FERMENTATION

1. Introduction

Fermentation is most commonly defined as the anaerobic evolution of carbon dioxide [124-38-9] from microorganisms (such yeast or bacteria) growing on energy-rich nutrients (such as sugar, malted grain or fruit) to produce ethanol [64-17-5] and/or organic acids (such as beer, wine, or vinegar). The term is derived from the Latin verb *fervere*, which means "to boil" and describes the bubbling gas emissions commonly observed during the process. More generally, fermentation is defined as the overall activity of microorganism cultivation in a container. Although often used interchangeably, the word "fermentor" refers to the vessel and the word "fermenter" refers to the organism itself.

Anaerobic processes now are in the minority with aerobic processes much more commonplace. Although a large number of processes have been researched and developed to various extents, fewer have been commercialized. Culture types utilized in fermentation include single-cell bacteria, filamentous bacteria (actinomycetes), yeast, fungi, as well as mammalian, insect, and plant cells. The key components of a fermentation process include the (*1*) development of medium and conditions best suited to organism growth and production; (*2*) preparation of sterilized medium, fermentors, and related equipment; (*3*) propagation of active culture as seed (inoculum) for the production fermentor; (*4*) production fermentor growth and product accumulation; (*5*) product separation and isolation; and (*6*) treatment and waste disposal. The term "biotechnology" was introduced originally around World War I (WWI) to describe the transformation of raw materials to fermentation products by living organisms. Subsequently, it has been more closely associated with genetic engineering products of the late

twentieth century and the process of redesigning what Nature has provided to improve its benefits. The term "biochemical processing" or "bioprocessing" broadly includes fermentation (both aerobic and anaerobic) and animal cell cultivation as well as whole cell and enzymatic biotransformations.

Teams of microbiologists, biochemists, chemical engineers work on the development of a fermentation process. Best results are obtained using cross-disciplinary and integrated approaches to problem solving. In addition to the microbial reaction of interest, the nature of the microenvironment around the cell, and the microbial reaction to this environment must be considered (1). The total process impact of any proposed solution should be considered before changes are implemented.

Many fermentation processes are conducted with cells circulating in liquid medium (submerged culture); several processes utilize cells immobilized within or upon either an inert support (immobilized culture) or a nutritive substrate [surface or solid state culture (2,3)]. Although many fermentation characteristics are common to these various modes of operation, the major focus of this article is on aerobic submerged culture.

2. Types of Products

The principal product categories for fermentation products fall into the broad areas of primary and secondary metabolites, biomass, biotransformed compounds (synthesized by enzyme or whole cell biocatalysts), and large molecular weight biologicals (such as therapeutic proteins, therapeutic enzymes, antibodies, DNA, and vaccines).

2.1. Primary Metabolites: Organic Acids, Solvents, Amino Acids, Polyols. Primary metabolites are intermediates of pathways directly involved in growth processes. They are small molecules, typically under 1500 Da in molecular weight. Beer, wine, and other distilled beverages are produced through finely tuned fermentation processes employing highly developed strains. Nonalcoholic beer can be manufactured via arrested fermentation, in which the fermentation is halted before significant alcohol is produced. Examples of higher volume organic acids and salts include citric acid [77-92-9] (a food and beverage acidulant), acetic acid [64-19-7] (vinegar), lactic acid [598-82-3] (for food preservation such as pickling), propionic acid [79-09-4] (food preservation), butyric acid [107-92-6] (a dietary supplement), succinic acid [110-15-6] [a flavoring agent; alternative fermentation process to petrochemical route developed using agricultural waste carbon sources and consuming or fixing carbon dioxide (4)], itaconic acid [97-65-4] (an intermediate in polymeric resins and fibers), and sodium gluconate [527-07-1] (an industrial cleaner). Key solvents examples include ethanol (fermented from cornstarch instead of petrochemically derived), 2,3-butanediol [513-85-9] (a precursor to 1,3-butadiene [106-99-0] for rubber manufacture), acetone [67-64-1] and isopropyl alcohol [67-63-0]. Glycerol [56-81-5] (used for sealing compounds, antifreeze, and personal care products) is produced by altering the yeast fermentation process to favor its accumulation. Major amino acids, commonly produced using *Corynebacterium glutamicum*, are L-glutamic acid [56-86-0] (monosodium glutamate [142-47-2], MSG, for flavor enhancement),

L-lysine [56-87-1] (key supplement in feed grains for livestock and poultry), L-phenylalanine [150-30-1] (one component of the artificial dipeptide sweetener, aspartame [22839-47-0]), D,L- methionine [348-67-4, 63-68-3], and L-aspartic acid [56-84-8]. D-Sorbitol [50-70-4] (a low calorie food sweetener and precursor for vitamin C [50-81-7] synthesis) is the most common polyol produced via fermentation. Additional primary metabolite classes include vitamins {vitamin B_{12} [68-19-9] and riboflavin [83-88-5] (vitamin B_2)} and nucleotides (5′-inosinic [131-99-7] and 5′-guanylic [85-32-5] acids) that are used to enhance meat flavor. Finally, water-soluble viscous polysaccharides (such as xanthan [11138-66-2] and gellan [71010-52-1] gums used for thickening of foods) and biodegradable polymers (such as polyhydroxyalkanoate and polylactic acid), are synthesized in fermentations possessing complex broth rheology.

2.2. Secondary Metabolites: Antibiotics and Other Natural Products. Secondary metabolites also are known as idiolites (peculiar metabolites). Most have molecular weights <1500 Da (5) and have unusual extended ring structures. They are not involved in growth processes, are strain specific, and are produced as a mixture of a chemical family with slight differences in side chains (structural analogs). Sometimes the natural compound has been modified via chemical synthesis or biotransformation (semisynthetic compound) to improve potency and selectivity. It often has been debated whether secondary metabolites have survival functions in nature or sometimes serve no role whatsoever (6–8). The "no role" argument was supported when expanded detection methods yielded several nonantibiotic products, the production of which would give the organism absolutely no advantage in nature.

Most commonly, the term secondary metabolites are meant to indicate antibiotics or antiinfectives. These compounds are produced by microorganisms that kill other microorganisms at low concentrations according to the definition introduced by Selman Waksman (Rutgers University) ~1941. About two-thirds of known antibiotics are made by actinomyces. Other types of nonantibiotic secondary metabolites include cholesterol-lowering drugs (such as lovastatin [75330-75-5]; (9)), immunosuppressants (such as cyclosporin [59865-13-3]), enzyme inhibitors (such as acarbose acid [56180-94-0] and clavulanic acid [58001-44-8] (9), herbicides (such as bialaphos [35597-43-4]), and anticancer–antitumor compounds (such as taxol [33069-62-4] and daunorubicin [20830-81-3]).

2.3. Biomass. The production of biomass has been undertaken to manufacture cell mass for the food industry. Food manufacturing applications include starter cultures such as Bakers' yeast for cooking and cheese cultures. Animal feed applications include Brewers' yeast, a by-product of the brewing industry, obtained via broth flocculation (10). In addition, lactic acid bacteria are used to inoculate silage, corn and hay crops harvested for animal feed and then fermented to lower pH, thus inhibiting the growth of microorganisms and preserving nutritional value. Cell mass from one fermentation even has been used as a nutrient source for another fermentation (11).

Single-cell protein (SCP) has been developed as an animal feed (Pruteen, ICI, UK) and potential human food source [Quorn, Zenaca, UK, (12)]. Using various cultures such as *Methylophilus methylotrophus*, Pruteen is grown on normal paraffins (hydrocarbons), methanol or other substrates in the presence of a nitrogen source such as ammonia [7664-41-7]. In contrast, Quorn is comprised of the

fungus *Fusarium graminearium*, processed to have a meat-like texture and flavor similar to that of mushrooms. Since ~50–60 wt% of the cell's dry weight is protein, single cell protein is a potentially rich source of nutrition. Most recently live bacterial cultures have been used as animal feeds to competitively exclude and control salmonella (and thus avoid the use of antibiotics) and as human dietary supplements for healthier low fat lifestyles.

