from BSE (bovine spongiform encephalopathy) and other TSEs (transmissible spongiform encephalopathies, including the human form of BSE) in biological drugs made using animal-derived products. This concern drives process development toward using raw materials free of animal products (7) and drives new purification technology development toward the removal of prions, the most likely TSE agents (8).

Another important regulatory challenge for biological processes is the need to demonstrate process capability for viral clearance (9).

Mastery of the five areas is essential for a process development team to be highly effective and efficient in the biopharmaceutical industry. We present below some practical industrial examples to illustrate how these elements are applied in microbial process development.

Biology of Microbial Expression Systems

Case Study 1, Optimization of Nutrient Conditions for an *E. Coli* Expression System: One essential consideration for microbial process development is identification of an optimal nutrient condition. To achieve high-productivity fermentation, it is important to understand microbial metabolism and to detect potential sources of metabolic inhibition.

Microbial expression systems are two-way feedback systems with an input of nutrients and an output of metabolites and products. Nutrients and metabolites can support as well as inhibit cell growth and protein expression at certain concentrations. Figure 1 illustrates the two-way feedback process of a microbial expression system.

In our first example, a recombinant *E. coli* strain was used to express a protein product. The initial conditions for this strain were M9 medium supplemented with 2% casamino acid, 20 mg/L proline, and 20 mg/L tryptophan. The protein activity yield was 37 U/mL (normalized). Because it is highly desirable to keep glucose as a single carbon source for this strain, our development emphasis was on the optimization of nitrogen sources. We used a screening experiment to select from four nitrogen sources: yeast extract, ammonium phosphate, proline, and tryptophan. To screen the compositions efficiently, a fractional factorial experiment (DOE) was used as shown in Table 1. The actual design will be discussed in detail in later sections. After the experiment was complete, the result was statistically analyzed and presented as in Table 2.

This experiment let us quickly eliminate proline and tryptophan because they had no significant impact. Eliminating those two components simplified the selection of the media composition. We chose yeast extract and ammonium phosphate as batch process nitrogen sources and selected ammonium phosphate as the sole feed media for a fed-batch process. Our fed-batch culture achieved a maximum protein yield of 350 µg/mL, about a

Table 1: Nitrogen composition experiment

Nutrients Concentration Level	Yeast (g/L)	Ammonium Phosphate (g/L)	Tryptophan (mg/L)	Proline (mg/L)
1	0	0	0	0
2	0.5	0.5	10	10
3	2.0	2.0	50	50

Table 2: Media composition experiment results

Nutrients	Statistical Significance
Yeast	medium
Ammonium phosphate	high
Tryptophan	low
Proline	low

Figure 1: The two-way microbial feedback process

tenfold increase in yield compared with that of the original process.

Further, we studied this expression system's potential metabolic inhibitors: acetate accumulation and inhibition. Based on bacterial physiology, *E. coli* may accumulate metabolic acetate during its fermentation. The accumulated acetate, if it is not assimilated quickly, may inhibit cell growth and prevent further expression of a targeted protein product. In a series of experiments, we added various amounts of acetate during the exponential growth of a culture. The results in Figure 2 indicate that acetate at 20 mM inhibited cell growth.

Based on *E. coli* physiology, we also know that these cells assimilate acetate as a carbon source if acetate concentration is not high enough to become inhibitory. This assimilation was demonstrated by the experiment in Figure 3. As shown, the *E. coli* strain assimilated acetate at a concentration up to 2g/L, resulting in a twofold increase in product yield. In fact, acetate assimilation occurred only after glucose was consumed. Therefore, completion of glucose consumption is essential to allow assimilation of the accumulated acetate.

Using the above information, we designed a computer-controlled fed-batch program that provides a continuous but limited glucose feed scheme. In this way, the cells were always just short enough on glucose to allow complete assimilation of acetate. This nutrient feeding strategy supported a high-density culture with continuous clean-up of potentially inhibitory metabolites. The yield of our enhanced fed-batch process reached 990 µg/mL (7). This is a good example of applying cell physiology and control strategies to improve productivity. The significant increase in volumetric productivity with the limited fed-batch cell culture is shown in Figure 4.

In addition to nutrient composition, other factors to be carefully investigated include the timing and concentration of the inducing agent and environmental conditions such as temperature and pH.

