Fermentation Fundamentals: Brewing Bugs for Bioengineering

Sujata K. Bhatia, M.D., P.E.
Harvard Univ.

A fermentation process leverages live cells to manufacture a variety of products. Choosing the correct host cell, as well as the optimal production equipment and method, are the fundamental first steps of these complex processes.

Fermentation, broadly defined as the cultivation of live cells to produce useful molecules, is arguably the oldest chemical engineering process on earth. The word fermentation is derived from the Latin verb *fervere,* which means "to boil." Fermentation, with its bubbling culture media and animated cells, also conjures images of commotion, excitement, and agitation. Living cells are themselves miniature chemical reactors, capable of performing complex reactions and manufacturing valuable products, such as pharmaceuticals, plastics, and biofuels *(1)*.

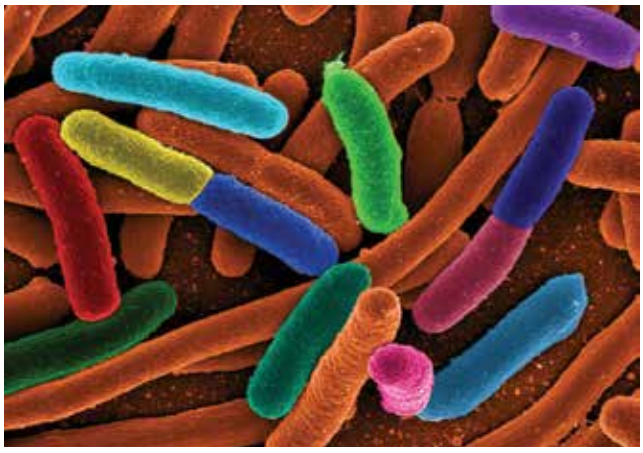
Both prokaryotic (bacterial) cells and eukaryotic (yeast and mammalian) cells are incredibly versatile for chemical production. Yet live cells also demand exquisite control systems in order to optimize cellular growth, maximize the quantity of the desired output, ensure product quality and reliability, and minimize side reactions. The biochemical engineer must therefore carefully choose both the host cell and the fermentation process for cellular cultivation.

This article describes the fundamentals of host cell selection, process design considerations, and equipment selection for fermentation processes. It compares and contrasts bacterial cells, yeast cells, and mammalian cells in fermentation. It also discusses the U.S. Food and Drug Administration's (FDA) requirements for fermentation equipment in pharmaceutical and biologic production, according to principles of good manufacturing practice (GMP). Finally, the article discusses important challenges and future directions in research and development of fermentation processes, including algal fermentation.

Choosing a host: The cellular reactors

Fermentation relies on live cells to carry out complex chemical synthesis and breakdown reactions. Bacteria, yeast, and mammalian host cells are all commonly employed in biochemical engineering processes, and each of these cell types has distinct capabilities, advantages, and disadvantages. In general, the biochemical engineer faces a trade-off between low-cost, well-characterized, conveniently grown prokaryotic cells on the one hand, and highly versatile, complex, reliable eukaryotic cells on the other. The desired product will ultimately guide the selection of the host cell.

Bacterial cells maximize convenience, speed, and



▲ **Figure 1.** *Escherichia coli* bacterial cells exhibit a relatively high growth rate and are the most frequently used bacteria for fermentation.

cost-effectiveness. The most frequently used bacterium for fermentation is *Escherichia coli* (Figure 1). These bacteria are advantageous because their physiology and genetics are far better understood than those of any other organism; their genome has been mapped *(2)*. *E. coli* exhibit a relatively high growth rate, doubling every 20 to 30 min, and survive on simple and inexpensive culture media. During fermentation, *E. coli* are able to grow to high cell concentrations (>50 g dry weight of cells per liter of culture media) and are extremely productive, with the ability to produce 25–50% or more of their total protein as the desired protein product *(3)*. *E. coli* can therefore achieve high productivity without sacrificing cell growth.

However, these cells do not normally secrete and release the proteins they produce; this lack of secretion complicates recovery and purification of desired products. *E. coli* may actually degrade proteins or form insoluble bodies that further complicate product recovery. Most importantly, *E. coli* and other bacterial hosts cannot perform glycosylation reactions or other modifications to proteins after translation has occurred.

Bacterial cells are typically chosen as fermentation hosts for the production of specialty chemicals, biofuels, and other non-food, non-biologic products.