Cell mass from *Bacillus thuringensis* (BT) contains an endotoxin (20–30 wt% of its dry cell weight) active against insects upon ingestion. Recombinant DNA expression of this BT toxin in *Pseudomonas fluorescens* results in even higher accumulated concentrations in inclusion bodies and has been more effective than the natural cells when sprayed on crops as an insecticide.

In the field of bioremediation, cell mass is used to degrade toxic chemicals by bioconverting substrates into less harmful compounds (algae and bacteria for activated sludge), consumption of residual oil (*Pseudomonas putida*), destruction of microbial and synthetic polyesters by fungi (13), and extraction of metals from low grade ores (acid or base produced by *Thiobacillus* strains releases soluble metal). In addition, the biodegradation of dairy waste high in fat has been accomplished by the combination of lipases that digest the fat and bacteria which use the fatty acids liberated for growth (14).

2.4. Enzymes (and Whole-Cell Biocatalysts). Bioconversions or biotransformations are fermentation processes performed by organisms in which the products and substrates are similar. They utilize enzymes (protein catalysts) either as whole cells, isolated enzymes, or enzyme preparations (crude, pure liquid, or solid). Enzymes used as whole cells must have a cell wall that is permeable to the substrate of interest. Each of these sources of enzymes can be used in either free or immobilized forms or in either aqueous or solvent solutions. The nature of the enzyme source depends on the application as well as influences process cost and product impurities from side reactions. Batch-to-batch and vendor-to-vendor specific activity (activity per unit weight of enzyme preparation) variations can be difficult to minimize. Enzyme selection is affected by process economics, specifically product recovery, enzyme recycle/reuse, coenzyme requirements, side reaction tolerance, and activity stability.

There are >2000 known microbial and mammalian enzymes, excluding catalytic antibodies (Abzymes), which have been created for specific substrate specificity. They catalyze a broad spectrum of reactions and current areas of application are varied (15). One-third of all enzymatic reactions require cofactors such as adenosine triphosphate (ATP) [51963-61-2] or reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H) [606-68-8], [1184-16-3], which then need to be regenerated. The production and properties of proteases (16) and lipases (17) have been well studied.

The current areas of enzyme application are varied (18,19) with the major enzymes used industrially being proteases (added to detergents for protein stain removal) and amylases (for starch breakdown to dextrins, corn syrup extraction from wet grain, and bread shelf-life improvement). In addition, glucose [50-99-7] is converted to high fructose corn syrup (HFCS) by immobilized glucose isomerase [9001-41-6], juice and wine clarification is done by pectinase [9032-75-1], progesterone [57-83-0] is converted to cortisone [53-06-5] by *Rhizopus nigricans*, and ethanol is converted to vinegar by *Acetobacter* and *Gluconobacter* cultures.

Microbial rennins [9042-08-4] (also known as chymosin, a protease) have been developed for cheese manufacture to replace animal-sourced rennin. Finally, due to the European ban on animal proteins in animal feeds, demand for plant-protein hydrolyzing enzymes such as phytase [37288-11-2] has increased [Phyzyme XP, Danisco, Denmark, (20)].

In the preparation of chiral synthons for specialty and fine chemicals, several examples of enzymes use are evident. The production of sorbose [87-79-6] from sorbitol by *Gluconobacter oxydans* is a key step in the synthetic vitamin C process. The stereospecific coupling of racemic mixtures of phenylalanine and aspartic acid is accomplished enzymatically to produce aspartame and the bioconversion of acetoin [513-86-0] to diacetyl [431-03-8] (the flavor of butter) is done by lactic acid bacteria. Acrylonitrile [107-13-1] is enzymatically hydrolyzed to acrylamide [79-06-1] (a polymeric building block for materials such as nylon [63428-83-1]). A stereospecific enzymatic hydroxylation is part of the chemical synthesis of L-carnitine [541-15-1], a natural amino acid able to reduce circulating fat in the bloodstream. Penicillin G [61-33-6] or V is biochemically hydrolyzed to form 6-aminopenicillanic acid [66376-36-1] (6-APA), which then is used for synthesis of other beta-lactam antibiotics (21). Several other bioconversions to obtain derivatives of penicillins (22) and derivatives of rifamycin [13292-46-1] (23) have been developed.

Enzymes have been used in the preparation of protein pharmaceuticals such as the modification of porcine insulin to human insulin (18) and in gene splicing for the construction of plasmids in biotechnology.

2.5. Biologicals. Biologicals are large molecular weight (often >5 kDa) therapeutic proteins or vaccines with complex structures that are produced by living systems but are not metabolites. Therapeutic proteins can be constitutive (naturally occurring in the host cell) or recombinant (not normally synthesized by the host cell). Most commonly, the recombinant protein production route in genetically engineered cells has been selected since it results in higher product concentrations and permits alternate forms of the natural protein to be expressed. Therapeutic proteins include growth hormone, human insulin, interferons, erythropoietin, factor VIII, colony stimulating factors, interleukins, glucocerebrosidase, and humanized–human monoclonal antibodies [eg, for rheumatoid arthritis and pediatric respiratory syncytial virus (RSV) infection]. Proteins also are produced for diagnostic assays.

Vaccines can be of three major types: live attenuated viral (measles, mumps, rubella, oral polio, chicken pox), inactivated attenuated viral [hepatitis A (24)], cellular–subcellular component [polysaccharide conjugate vaccines (25)] or recombinant epitope [hepatitis B (26)]. Viral vaccines are grown in mammalian cell culture using anchorage-dependent primary cell lines (cells that require surface attachment to grow and that eventually reach senescence after a set number of generations). Attenuated or weakened viruses (viruses cultured for generations to select for strains weakened in their ability to cause disease) are used to inoculate these cultures. (The term "attenuated" was first coined by Louis Pasteur in 1880 when he was working on using attenuated bacteria for vaccines for anthrax, tetanus, and rabies.) Cells then are lysed, viruses or antigens purified to varying degrees, and finally inactivated as necessary. In contrast, only the genetic information necessary to create the viral epitope

(the subunit of the virus which causes an immune response) is expressed in a recombinant epitope vaccine.

3. Advantages Over Organic Synthesis

Biochemical routes can have strong advantages over synthetic organic routes to synthesize molecules of commercial interest. Often the most economical way to manufacture complex molecules like enantiomers utilizes whole cells or enzymes. For some large volume products such as penicillin, chemical syntheses have never been more economical than fermentation; for others the evaluation has depended largely on the availability of raw materials and the development stage of the processing technology.

Fermentation-derived compounds can have greater specificity for the desired target since only the desired enantiomer is produced that avoids yield losses incurred by discarding the wrong enantiomer. A single step can replace several steps of a chemical synthesis, reducing the number of overall reaction steps. Biochemical conversion is able to be achieved at positions difficult to alter chemically due to lack of activation. In addition, natural carbon sources, such as corn starch, already have an oxygen on each carbon atom and thus avoid having to oxidize petrochemical feedstocks (27). Mutation and genetic engineering techniques can enhance the versatility of biochemical routes, increasing the variety of reactions performed (28,29).

Adverse and extreme chemical synthesis conditions are reduced substantially since biochemical reactions are performed at moderate conditions of pH, pressure, and temperature. Biochemical routes utilize cheaper and more environmentally friendly raw materials with the toxic and hazardous reagents often necessary in chemical synthesis less common. Natural resources are conserved as in the specific case of the anticancer drug taxol [33069-62-4], where large scale harvests of the Pacific yew tree were avoided through the introduction of biotransformations into the synthetic route and the development of a plant cell fermentation process. Due to these attributes, whole cells and enzymes are referred to as “green” catalysts (30), although they are autocatalytic in nature.