Table 3: Experimental Design $L_8(2^7)$

Experiment #	Temperature °C	K_2HPO_4/Glucose M/g/L	Peptone g/L	Salts Dx	$(NH_4)_2SO_4$ g/L
1	28	0/40	0	0	0
2	28	0/40	5	2	5
3	28	0.2/20	0	0	5
4	28	0.2/20	5	2	0
5	32	0/40	0	2	0
6	32	0/40	5	0	5
7	32	0.2/20	0	2	5
8	32	0.2/20	5	0	0

Process Engineering and Manufacturing Ability

Case Study 2, Using Process Control to Achieve High Specific Yield (product/cell): A recombinant *E. coli* expressing gamma interferon (INF-γ) was studied in this case. The original process was an incremental-time–based manual fed-batch process, a very common type in biologics production. The periodic fed-batch procedure generated a variable nutrient condition in the culture causing variable cell growth rates during the process. We thought this process could be improved by a computer-controlled engineering solution.

The culture yields were as follows (IU is international unit of interferon activity):

cell density = 26.3 g/L DCW (dry cellular weight)

IFN yield = 1.55×10^8 IU/L

IFN productivity = 0.65×10^7 IU/g DCW.

A computer-controlled feed program with an exponential feeding rate was implemented for this fed-batch process. In this case, we simply set up the exponential feeding rate based on cell growth rate and nutrient consumption. The resulting INF-γ yield with the new process was 2.2×10^7 IU/g DCW, more than a threefold increase in productivity. In addition, the program reduced manual operations and resources while increasing productivity. This powerful example illustrates how a process engineering solution can contribute to the production bottom line.

Case Study 3, pH Control To Improve Antigen Yield in Recombinant Hepatitis B Vaccine Production By *S. cerevisiae*: The antigen yield of a

manufacturing process was highly variable with different lots of yeast extract used in its fermentation media. Preliminary analysis indicated that yeast extract lots causing relatively less variability of process pH seemed to be producing higher yields. The process pH was not controlled, but monitored.

One strategy to deal with the variability was to screen those yeast extract lots for better characteristics in flasks before using them in production. This approach minimized disastrously poor yield cases, but it could not ensure a consistently desirable antigen yield. The process achieved antigen yields from 2.0 to 3.5 g/L.

To further improve the situation, we needed a better control mechanism. One hypothesis was that the antigen yield variability was not directly caused by varying yeast extract quality, but was actually caused by the resultant variations in process pH. In other words, the yeast extract affected pH, which indirectly affected the antigen yield. A tighter pH control was implemented for this process, leading to a significant improvement: The antigen yields reached 4.5 to 5.5 g/L, although the cell density did not change significantly.

The above cases illustrate the importance of process controls. Another important engineering aspect is a fermentor's mixing capacity. This generally determines process scale-up capability. The mixing condition directly affects two important process factors: oxygen transfer and heat transfer. For oxygen transfer, the following estimates are useful for scale-up considerations:

Maximum Oxygen Transfer Rate
(OTR = mmole O_2/L/h, use air only)

Bench Fermentor	Pilot Fermentor	Large-Scale Fermentor
300	100–150	50–100

Based on the ratio of these parameters, we can realistically use scale-down modeling (bench processes) to support production design.

Case Study 4, Heat Generation in Recombinant *Pichia Pastoris* Fermentation: A *Pichia pastoris* fermentation was successfully developed to produce a recombinant protein in a high–cell-density culture, with plans for scale-up to production-scale fermentation. During the initial scale-up run, cell growth suddenly stopped three to four hours postinduction when methanol was added as the induction reagent.

The investigation found that the fermentation process generated a lot of heat, causing temperature controls to fail and cell growth to stop. This problem was resolved by adding a chilled glycol system to improve the cooling capacity of the production fermentors.

A good approach to deal with engineering issues is to review the process design with operations experts before finalizing process development.

Methodologies for Process and Project Development

Case Study 5, Recombinant *Bacillus* Producing Dual Proteins PI and PIII: In this case, the goals and strategy were to identify optimal conditions for protein formation (not for high spore counts), to study various factors in the protein formation phase, and to establish scale-up factors for optimizing the large-scale process.

The following methodologies and approaches were applied:

- Experiments were designed to isolate the growth phase from the protein formation phase.
- A scale down model was established that is consistent with large scale.
- DOE was applied for a multiple-factor design.

To isolate the protein synthesis phase, we first grew the culture in a

Table 4: Statistical significance analysis of the experimental results

Factors	High Yield Level	Sum of Squares	F Ratio	Probability > F
Temperature	Low	1.62	6.6575	0.0818
K_2HPO_4	Low	2.88	11.8356	0.0412
Glucose	High	2.88	11.8356	0.0412
Protein	Low	21.78	89.5068	0.0025
Salts	Low	0.405	2.4923	0.2552
$(NH_4)_2SO_4$	Low	16.25	66.7603	0.0038