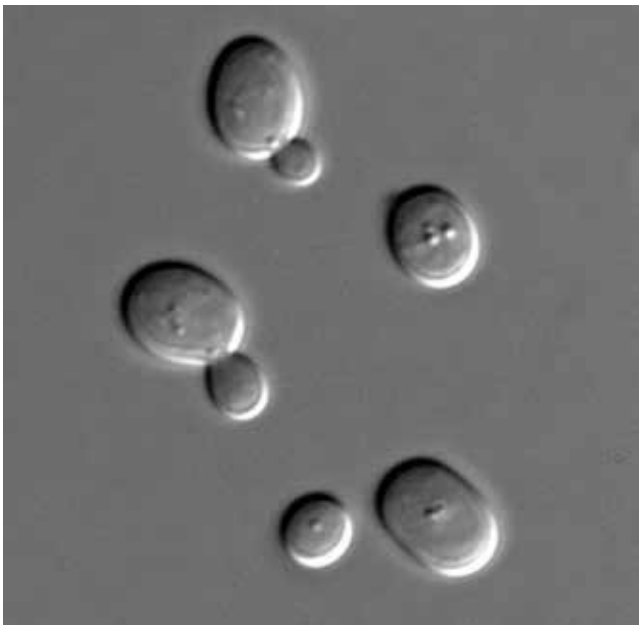
Yeast cells are able to carry out more complex reactions than bacterial cells, but there are sacrifices with regard to speed and cost. *Saccharomyces cerevisiae*, commonly called baker's yeast, are frequently employed in fermentation (Figure 2). A particular advantage of *S. cerevisiae* yeast cells is that the FDA considers these organisms to be generally regarded as safe (*i.e.,* they have GRAS status) *(4)*, so that food additives, food ingredients, and nutritional supplements manufactured via yeast fermentation may readily gain FDA approval. Yeast cells are relatively low-cost and have a reasonably high growth rate, doubling every 1 to 2 hr, and they can perform simple glycosylation reactions.

However, it is difficult to achieve protein production levels in yeast that are as high as those in *E. coli.* In addition, bottlenecks in secretion can occur with yeast production hosts, limiting cell productivity and complicating product recovery *(5)*.

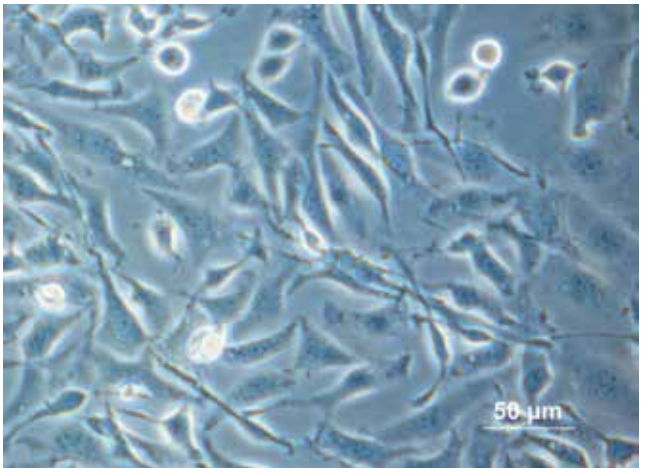
Because of their GRAS status, yeast cells are often chosen as fermentation hosts for food and nutritional products.

Mammalian cells for fermentation processes are typically selected from cell lines that have been immortalized. Normally, mammalian cells do not proliferate indefinitely, but immortalized cell lines carry mutations that allow for an indefinite number of cycles of cell division and proliferation. This enables the cultivation of immortalized cell lines for prolonged periods *in vitro.* Immortalized cell lines for mammalian fermentation cultures include Chinese hamster ovary (CHO) cells (Figure 3) and baby hamster kidney fibroblast (BHK-21) cells. Mammalian cells can perform complex glycosylation reactions, and readily excrete most proteins, which avoids the bottlenecks associated with yeast. Of all the fermentation hosts discussed in this article, mammalian cells produce a product that most closely resembles its natural counterpart.

Mammalian cells, however, are by no means convenient for fermentation. Mammalian cells grow slowly, doubling approximately every 24 hr, and the media ingredients for mammalian culture are very expensive. Mammalian cells cannot achieve high protein production levels — less than



▲ **Figure 2.** *Saccharomyces cerevisiae* yeast cells can carry out more-complex reactions than bacterial cells and are classified as generally regarded as safe (GRAS).



▲ **Figure 3.** These Chinese hamster ovary (CHO) mammalian cells are part of an immortalized cell line — they carry mutations that allow them to divide and proliferate indefinitely.