For fine chemical synthesis, the main drawback of biochemical processes when directly compared to synthetic processes is their dilute nature with typical product concentrations less than the peak values of 10–15 wt% observed for high volume primary metabolite organic acid and alcohol fermentations. A second drawback can be the efficiency and speed of the discovery of the biochemical route itself. Although in general there is a high ratio of biocatalyst surface area to liquid volume which facilitates rapid uptake of nutrients in biochemical systems (31), limitations in liquid mass transfer, mixing and possibly heat transfer at lower cultivation temperatures can challenge scale-up performance.

4. History

4.1. Early Times. Ancient fermentations were prominent in several civilizations (Oriental, African, Aryans, Egyptians, Sumerians) (32,33). Reliable

procedures were developed for making items such as soy sauce, bread, beer, wine, sake, vinegar, pickled foods, cheese, kefir (a fermented milk drink), and yogurt prior to the emergence of microbiology or even the development of a thermometer. Both single and mixed-culture biochemical systems were employed. For example, Koji-kin or Koji mold, consisting of *Aspergillus oryzae*, (1) ferments soybeans directly to produce miso or soy sauce or (2) ferments rice starches into sugars that then are fermented by yeast to produce sake. Active enzymes from malted barley (diastatic malts) degrade starches that then are fermented by yeast to produce beer. These varied food-based fermentations were conducted to enhance shelf-life and provide an improved variety of flavors for a typical diet of the period (34).

4.2. 1700s–1900. Alcoholic beverages and vinegar comprised much of the world's fermentation capacity prior to 1900 with large scale breweries appearing as early as the 1700s. By the nineteenth century, fed-batch operation and aeration was used for growing yeast cell mass. The first bioconversion was the transformation of ethanol (wine) to acetic acid (vinegar from *vinaigre*, which means sour wine) using *Acetobacter* and *Gluconobacter* species. The first immobilized bioconversion appeared in the 1820s and used wood chips to immobilize cells. In Japan, black shoyu was prepared first by conducting a surface anaerobic fermentation of mold-hydrolyzed grains, then transferring the contents to 10,000 gal open top fermenters for 1 year (35). Machinery, such as steam power and power-transmission systems developed in the nineteenth century, was adopted for fermentation use.

Early microbiological discoveries were key to the history of fermentation. Around 1677, Anton van Leeuwenhoek skillfully ground his microscope lens to obtain magnifications of 200-fold, enabling him to see bacteria. In 1837, Louis Pasteur offered the first proposal that yeast is responsible for conversion of sugars to ethanol and carbon dioxide, but it was challenged since the process was thought to be too simple to be done by a living organism. Pasteur also recognized that living organisms only produce molecules of a certain optical symmetry. Investigations of contaminations during yeast fermentations by Pasteur in 1861 identified a bacterial strain that converted sugar to lactic acid. In the same year, Pasteur also discovered that more alcohol was produced when sugar was fermented anaerobically and that butyric acid fermentations required anaerobic organisms. From his work on both the yeast and butyric acid fermentations, Pasteur hypothesized that fermentation was a biological process performed by microorganisms (germs), either anaerobically or aerobically depending on the specific system. By 1870, the concept of abiogenesis or spontaneous generation of life from nonliving materials, was being disproved for newly discovered microorganisms as it had been previously in 1665 for complex organisms. In 1899, the first recognition of the dependence of viruses on cells for production was made by Martinus Beijerinck.

Other accomplishments aided progress in fermentation development. In 1878, Joseph Lister demonstrated that the lactic acid fermentation of milk caused souring, isolated the first pure culture (*Bacterium lactis*), and subsequently developed techniques for streaking and culture enrichment. In 1881, Robert Koch demonstrated the growth of bacteria on solid media, in 1884 Christian Gram developed the gram stain to help identify bacteria, and in 1887

Julius Richard Petri developed the Petri dish. Finally, in 1897, Eduard Buchner (1907 Nobel Prize in Chemistry) discovered that yeast extract (an enzyme preparation from hydrolyzed yeast cells) combined with sugar could produce carbon dioxide and alcohol, the first cell-free bioconversion. This latter discovery proved that whole cells were not necessarily required for fermentation and thereby inaugurated the field of enzymology.

Around 1894 in Japan, Jokichi Takamine isolated a powerful starch digesting fungal enzyme from rice malt, call Takadiastase (taka—best, diastase—enzyme). He produced it using large-scale surface culture both in Japan and the United States (where he immigrated with his American wife).

4.3. 1900–1945. Developments in fermentation and biochemical processing during this period were driven by World War I (WWI) and World War II (WWII). In the early 1900s, fed-batch cultures were devised for Bakers' yeast production to shift malt use from ethanol production to cell mass so that yeast might be used for human food and animal feed due to shortages during WWI. To cheapen biochemical synthesis costs in Germany, ammonia was used instead of organic nitrogen sources. Glycerol and acetone were required for munitions (explosives) manufacture in Germany and England, respectively (31). In 1911, Carl Neuberg developed the glycerol fermentation in Germany by adding sodium bisulfate [10034-88-5] to alcohol fermentation by yeast to enhance formation of this by-product. In England, Chaim Weizmann developed the first truly aseptic fermentation in 1916, the relatively simple Weizmann process for anaerobic acetone–butanol fermentation by *Clostridium acetobutylicum*, which utilized pure culture, sterile media, short cycle time, and had no mid-cycle additions. (Initially acetone and then later on butanol were needed for British WWI munitions preparations.)

In parallel to WWI demands, denatured ethanol production for industrial usage was bolstered due to its newly acquired tax-free status in the early 1900s. This economic driver directly resulted in the construction of several alcohol plants worldwide by the 1920s. Papain was used to remove protein hazes from beer ~1911. In 1911, J. N. Currie first produced large quantities of citric acid from *Aspergillus niger* fermentation by using a growth-limiting iron-rich medium. Later in 1919, Pfizer (New York) conducted large quantities of citric acid tray fermentations to avoid dependence on European sources of lemon and lime concentrates that were not available due to WWI. In 1914, E. Adern and W. Lochett pioneered the first large scale activated sludge process for sewage treatment to minimize disease outbreaks in industrial cities complete with aeration, suspended cells, and biomass recycle. By the mid-1920s, several multiple large (about 50,000 gal each) submerged fermentor installations were in place and operating successfully for solvents and organic acids, primarily anaerobic fermentations. In the 1930s, pectinases first were used for fruit juice clarification and *Acetobacter suboxydans* first was used for the oxidation of sorbitol to sorbose in vitamin C synthesis.

By the 1930s, the needs of WWII led to a worldwide emphasis on the study of fermentation of natural feedstocks (such as sugar and sugarcane) to produce fuels and solvents. Emphasis rose in Germany when the Allies' blockage prevented importation of the necessary fats for synthetic routes. Fed-batch yeast cultivation processes developed earlier used open style large fermenters, cooling

coils, and subsurface aeration with sparge tubes and glass wool filtration to trap microorganisms from the air. In the 1940s, the recovery of carbon dioxide as an ethanol fermentation by-product was undertaken to use it for compressed gas, "dry" ice and beverage carbonation.

In the meantime, Alexander Fleming had discovered lysozyme, an antibacterial enzyme, in 1922 and then he discovered the first penicillin strain by 1929 (31). Penicillin was initially grown in a tray form (surface culture) in shallow pans [including bedpans when glass was scarce during WWII (36)] placed in incubators with the medium manually refreshed periodically. Surface culture cultivation was already well-established worldwide for diastatic enzyme and organic acid processes.

At the time ~1938, when the need to produce large quantities of penicillin was becoming apparent, the *Aspergillus niger* citric acid fermentation (also being done as surface fermentations in trays as early as 1911) was being converted to submerged fermentation following the already well-established submerged process for alcohol. Molasses substituted for refined sugar for citric acid production later became key to large scale penicillin production. In addition, other surface cultivation processes being converted to submerged processes were gluconic acid production by *A. niger* ~1937, riboflavin production by *Bacillus subtilis* around 1940, and fumaric acid [110-17-8] production by *Rhizopus nigricans* around 1943.