Figure 2: Acetate inhibition of cell growth

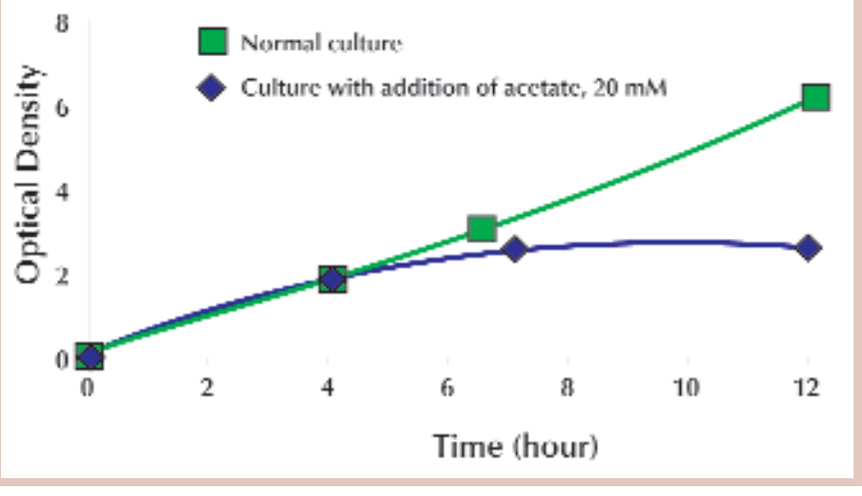
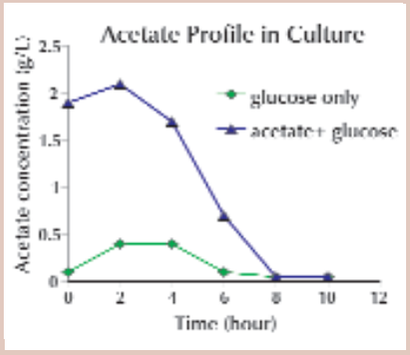


Figure 3: Assimilation of acetate by *E. coli*



fermentor, then transferred culture samples at its peak growth phase to flasks with predefined nutrient and operational conditions. Figure 5 illustrates this approach. To set a realistic flask scale down model, we considered the following parameters:

- Maintaining the growth profile and protein expression ratio
- Ensuring adequate O_2 supply so that oxygen is not a limiting factor
- Ensuring adequate buffer capacity to maintain pH.

Using DOE, we investigated several critical parameters including temperature, various nitrogen sources, glucose, and salts in one set of experiments. The design is shown in Table 3. After the experiment, the results were analyzed and described in Table 4.

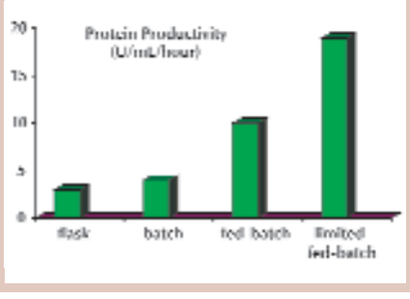
The temperature found to be optimal for protein expression was 28 °C, whereas the optimal growth temperature is 32 °C. Additional K_2HPO_4 was not needed in the media because a certain amount of PO4-4 was present in the buffered initial media. A high glucose level (40 g/L) actually encouraged protein expression, but it did not further growth and spore formation.

It was very clear that additional peptone was detrimental to protein expression. This confirms that in a batch culture, protein expression becomes efficient after the organic nitrogen sources are depleted. Inorganic nitrogen sources such as $(NH_4)_2SO_4$ were also found to be detrimental. So all nitrogen sources had to be depleted before the targeted protein could be efficiently produced. In addition, this experiment showed that no additional mineral salts were needed for this composition.

With completion of small-scale optimization, we prepared for process scale-up based on a critical criterion: meeting the demand for a high oxygen-transfer rate (OTR) because a limited oxygen supply can severely reduce protein formation. There are two common methods for estimating OTR and the oxygen-uptake rate (OUR): the steady-state and dynamic methods. We applied a steady-state method in this study and obtained a maximum OUR of 108 (mmole/L/h). The fermentation scale-up was successfully completed, showing a yield consistent with that of the small-scale data.

Development efficiency and outcome can be greatly improved when using smart methodologies. Effective methodologies include small-scale process modeling, statistical DOE for screening and optimization of critical parameters, FMEA for evaluating development risks and priorities, and design control procedures for managing the overall flow of process development.

Figure 4: Comparison of volumetric productivities with different culture processes



Biologics Quality System

For high-quality processes, it is important to manage quality issues carefully according to FDA and ICH guidelines governing biologics processing and production. Specific regulatory requirements apply to each stage of process development, from preclinical through commercial manufacturing. Effective documentation of important data, analyses, and materials at each development stage is essential.