5% of the total protein they produce is the desired protein product *(3)*. Finally, since immortalized cells are mutants, these mammalian cells require extreme care during purification processes to avoid any contamination of the desired product with genetic material from the mutated cells. This makes mammalian cell fermentations incredibly costly in terms of both time and resources.

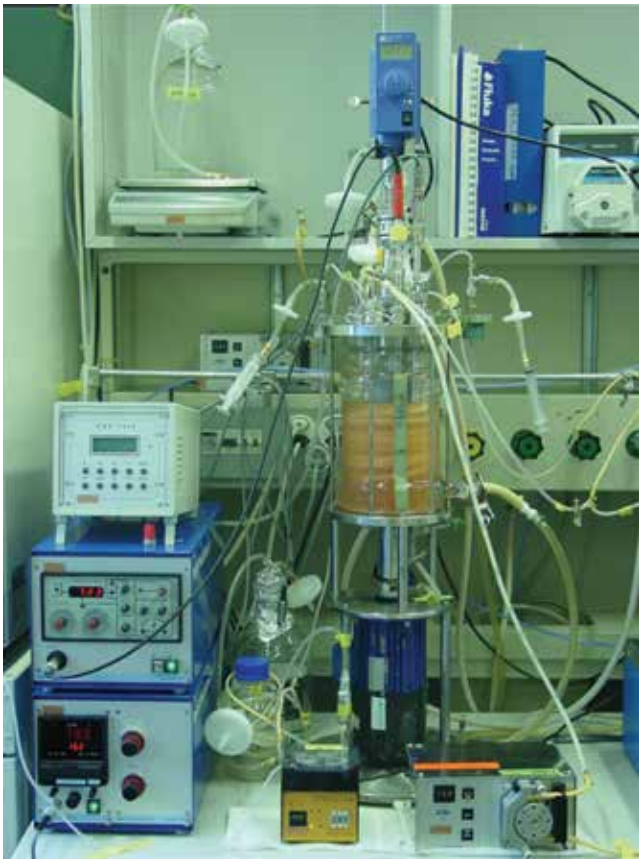
Mammalian cells are chosen as fermentation hosts when the authenticity of the product must be complete and guaranteed. Mammalian cells should be seriously considered for production of human therapeutic proteins and other biologic therapeutics.

Scaling it up: The fermentation reactor

Fermentation reactors, often called bioreactors, can operate at the laboratory scale (Figure 4) up to the pilot-plant or plant scale (Figure 5). The traditional fermenter is a stirred tank reactor. A sparger supplies gas under pressure to the tank, and an impeller disperses gas bubbles throughout the tank. A disc stirrer or turbine stirrer is used for mixing, and baffles are included to facilitate gas dispersion and mixing *(3)*. The fermenter will usually contain four baffles, each with a width of 8% to 10% of the reactor diameter. Fermenters are typically constructed with a height-to-diameter ratio of 2:3, and the impeller diameter is usually 30% to 40% of the tank diameter.

Fermentation processes can be designed for batch, fed-batch, or continuous operation. A batch process, with no inflows of nutrients nor outflows of waste products, allows maximum containment of the live cells, but minimal control over cellular proliferation. During batch fermentation, cell growth rates start off slow in a lag phase, and then accelerate quickly into an exponentially increasing growth phase. Eventually the cells exhaust the available nutrients, and cellular growth levels off into a final stationary phase.

Batch processes are associated with significant periods of nonproductive time, as the batch culture must be stopped once the cells reach the stationary phase. The product must be removed and the media must be replaced before the fermentation can be restarted. The environment within a batch reactor is difficult to control as well: heat output, acid or base production, and oxygen consumption vary from low rates at the beginning of the fermentation to very high rates during the exponential growth phase *(6)*. Finally, batch fermentation carries the risk that the desired product may itself be depleted, as cells may utilize the product as an



▲ **Figure 4.** Mammalian cell culture in a bench-scale bioreactor.

▲ **Figure 5.** This mobile pilot-plant fermenter has a 90-L capacity for the production of cellulosic ethanol. Source: U.S. Dept. of Energy.

energy source once the available nutrient supplies have been exhausted.

A continuous fermentation process, with both inflow of nutrients and outflow of waste products, has very significant productivity advantages. The continuous fermentation operates at steady state: production and consumption rates of all materials are constant. Since the continuous process avoids the stationary phase of cell growth, there is no need to stop and restart the process; continuous operation thus minimizes nonproductive time. For a culture with a 1.5-hr doubling time and a 20-hr batch cycle, a continuous system has a 14-fold productivity advantage over a batch system *(3)*. Moreover, a continuous fermenter does not experience swings in heat output, acid or base production, or oxygen consumption by cells, so the environment within a continuous bioreactor is much more easily controlled.