Based on the success of penicillin in early patients, several companies obtained cultures and started production programs to meet the wartime production requirements. The first submerged fermentations for penicillin were conducted ~1942/1943 to improve production under aseptic conditions. In contrast to the earlier fermentation processes, penicillin cultivation required an absolutely pure culture. It was susceptible to contamination due to its rich, nonselective medium, fed-batch operation, high airflow rate and long cycle time. Initial attempts at submerged penicillin culture tried to mimic static culture, using gentle agitation conditions. It soon was discovered that higher impeller speeds and shear gave better results (37). Despite tremendous difficulties, the submerged penicillin process was successfully implemented to provide sufficient quantities for WWII D-day invasion casualties. Key developments included the discovery of strains more suited to submerged cultivation, the resolution of raw material variations, and the reliable supply of large quantities of sterile air for broth aeration.

As modern industrial microbiology developed, it became possible to obtain pure cultures reliably and thus discover and develop new strains (38). The term "soil microbiologist" was used by the end of this period since many new cultures were isolated from soils, typically containing ~10 million microbes/g. The antibiotic streptomycin [57-92-1] was discovered by Selman Waksman (Rutgers University, NJ) in 1944 (1952 Nobel Prize in Medicine/Physiology) by screening soil bacteria to find *Streptomyces griseus*. This success indicated that systematic screening for new antibiotics was feasible (39), a strategy that was widely adopted post-WWII.

4.4. 1945–1960. The year 1947 has been referred to as the "dawn of biochemical engineering" (33) with the introduction of formal university biochemical engineering training (40) and the increasingly important role of

biochemical sciences being recognized (41). Also in the 1950s, the *Journal of Biotechnology and Bioengineering* was founded. The "biologist-engineer team" (37) was firmly established. As a result of these joint efforts, key developments in the sterile aeration/mixing of viscous non-Newtonian mycelial cultures fluids (42,43), culture mutation–selection and product isolation were realized. Specific equipment was devised including ring spargers, larger mixer shafts for higher horsepower input, aseptically operating agitator seal stuffing boxes, oil-free sterile air compressors and glass wool for sterile air depth filtration. Multiple Rushton radial flow impeller configurations (flat disk with perpendicular blades) were introduced for fermentation applications. The pilot plants that had been erected starting in 1941–1942 now were complete and being actively used in development programs to mimic larger fermentation and isolation installations.

Throughout the post-WWII 1950s other antibiotics were commercialized such as novobiocin [1476-53-5], erythromycin [114-07-8], streptomycin, tetracycline [60-54-8](44), neomycin [1405-10-3], chloramphenicol [56-75-7], vancomycin [123409-00-7], kanamycin [25389-94-0] and nystatin [1400-61-9] (the first fungal antibiotic). Worldwide demand soared for streptomycin as its success in the treating infections became apparent. Antibiotic screening and production efforts were occurring worldwide in Japan, Switzerland, Russia, and England as well as in the United States. Selman Waksman (Rutgers University, NJ) was the leading American discoverer of 18 antibiotics, and Hamao Umezawa (Institute of Microbial Chemistry, Tokyo, Japan) rose to become another world authority in development of new antibiotics via fermentation. Antibiotics began to be used in animal cell cultivation to maintain sterility which permitted breakthroughs in cultivating viruses in specific cell lines rather than in whole animals. Specifically, in 1949, John Enders, Thomas Weller and Frederick Robbins demonstrated growth of the polio virus in test tube cultures of human tissues.

Vitamin B12 was discovered to be produced by *S. griseus* through screening soil microorganisms in comparison to the antianemia activity known to be present in liver extracts. It was subsequently produced at the large scale by bacterial fermentation in aerated stirred tanks (45). Amino acid production was enhanced by the successful commercialization of the 1957 discovery by Shuko Kinoshita and co-workers (Kyowa Hakko Kogyo) that *Corynebacterium glutamicum* excreted L-glutamate, previously isolated from seaweed.

A bioconversion, discovered by Peterson and Murray (Upjohn Laboratories, Kalamazoo, Mich.), was used to produce cortisone from the hydroxylation of progesterone (46). This single bioconversion reduced the process complexity from 37 to 11 steps and price from $200 to $6/g (during the 1950s), and then further to $0.46/g in 1980s with improved process development. Also during this period, renewable energy applications were being developed using cellulolytic enzymes (cellulase) to enzymatically convert renewable biomass to inexpensive fermentable sugars (47).

Meanwhile, two biochemists, James Watson and Francis Crick developed the double helix DNA model with the structure of DNA being elucidated in 1953 (1962 Nobel Prize in Medicine/Physiology).

4.5. 1960–1970. The number of products grew dramatically as additional antibiotics (streptavidin [9013-20-1]), enzymes (glucose isomerase, rennin),

microbial insecticides (*Bacillus thuringiensis*), purine nucleosides (inosine [58-63-9] and guanosine [118-00-3]) and other flavor and perfume compounds were introduced and commercialized. The low prices for carbon feedstocks useful for fermentation (such as methanol [67-56-1], agricultural by-products and hydrocarbons) led to the production of biomass as a source of protein for animals. At Imperial Chemical Industries (ICI, UK), a large 1,500,000-L continuous fermenter was implemented to produce biomass at a competitive price, which was a tremendous undertaking (although problematic due to its size and now retired and dismantled). The steam-sterilizable (or autoclavable) galvanic dissolved oxygen electrode for biochemical engineering usage was introduced (48) and quickly implemented to monitor broth dissolved oxygen levels during aerobic fermentation.

Suspension and anchorage-dependent animal cell cultivation processes for vaccines were implemented. For polio, the killed virus Salk vaccine was produced and tested on a large scale in the mid-1950s, followed closely by the attenuated live virus Sabin vaccine that was approved in 1960. The live virus mumps vaccine was developed in 1967 and the smallpox vaccine was widely distributed by WHO in 1967. Later on, anchorage-dependent cells were attached to microcarriers (cross-linked dextran beads charged with tertiary amine groups) with a higher surface to volume ratio than previously used flat surfaces (49,50).

4.6. 1970–1995. During this period, substantially improved cultures and fermentation processes for primary and secondary metabolites resulted in excess fermentation capacity for newer products or for contract manufacture. In other cases, fermentation capacity expansions and new plant construction were required. Special sensors were developed for on-line and off-line measurements for improved process monitoring and control. Additional beta-lactam antibiotics (such as cephamycins [35607-66-0], carbapenems [328-50-7], clavulanic acid [58001-44-8] and nocardicins [118246-74-5]) besides the penicillins and cephalosporins [61-24-5] were discovered. The microbial polysaccharide, xanthan gum, was approved as a food additive in 1983 and commercialized in 1986 (51). Contract companies such as Panlabs (Bothel, WA), specializing in the microbiological aspects of antibiotic process development (screening, mutation), sped the scale-up and production of these new compounds.

Advances in genetic and metabolic engineering exploded with a heretofore unprecedented pace of biology research. Traditional microbiology merged with molecular biology to produce several new products of the biotechnology era (31). In 1973, the first recombinant DNA plasmids (plasmid DNA enzymatically broken and recombined with DNA of a different origin) were constructed (52) to demonstrate the initial strategy for gene transplantation to produce foreign proteins in bacteria. Herbert Boyer (University of Southern California) produced the first synthetic insulin. Sequencing of DNA base pairs was established in 1975 to confirm that precise alterations of genetic material were able to be implemented. In 1988 Kary Mullis (1993 Nobel Prize in Chemistry) published the details of the polymerase chain reaction (PCR) to exponentially amplify a specific region of DNA by severalfold.