Case Study 6, Raw Material Considerations in Production of a Bacterial Vaccine: To improve manufacturing consistency and productivity, a defined medium was sought to replace the complex medium used in a bacterial vaccine production process. The new medium was demonstrated to improve yield at both the bench and pilot scales. Several pilot batches were potent and consistent in clinical trials. However, when the new process was transferred to the factory, the defined medium proved not to be robust because it failed to support growth to the anticipated yield.

Investigation of this problem revealed that the organism involved was extremely sensitive to trace elements in the defined medium. Material specifications describing the purity and grade of ingredients used in research were inconsistent with those used in the factory. We believed that trace metal differences between the two material specifications caused the failure in the factory demonstration. Our solution was to modify the medium formulation to include a small amount of a complex component. The modified medium did produce an expected yield in the factory.

The original problem was resolved, but this situation significantly delayed the timeline for the new process's commercial implementation. This case demonstrates the importance of establishing quality-control specifications for all raw materials used in process development.

Emerging Regulatory Challenges

Regulatory challenges for the industry arise when an issue may significantly affect product quality across the industry. Currently, because of the concern about BSE/TSE contamination, regulatory agencies across the world require that biopharmaceutical

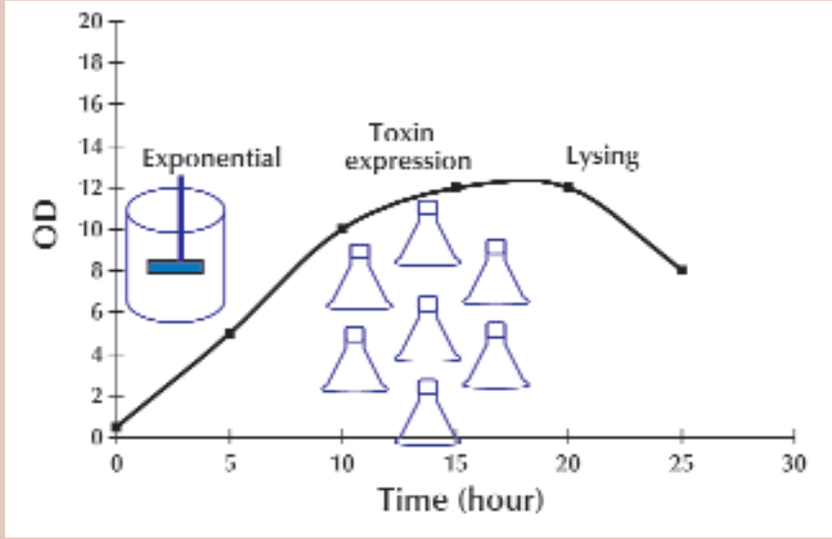
processes demonstrate prion removal if animal-derived products are used (**9**, **10**). In such cases, demonstrating viral clearance and inactivation may be another challenge (**8**). New viruses posing potential hazards to humans may be identified as science advances, so each process may need to demonstrate its ability to remove a wide range of viral contaminants.

According to a recent FDA recommendation, licensed biologics manufacturers must evaluate all bovine-sourced materials in use. Working cell banks (WCBs) established using bovine-derived materials from countries on the USDA's high-risk list must be replaced. Full compliance with the TSE guideline is required for ruminant materials used in WCBs, fermentation, and routine production. For process development, we face two challenges: First, we need to remove animal-derived materials from current commercial processes; second, we must develop animal-product–free processes for new products.

Case Study 7, Animal-Product–Free (APF) Microbial Process Development: Peptone derived from animal meat was used in a bacterial master cell bank, and it was used as the main nitrogen source in production fermentation as well. The goal in this case was to remove peptone from the process. First, a working cell bank without peptone had to be generated. Experiments showed that a soy protein nutrient could serve as a robust nitrogen source for the growth of the target microorganism. Cells were then passed through a soy protein–based media, and a new working cell bank was generated. Cell bank quality tests for purity, growth profile, plasmid retention, and protein expression were performed.

Next, the peptone was replaced with soy-based protein media in production fermentation. The medium was formulated so that the growth profile and product expression remained comparable. Using the new working cell bank, three production fermentation runs were performed, and the resulting materials were purified. The final products met all product-release criteria, and the product was characterized by advanced analytical methods to demonstrate that it was comparable to the original product.

Figure 5: Phase-Specific-Study Approach: Isolate growth phase from protein formation phase by transfering one-source end-of-growth culture to various flask cultures



A Challenging Environment

Although the above examples describe different processes, the five key drivers they illustrate are usually embedded in every development project. In fact, an important goal of process development is to successfully integrate all of those elements. Today's biopharmaceutical process development efforts take place in a complex environment characterized by competition for quality and efficiency. Integrating the five knowledge areas identified above in practical development activities will be the key to successful process development of biopharmaceutical products.

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