However, continuous bioreactors do have notable disadvantages. While the production of desirable products is more efficient in the continuous reactor, the production of growth-associated byproducts is also more efficient *(6)*. Continuous systems may also be more susceptible to contamination, and the cells themselves may be more difficult to contain.

A fed-batch process is intermediate between a batch process and a continuous process. Fed-batch fermenters, which enable inflow of fresh nutrients with continuous or periodic withdrawal of broth, can overcome the major limitations of batch processes without the disadvantages of continuous processes. Fed-batch fermentation can extend the productive period of a traditional batch process and achieve a substantial level of process control, yet it does not require any additional special equipment beyond that required for batch fermentation *(7)*. The fed-batch process can be designed to keep the cellular growth rate constant, and to maintain the nutrient supply at a low, constant concentration. The constantly replenished nutrient supply enables cells to proliferate and reach high cell densities within the bioreactor, and controls deviations in the growth pattern of the cells. Fed-batch fermentations also limit byproduct formation, control product concentration, and avoid product depletion. Most importantly, fed-batch processes allow containment of organisms and avoid contamination.

Making it right: Good manufacturing practice

The goal of GMPs is to ensure the quality of medicines, as well as some food products *(8)*. Because fermentation is increasingly being applied to the production of small-molecule drugs, biologic therapeutics, vaccines, nutritional supplements, and other medicinal and food products, it is critical for chemical engineers to consider GMP guidelines when selecting fermentation equipment.

GMP regulations are issued by the FDA and are laid out in Section 21 of the Code of Federal Regulations, Parts 210 and 211. They are enforced via inspections of manufacturing facilities; failure to comply with GMP requirements can result in regulatory actions against manufacturers, and can even jeopardize FDA approval of a new drug.

Chemical engineers must recognize that GMP regulations are continually evolving to meet the demands of new technologies; for this reason, GMP is often denoted as cGMP, meaning current good manufacturing practice. Also keep in mind that GMP regulations represent the minimum requirements for a compliant process; many companies choose to exceed these standards.

To allow manufacturers the maximum flexibility in equipment selection and process design, the FDA does not

Literature Cited

1. **Bhatia, S. K.,** "Biology as a Basis for Biochemical Engineering," *Chem. Eng. Progress,* **109** (7), pp. 40–44 (July 2013).
2. **Perna, N. T., *et al.,*** "Genome Sequence of Enterohaemorrhagic *Escherichia coli* O157:H7," *Nature,* **409,** pp. 529–533 (Jan. 25, 2001).
3. **Shuler, M. L., and F. Kargi,** "Bioprocess Engineering: Basic Concepts," Prentice-Hall, Englewood Cliffs, NJ (1992).
4. **U.S. Food and Drug Administration,** "Microorganisms and Microbial-Derived Ingredients Used in Food (Partial List)," FDA (Aug. 21, 2013).
5. **Niebauer, R. T., and A. S. Robinson,** "*Saccharomyces cerevisiae* Protein Expression: From Protein Production to Protein Engineering," in "Protein Expression Technologies: Current Status and Future Trends," Fracois Baneyx, ed., Taylor and Francis, Norfolk, U.K. (2004).
6. **Stanbury, P. F., *et al.,*** "Principles of Fermentation Technology," 2nd Ed., Butterworth Heinemann, New York, NY (1999).
7. **Longobardi, G. P.,** "Fed-Batch versus Batch Fermentation," *Bioprocess Engineering,* **10,** pp. 185–194 (May 1994).
8. **U.S. Food and Drug Administration,** "Facts About Current Good Manufacturing Practices (cGMP)," www.fda.gov/drugs/developmentapprovalprocess/manufacturing/ucm169105.htm (May 2, 2013).
9. **U.S. Food and Drug Administration,** "Questions and Answers on Current Good Manufacturing Practices, Good Guidance Practices, Level 2 Guidance – Equipment," www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm124777.htm (Sept. 25, 2013).
10. **U.S. Food and Drug Administration,** "Code of Federal Regulations, Title 21, Part 211, Section 211.65," www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=211.65 (April 1, 2013).
11. **Muchemu, D. N.,** "Change Control for FDA Regulated Industries: A Risk Assessment Approach," AuthorHouse, Bloomington, IN (2007).
12. **Pienkos, P. T., and A. Darzins,** "The Promise and Challenges of Microalgal-Derived Biofuels," *Biofuels, Bioproducts and Biorefining,* **3,** pp. 431–440 (May 28, 2009).
13. **Wilson, S. A., and S. C. Roberts,** "Recent Advances towards Development and Commercialization of Plant Cell Culture Processes for the Synthesis of Biomolecules," *Plant Biotechnology Journal,* **10,** pp. 249–268 (April 2012).