Recombinant techniques were applied to raise secondary metabolite production titers and vary structures (53). Ichiro Chibata (Tanabe Seiyaka, Japan) utilized recombinant techniques to improve tryptophan [73-22-3] titers

and recombinant technology was applied to several amino acid biosynthesis processes (54). Recombinant plants were developed that were resistant to herbicides used for weeds, pesticides, drought, salt, cold/frost, and mercury. The field of technical bioenergetics arose to harness the conversion of energy by living organisms to produce renewable fuel (55).

Proteins normally produced in more complex and difficult to culture animal cells were able to be manufactured in bacteria, yeast and fungi. Using high cell density fed-batch fermentations, cells were grown, and then induced to produce the product of interest. Multiple copies of genes were inserted for the desired protein to enhance its production, either to obtain the protein directly or to enhance productivity of a metabolite by increasing concentrations of enzymes in a synthetic pathway (metabolic pathway engineering).

In 1975, George Kohler and Cesar Milstein (1984 Nobel Prize in Medicine/Physiology) developed hybridomas (physically fused antibody-producing B-lymphocyte cells with immortal myeloma cells). This development permitted generation of a single unique type of antibody (monoclonal) raised against a specific desired antigen.

Several start-up companies were established to commercialize these developments. Specifically, Cetus (now Chiron, Emeryville Calif.) was founded in 1972 to specialize in the genetics of industrial microorganisms, Genentech (San Francisco, Calif.) was founded in 1976 as an entrepreneurial biotech company to exploit "genetic engineering technology", Biogen was founded in 1978 and now claims to be the world's oldest independent biotech company, Amgen (Applied Molecular Genetics) was founded in 1980, and Genencor (joint venture between Genentech and Corning) was founded in 1982. The first biotechnology company in the United Kingdom, CellTech, was founded in 1980.

Animal (bovine, pig, and fish) growth hormones were developed initially to increase milk production and/or body weight. Shortly thereafter, human therapeutic proteins entered the market (56,57) which included human insulin in 1982 (Eli Lilly/Genentech), interferon in 1986 (Schering Plough, Hoffman-LaRoche), human growth hormone in 1985 (Genentech), hepatitis B vaccine in 1986 (Chiron/Merck), allograft rejection antibody in 1986 (Johnson and Johnson), tissue plasminogen activator in 1987 (TPA, Genentech), erythropoietin in 1989 (Amgen), granulocyte-colony stimulating factor in 1991 (Amgen) and interleukin-2 in 1992 (Cetus/Chiron). These recombinantly produced proteins were alternatives to the inefficient isolation of the previously discovered desired natural proteins from animal body parts.

An emerging acceptance of biochemical enzymatic transformations as "routine" parts of chemical syntheses for a wide spectrum of reactions (18,58) arose along with novel approaches such as using proteases to synthesize rather than break down peptides (59). The enzyme industry was enhanced by a newly found ability to produce large quantities of enzymes (such as recombinant renin (chymosin) for cheese) via genetic engineering. Improvements in the production and alteration of enzyme activity, specificity and stability were attained by selective replacement of amino acids at the active site using directed evolution or site-specific mutagenesis to reengineer enzymes to match specific process needs or to increase their activity against unnatural substrates (60). Immobilized enzymes and whole cell systems began to replace some fermentations. During

the mid-1970s high fructose corn syrup was produced in high volumes using glucose isomerase to transform glucose into its sweeter isoform fructose [7660-25-5]. A decade later in the mid-1980s, catalytic "enzyme-like" antibodies were raised against the stable transition state analogue of the reaction of interest (61,62).

The extensive developments over this period are evident when comparing a checklist of fermentation products and producers in 1977 (63) with a summary of products and bioprocesses using genetically modified organisms (GMO), including bioengineering issues and trends though 1995 (64). As a result of changing product types (and a 1974 plea from noted scientists concerned about the creation of harmful recombinant organisms), additional regulations were initiated such as the 1976 recombinant DNA guidelines from the US National Institute of Health (NIH), the 1976 Good Manufacturing Practice (GMP) and Good Laboratory Practice (GLP) guidelines from the US Food and Drug Administration (FDA), and various environmental acts from the US Environmental Protection Agency (EPA) throughout the 1970s. These regulations were updated and augmented continually through to the present day. In 1980, the United States Supreme Court ruled that laboratory-altered microorganisms were patentable (Diamond vs. Chakrabarty), the first of many high commercial impact biotechnology court rulings.

4.7. 1995—Present. During the past few years the explosion in information science has influenced biochemical processing dramatically. Genome sequencing (decoding of the human genome of ~30,000 genes) has expanded the number of new targets for new drugs and driven the implementation of new drug discovery technologies. Functional genomics has developed huge sequence databases and, with the power of advanced computers, has determined the biological function of gene protein (specifically its role in disease) to improve attempts to create a therapy. Bacteriophages (bacterial viruses) have been developed as enzymatic tools for molecular biology using phage display techniques in which foreign gene products are fused onto the phage outer-surface coat proteins (65). The use of recombinant DNA technology to manipulate cell functions (66) has been used to develop new metabolic pathways to generate new products or to eliminate by-products to increase titers of desired products (67–69).

Although there have been continuous advances in the discovery and production of novel anti-infectives, enzymes and therapeutic proteins, new types of drug products have emerged that have demanded new types of process development efforts. These categories include: DNA vaccines (recombinant plasmids injected into human cells to stimulate antigen production have required techniques for amplifying levels of a specific plasmid above its counterparts); gene therapy (use of recombinant retroviruses to transfer healthy copies of a defective gene has required large scale cultivation of the host viruses and cells); and murine (100% mouse), chimeric (30% mouse/70% human), humanized (5–10% mouse/90–95% human) or fully human antibodies (recombinant antibodies, with key portions altered to match those of the natural human antibody rather than its mouse antibody counterpart have required methods to produce high titers in animal cells). Controversial stem cell technology has required cultivation techniques for a primitive cell line obtained from embryos that can develop into most of the cell types found in humans. Finally, antisense

compounds, such as fomivirsen [144245-52-3] (Isis Pharmaceuticals, Carlsbad, CA), which are "senseless" since they lack Watson–Crick hydrogen-bonding sites, prevent the attacking bacteria or virus from producing disease-causing proteins and have required the synthesis and incorporation of unusual purine bases to insert within RNA.

Food-based therapies or "functional food" enhancers, known as nutriceuticals, have gained tremendous popularity. These include a fermented lactic acid drink to moderate blood pressure (Calpis Co., part of Ajinomoto, Japan); production of glucosamine [66-84-2] (to reduce arthritis) in a cost-effective microbial fermentation process as an alternative to chemically treating shellfish (Bio-Technical Resources in partnership with Cargill, Minneapolis, Minn.); probiotic bacteria (Generally Recognized As Safe (GRAS) strains that survive digestion and confer a demonstrated medical benefit) to restore health to human gastrointestinal systems using a fermented capsule containing live bacteria (Nutraceutix, Redmond, Wash.); production of omega fatty acids (DHA, docosahexenoic acid [6217-54-5]) for infant formula and cognitive decline through fermentation of algae instead of processing fish oil; and a new strain for the Japanese fermented soybean "natto" production with a six-fold higher thrombolytic activity to improve longevity (Asahi Industry Co., Ltd., Tokyo, Japan).

Alternate fermentation methods have arisen aimed at conducting fermentations on a smaller scale for screening and process development purposes. These methodologies include scale down, "well plates" and microfermenters on a silicon chip. The idea is to set up 10–10,000-fold smaller systems to mimic operating conditions of production scale fermentors to simulate mixing, mass transfer, and heat transfer performance for troubleshooting of production problems, process development and/or process validation. Several multiple smaller units also have been useful to execute statistically designed parameter screening experiments.

Bacterial and fungal fermentations have been conducted in space to study growth and antibiotic production (70,71). In instances where lack of gravity positively affected cell growth, production improvements of 75–200% were observed (72). Normal and cancerous animal cell lines also have been cultivated in space to study their growth and assembly into tissue (73).

5. Fermentation Companies, Products, Market and Economic Aspects

Fermentation products span the gamut from high value, low volume products to low value, high volume products (Table 1). Products that have a high market value are normally required in smaller quantities. Costs generally are inversely proportional to annual tonnage demand (Fig. 1) since higher demands force greater production efficiency for both the strain (maximal specific productivity) and the process (maximal volumetric productivity). Specifically, the price of penicillin decreased >1000-fold as production quantities increased >1000-fold during a 50-year period.

Depending on the nature of the product, the dominating factors of inventory cost and the relative proportion of costs incurred by either the fermentation or isolation stages can vary considerably (Table 1). Although medium costs are a

Table 1. **Comparison of Estimated Typical Parameters for Various Classes of Fermentation Products**[a]

Product Class	Batch size (kg)	Product selling price ($/kg)	Media cost ($/L)	Titer (g/L)	Purification/ fermentation cost	Manufacturing scale (L)	Example product span	Annual world market (billion $/year)	Annual world tonnage (million metric tons/ year)
primary metabolites (organic acids, amino acids, solvents, vitamins)	100–10,000	0.1–10,000	0.10–0.25	0.1–150	0.4	50,000–250,000	ethanol to vitamin B12	10	2–3
secondary metabolites (antibiotics)	100–1000	10–1000	0.25–2	1–10	0.6	50,000–250,000	penicillin to lovastatin	30–35	1
cell mass	100–10,000	0.1–10	0.10–0.25	50–300	0.1–0.3	50,000–250,000	Bakers' yeast to *B. thuringienisis*	0.5	0.1
industrial enzymes	10–1000	10–100,000	0.25–2	0.1–100	0.3–0.7	10,000–100,000	glucoamylase to lactate dehydrogen-ase	2	0.15
biologicals (therapeutic proteins/ vaccines)	0.001–0.1	10,000–1,000,000	1–30	0.1–2	0.9	10–10,000	insulin to humanized antibody	30–35	0.00005

[a]Based on information from (31,74) and corporate website sources.

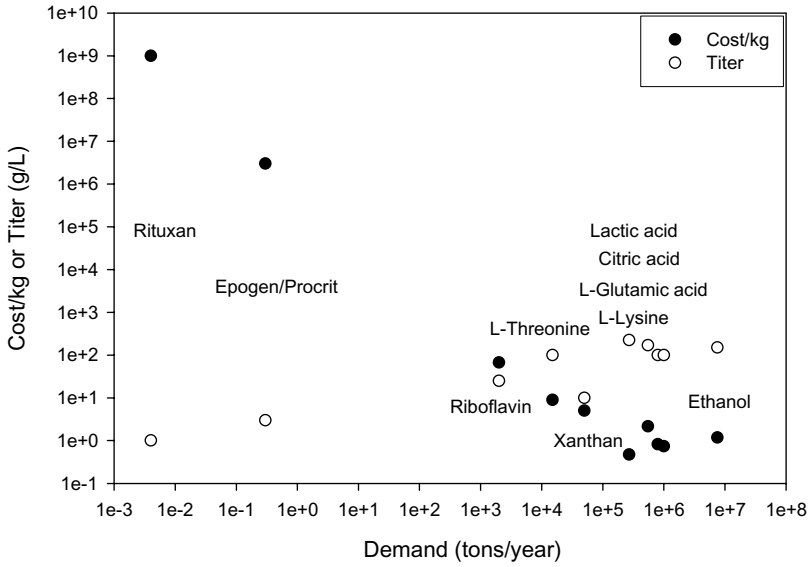

Fig. 1. Relationship of annual worldwide production volume (demand) to typical product titer, unit cost and worldwide market value (based on data from 1998–2003). Data is from (31,74,75) and *Chemical Market Reporter*.

significant proportion of inventory cost for high volume commodity chemicals, this proportion is less for lower volume materials in which purification costs often dominate. Inventory cost is affected by requirements for product purity, process characteristics, raw materials, waste disposal, capital (including automation requirements), and operating costs such as labor and energy. The key factors that influence profitability are the strain yield and product complexity.

Excluding biologicals (specifically vaccines and therapeutic proteins), >60% of approved and prenew drug application (NDA) candidates and nearly one-half the best selling pharmaceuticals are either natural products (or molecules related to them) (32). This trend is continuing with 17 of 37 (46%) of 2001 newly approved pharmaceutical products being biologically-based. There are >1600 biotech companies in the US alone (31).

To maintain filled pipelines, larger fermentation companies have expanded from small molecules and in some cases vaccines into therapeutic proteins (biotechnology products) either by acquisition/alliances (76) or by developing in-house discovery and production capabilities (Table 2). In 2001 >480 Pharma-biotech alliances existed worldwide owning one-third of the new entities in clinical trials and they are increasing consistently (81). Pharma companies benefited by outsourcing development work, while biotech companies gained sales and marketing forces for their products. Conversely, some biotech companies such as Amgen are expanding into small molecules. Interestingly some larger suppliers of fine chemicals are providing contract manufacturing for biologicals (82) and several traditional chemical companies are exploring biotechnology to improve existing fermentation routes or establish new ones (27). There

Table 2. **Examples of Fermentation, Biotechnology and Enzyme Companies and Their Product Areas[a] and Their Key Products Areas[b]**

Company (location)	Abbreviation and/or comments	Primary metabolites (A)	Secondary metabolites (B)	Biomass (C)	Enzymes (D)	Biologicals (E)	Contract sectors
Abbott (Abbott Park, IL)	acquired pharamaceutical business of BASF		antiinfectives		nutritionals	antibodies diagnostic proteins	A, B, D, E
Ajinomoto (Tokyo, Japan)		amino acids flavors nucleic acids nutraceuticals			nutraceuticals		
Alltech (Nicholasville, KY)		beverages (alcohol) flavors	antibacterials	Brewers' and distillers' yeast Probiotics Silage inoculants	surface bulk enzyme fermentations		
Altus Biologics (Cambridge, MA)	protein crystallization methodology		amino acids		industrial and diagnostic enzymes		D,E
Amano (Nagoya, Japan)					bulk, fine chemical and diagnostic enzymes		
Amgen (Thousand Oaks, CA)	merged with Immunex					therapeutic proteins antibodies	
Archer Daniels Midland (Decatur, IL)	ADM	solvents amino acids organic acids polyols vitamins xanthan gum glucodeltalactone nutraceuticals		animal feeds	food and industrial enzymes		
Avecia (Manchester, UK)		nucleic acids			fine chemical enzymes		A, B, D, E
Aventis (Strasbourg, France	formerly Hoechst and rhone–Poulenc and Aventis Pasteur	vitamins	antiinfectives			therapeutic proteins human vaccines	
Baxter (Deerfield, IL)	acquired North American vaccine		secondary metabolites			therapeutic proteins human vaccines	

Table 2 (*Continued*)

Company (location)	Abbreviation and/or comments	Primary metabolites (A)	Secondary metabolites (B)	Biomass (C)	Enzymes (D)	Biologicals (E)	Contract sectors
BASF (Mt. Olive, NJ)	acquired Takeda	amino acids vitamins polyhydroxyalkanoate polyesters nutraceuticals	antiinfectives				
Bayer (Leverkusen, Germany)	merged with Miles Labs		antiinfectives secondary metabolites			therapeutic proteins animal vaccines	
Biogen (Cambridge, MA)	merged with IDEC					therapeutic proteins Antibodies	
Bristol-Meyers-Squibb (New York, NY)	BMS, acquired part of Imclone		Antiinfectives, secondary metabolites			Therapeutic proteins antibodies	
Cambrex BioScience (Baltimore, MD)	acquired Marathon and BioScience						E
Cargill (Minneapolis, MN)	joint ventures with Dow, DSM and Degussa	solvents organic acids xanthan gum polylactic acid biodegradable polymers		bacterial biomass for animal feed	food enzymes animal feed enzymes		
Centacor (Malvern, PA)	acquired by Johnson and Johnson					therapeutic and diagnostic proteins antibodies	
Chiron (Emeryville, CA)	merger with Cetus					therapeutic and diagnostic proteins human vaccines	E
Danisco Cultor (Brabrand, Denmark)	partner with Diversa	polysaccharide gums flavors		food cultures probiotics	food, industrial, animal feed and molecular biology enzymes		