maintain a list of approved cGMP manufacturing equipment. Instead, the cGMP standards require that equipment be appropriately designed for its intended use, and that equipment be designed for thorough cleaning and maintenance *(9)*. The equipment surfaces in contact with the starting materials, in-process materials, or products must be nonreactive, nonadditive, and nonabsorptive to ensure that the equipment surfaces do not "alter the safety, identity, strength, quality, or purity of the drug product beyond the official or other established requirements" *(10)*. To guarantee inertness and reliable cleaning of equipment, engineers typically choose stainless steel, Hastelloy alloy, or glass-lined steel units as cGMP fermentation reactors.

GMP also requires documentation of any changes to the fermentation process or equipment; this is known as change control. Change control procedures apply to changes in operating conditions, standard operating procedures, manufacturing facilities, raw materials, production equipment, technical specifications, software, and quality assurance protocols *(11)*. A rule of thumb is that change control applies to any change that affects one of the five inputs of a process, also known as the five M's: man, material, method, machine, and Mother Nature. The goal of change control procedures is to limit risk by assessing the impacts of any process changes. Whenever engineers introduce a process alteration, the change must be documented and reported, and the adverse impacts on the safety, quality, efficacy, potency, and purity of the product must be evaluated and appropriately mitigated.

▲ **Figure 6.** Green algae in a fermentation bioreactor at the laboratory scale. Image courtesy of Umberto Salvagnin, Dept. of Sustainable Agro-Ecosystems and Bioresources, Centro Ricerca e Innovazione, Italy.

Forthcoming advances in fermentation

A well-designed, well-controlled fermentation process leverages live cells to manufacture specialty chemicals, polymers, therapeutic drugs, biologics, and foods and nutritional supplements. Depending on the desired product, the chemical engineer may select bacteria, yeast, or mammalian cells as production hosts, and can choose to design a batch, fed-batch, or continuous fermentation process.

Increasingly, fermentation processes are being recognized as critical components for the establishment of a sustainable bio-economy, and fermentation operations are being designed to tackle issues of energy and the environment. For instance, algal fermentation processes (Figure 6) are under development for the production of biodiesel, green diesel, green jet fuel, and green gasoline *(12)*. Plant cell fermentation processes are also under consideration for the manufacture of complex drugs and proteins *(13)*.

The main challenges for chemical engineers will be to increase the yield of these processes, improve the cost-effectiveness, and ensure that the manufacturing operations are scalable and reliable. With appropriate attention to engineering design, fermentation processes will address needs in healthcare, nutrition, and sustainability. CEP

SUJATA K. BHATIA, M.D., P.E., is a physician and bioengineer who serves on the biomedical engineering teaching faculty at Harvard Univ. (Phone: (617) 496-2840; Email: sbhatia@seas.harvard.edu). She is the Assistant Director for Undergraduate Studies in Biomedical Engineering at Harvard. She is also an Associate of the Harvard Kennedy School of Government for the Science, Technology, and Globalization Project, as well as a faculty member in the Harvard Kennedy School Executive Education program on Innovation for Economic Development. She received bachelor's degrees in biology, biochemistry, and chemical engineering, and a master's degree in chemical engineering, from the Univ. of Delaware, and she received an M.D. and a PhD in bioengineering, both from the Univ. of Pennsylvania. Prior to joining Harvard, she was a principal investigator at the DuPont Co., where her projects included the development of bioadhesives for wound closure and the development of minimally invasive medical devices. She has written two books, *Biomaterials for Clinical Applications* (a textbook that discusses opportunities for both biomaterials scientists and physicians to alleviate diseases worldwide) and *Engineering Biomaterials for Regenerative Medicine*. She received an award from the Harvard Univ. President's Innovation Fund for Faculty in recognition of her innovative approaches to biomedical engineering education, the John R. Marquand Award for Exceptional Advising and Counseling of Harvard Students, and the Capers and Marion McDonald Award for Excellence in Mentoring and Advising. She is a member of AIChE and is a registered P.E. in the state of Massachusetts.