Degussa (Hanau, Germany)		amino acids		food cultures probiotics	enzymes		
DSM (Basel, Switzerland)	merged with all/some of Gist-Brocades ChemFerm subsidiary purchased Roche vitamins	vitamins	penicillin and its precursors	yeast food cultures	fine chemical and food enzymes		D,E
Dupont (Wilmington, DE)	alliance with Diversa for enzymes	1,3 propane diol					
Eli Lilly (Indianapolis, IN)						therapeutic proteins antibodies	
Genencor (South San Francisco, CA)					bulk and specialty enzymes	therapeutic proteins, antibodies and enzymes Diagnostic proteins	
Genentech (South San Francisco, CA)	acquired by Hoffmann-LaRoche					therapeutic proteins and enzymes antibodies	
Genetics Institute (Andover, MA)						therapeutic proteins antibiodies	
Genzyme (Allston, MA)						therapeutic enzymes surgical polymers	
Glaxo-Smith-Kline (Uxbridge, UK)	GSK, formerly Glaxo Wellcome and Smith–Kline–Beecham					therapeutic proteins antibodies vaccines	E
Hoechst Marion Roussel		nucleic acids			industrial and diagnostic enzymes	recombinant DNA therapeutic proteins	
Hoffmann-LaRoche (Basel, Switzerland)	recently sold vitamins to DSM					therapeutic and diagnostic proteins antibodies	
Kyowa Hakko Kogyo (Tokyo, Japan)	created BioWa subsidiary for biologics	alcohols, amino acids, 4-hydroxy-L-proline nucleic acids				antibodies	

Table 2 (*Continued*)

Company (location)	Abbreviation and/or comments	Primary metabolites (A)	Secondary metabolites (B)	Biomass (C)	Enzymes (D)	Biologicals (E)	Contract sectors
Lonza (Slough, UK)	acquired Celltech manufacturing	vitamins					A, B, D, E
Merck (Whitehouse Station, NJ)			antiinfectives antiparasitics other secondary metabolites			human vaccines	
Meriel (Duluth, GA)	joint venture of Merck and Aventis		antiparasitics			vetinary vaccines	
Novartis (East Hanover, NJ)	merger of CIBA–Geigy and Sandoz		antiinfectives			Therapeutic proteins, antibodies	
Novo Nordisk (Bagsvaerd, Denmark)	also Novozymes				industrial and diagnostic enzymes	therapeutic proteins	
Pfizer (New York NY)	merged with Warner–Lambert and Pharmacia	organic acids biopolymers	antibiotics antiparasitics		bulk enzymes		
Pharmacia (Peapack, NJ)	merged with Upjohn and Monsanto					therapeutic proteins humanized antibody fragments Diagnostic antibodies	
Schering-Plough (Madison, NJ)			antibiotics cortisones			therapeutic proteins	
Tate and Lyle (London, UK)	merged with A.E. Staley and Haarmannn and Reimer	solvents amino acids organic acids polyols flavors		bacterial protein	bulk chemical transformations		
Wyeth (Madison, NJ)	formerly American Home products, acquired American Cyanamid and Ft. Dodge	vitamins	antiinfectives secondary metabolites			human and vetinary vaccines therapeutic proteins antibodies	

[a]Including contract facilities for production and research.
[b]From (77–80) and corporate website sources.

has been substantial merging and consolidation among companies within and across product areas (Table 2)

5.1. Primary Metabolites. The 1999 worldwide sales of amino acids are ~$3 billion/year for a production volume of 1 million tons/year (31). The largest volumes produced are glutamic acid, lysine, and methionine. Consumption of fermentation-derived amino acids is highly influenced by livestock and feed production as well as the price of naturally sourced amino acids (83). In the case of other primary metabolites, by 2000 the worldwide citric acid market was $1.4 billion/year with 400,000 tons/year produced, while the worldwide vitamin C market was much smaller at $0.06 billion/year with 60,000 tons/year produced (31).

About 92.5% of the 2000 annual US ethanol capacity is produced by fermentation rather than synthetically (84). For the 1.55 billion gal/year produced by fermentation for fuel, food and beverage usage in 2000, 90% is used for fuels (a substitute for fossil fuels, oxygen-containing molecules are needed for gasoline to satisfy US Clean Air Act of 1990 and ethanol competes favorably with methyl *tert*- butyl ether [1634-04-4] (MTBE), which has risks of groundwater contamination), 5% for industrial solvents/chemicals, and 5% for beverages. The current 2003 worldwide ethanol market is 2 billion gal/year and is expected to grow substantially as production processes become more commercially viable (85).

5.2. Secondary Metabolites. Although there are >6500 types of antibiotics described in the literature, only ~25% are characterized chemically and only ~2% are on the market. By 2000, the antibiotic worldwide market was $30 billion dollars and was composed of only ~160 antibiotics and derivatives (31). About 65% of the current antibiotic market is β-lactam antibiotics (86). New antibiotics continually are needed as resistance develops to existing ones and also since it can be difficult to treat some gram-negative infections and evolving new diseases. In addition, there are other several other nonantiinfective secondary metabolites (eg, immunosuppressants, hypocholesterolemic agents, enzyme inhibitors, antitumor compounds), some with individual annual sales of >$1 billion. About 500 new secondary metabolites are discovered each year (31).

5.3. Biomass. The market for biomass in its various forms (such as biopesticides, animal/human food, bioremediation) is relatively smaller. In 1998, biopesticides were selling at $125 million/year with ~3600 metric tons produced annually (27). This worldwide market increased to $2.8 billion/year as the organic food industry expanded (87).

5.4. Enzymes. As of 1985 there were ~1500 known enzymes with <20 in large scale commercial use and <100 prepared in kilogram amounts (58). By 1998, industrial enzyme worldwide sales rose to $1.6 billion/year and was growing at a rate of 6.5%/year (77). Usages were split as follows: 45% food, 34.4% detergents, 11% textiles, 2.8% leather, 1.2% pulp/paper, and the remainder for other applications including enzymatic synthesis for chiral drugs (77). By 2000, about 8% of enzyme sales of $1.8 billion were for fine chemical and/or pharmaceutical applications, a dramatic change from 1998 levels (78). Market growth has been estimated at 8–14% depending on the area (78).

5.5. Biologicals. Global sales of biologicals (therapeutic proteins including enzymes, antibodies and vaccines) currently are 8% ($31 billion) of the

$390 billion worldwide drug market in 2003 (82). Overall the market has been led by monoclonal antibodies and from the more mature insulin and erythropoeitin product classes. In 2000 the global vaccine market was $7 billion/year, in 2001 the therapeutic antibody market was $3 billion/year (88) with revenues expected to reach $5 billion/year in 2003 (89), and in 2002 the therapeutic enzyme market was $2.3 billion/year (90).

6. Microbiological and Fermentation Aspects

The overall goal is to overproduce the product of interest and minimize impurities due to differing minor by-products. In the case of secondary metabolites, the challenge can be reducing structurally similar analogs with small differences in side groups such as a hydroxyl group instead of a methyl group at a specific site. In the case of primary metabolites, product yields on substrates need to be optimized so that raw materials are not wasted down unwanted metabolic pathways. Finally, in the case of biologicals, differing glycoforms, clipped proteins due to proteolytic degradation, or improperly folded proteins all need to be avoided.

6.1. Strain. Prompt identification of the production strain is key. Extensive search efforts for new compounds from biological sources are executed with a variety of therapeutic targets (91). It is critical to design the screening assay carefully since hits can be low—on the order of 0.01%. High throughput screening in miniature microtiter plates using automated robotic systems efficiently identifies cultures producing desired compounds either as secondary metabolites, enzymatic transformations or recombinant protein clones (32). To minimize false negative assays, it is often necessary to ensure that any synthetic enzymes needed are induced by adding appropriate precursors, there is no catabolite repression, and the product is not degraded further after formation.

Microbial isolates from nature often are a heterogeneous population of clones with abilities to produce notably different titers of secondary metabolites (92). The highest producing colony must be isolated to assure consistent cultivation and to use for further strain development work. Mutation and strain improvement efforts are undertaken with multiple goals. It is important to select and prioritize the objective carefully since strain development is costly and time consuming despite the fact that microtiter plates have sped up the process considerably.

A mutation permanently alters one or more nucleotides at a specific DNA site either by substitution, deletion or rearrangement (38). Mutation is conducted by exposing cells to chemical mutagens or ultraviolet (uv) radiation (wavelengths $\sim$260 nm). Cells then are plated on a selective medium to favor survivors having the characteristic of interest or a nonselective medium for totally random approach. Survivors are tested against the objective of the mutation.

Mutations for secondary metabolites cultures are performed to overproduce the product of interest often by severalfold over wildtype levels. Alternatively, the goal can be to produce the product more efficiently by reducing carbon utilization, lowering analog levels, blocking a metabolic pathway leading to impurities or product degradation, or creating analogs (new metabolites) that might

have enhanced therapeutic value. In some cases, mutations are conducted to elucidate the secondary metabolite synthetic pathway. Mutations for primary metabolites are conducted to alter feedback resistance (such as regulation, inhibition and/or repression), which increases product concentration. Mutation also can be done to produce an auxotroph that requires specific nutrient(s) for cell growth or an idiotroph that requires specific nutrient(s) for secondary metabolite production. It is important to carefully decide when to switch to a new mutant during process development and/or production as well as to fully test the impact of the new mutant culture on subsequent purification steps before the transition is finalized.

Genetic engineering is being used in addition to mutation for secondary metabolites. Advances in the genetics of *Streptomyces* and other fungi are improving the toolbox contents available. It has been deciphered that many *Streptomyces* species have similar genetic maps, the genes associated with secondary metabolism are clustered, and additional gene copies of positively acting regulatory genes improve production severalfold (53,93). Genetic engineering has been used to introduce a macrolactone, normally synthesized by an actinomycetes organism, into a *Streptomyces* host and then to alter the associated genes to produce novel analogs (94,95). Hybrid antibiotics (eg, combining the ring structure of one macrolide with the sugar moieties of another macrolide) have been created both by feeding precursors to the organism and by genetic manipulation (96). Genome shuffling for strain improvement for natural products is increasing in prominence commercially (Codexis, Redwood City, Calif.). Finally, protoplast fusion can be used to obtain new antibiotics not produced by either parent or improve production culture robustness. Examples include crossing a recombinant strain which was a high producer with the ancestoral strain that had rapid growth, combining two mutant isolates, or combining two different strains (either producing mutants or blocked mutants).

For recombinant proteins, the selection of the host cell fundamentally influences major aspects of the process and the nature of the product, and the efficiency of the expression system determines the size of the facility (capital investment and operating costs). Although early cell line engineering can minimize future expenditures, it also can delay development and clinical material manufacture. Typical host cells include single cell bacteria (*Escherichia coli, Bacillus*), filamentous bacteria (*Streptomyces*), yeast (*Saccharomyces cerevisiae*), fungal (*Aspergillus*), animal (insect—*Spodoptera*, mammalian—Chinese Hamster Ovary (CHO) cells, hybridomas) and plant—*Nicotiana tabacum* (tobacco) cell lines. Recombinant protein expression in various hosts is well established (97).

When *E. coli* is used as the host cell, recombinant protein expression levels can be as high as 10–20 wt% of total cell protein with gene expression readily controllable for a wide variety of available cloning vectors. Since *E. coli* cannot always secrete proteins efficiently nor perform posttranslational modifications such as glycosylation (which can dramatically affect efficacy), the proteins can accumulate in inclusion bodies at high expression levels (98). Inclusion bodies are intracellular refractile bodies, specifically crystalline agglomerates of highly cross-linked insoluble protein, which are resistant to protease attack. Proteins in this form generally are not toxic to the host cell because they are not soluble in the cell cytoplasm. To obtain active protein, inclusion bodies must be denatured

then renatured to purify, which can be difficult and require large processing volumes due to the dilution necessary to remove the chaotropic (denaturing) agent.

Yeast expression levels are lower at 1–5 wt% of total cell protein. Proteins are secreted and some posttranslational modifications are performed including disulfide bond formation, endoproteolytic cleavage, glycosylation, and multimeric assembly. In some cases, yeast hosts overglycosylate proteins by adding complex mannose structures so patterns are not always similar to those of the natural mammalian cell protein (98). Fungi also have lower recombinant protein expression levels and are able to secrete efficiently, fold and glycosylate (but often also add complex mannose structures like yeast cell hosts). Fungal strains have been genetically altered to reduce native protease production to protect secreted proteins. Animal, insect, and plant cells generally are suitable hosts depending on the specific products. Although sometimes more expensive and difficult to cultivate, animal cells often are favored to produce recombinant proteins with similar *in vitro* and *in vivo* properties to their natural counterparts. The baculovirus expression vector system is commonly used to express recombinant proteins in insect cells but with glycosylation patterns that often are different than the desired product. In plant cell culture, since products accumulate intracellularly in vacuoles with secretion uncommon, product inhibition can limit production of recombinant molecules and xylose chains often are added to the final product (99).

Host cell selection influences proteolytic degradation which can occur both intracellularly and extracellularly during cultivation. It may be reduced by engineering of the host to minimize proteases (100–102), engineering of the product to improve stability, or altering of fermentation conditions (103). In addition, antibody fragments can be linked to polyethylene glycol molecules to increase the *in vivo* half-life of microbially produced antibodies relative to those produced in mammalian cell host systems (CellTech, UK).

Once a recombinant strain is constructed, the plasmid must be amplified to increase its copy number (the number of plasmids per chromosome). Copy numbers can vary from 1 to 200 copies of the gene of interest with the amount dependent on the selected cloning vector and the cell environment. It is necessary to understand the relationship between copy number and recombinant protein production for the system of interest since the highest copy numbers may not always result in the highest production levels. Substantial selection pressure exists on the cell to discard the foreign plasmid since it is typically present at high copy numbers. This burden to the recombinant cell relative to its nonrecombinant counterparts often causes a notably slower growth rate. Segregational instability occurs when the complete plasmid is lost during cell division; structural instability is when there is a change in plasmid structure due to the insertion, deletion or rearrangement. The cumulative impact of even a small amount of plasmid loss (0.01% per generation) to a large scale process with several (eg, 100) generations can be significant (1% overall loss).

The selection of the recombinant expression system must consider the type of inducing agent if required. In induced (regulated) expression systems, cells are grown under conditions that permit plasmid replication without recombinant gene expression. In noninduced constitutive (nonregulated) expression systems), recombinant genes are expressed as plasmids replicate. Selection pressure is

greater for these constitutive systems. In some cases, selective pressure can be applied via an antibiotic (since an antibiotic resistant gene is cloned into the host cell initially as part of the foreign plasmid).

6.2. Medium Development and Feeding. By necessity strain development proceeds in parallel with medium development and often is the key to realizing the full potential of a new mutant. In the most significant well-published example, it was process, media, and strain development together that improved penicillin G titers to 15000-fold greater than the wild-type original process. Economics and raw material consistency influence the selection of medium components.

Although cells typically are 70–80% water, they also require oxygen, carbon, nitrogen, and mineral sources. Each element can be used for cell mass, product, waste by-products, and cell maintenance. Nutritional requirements can be obtained via mass balance based on the efficiency of incorporation of that element (cell yield on element), desired cell mass and cell elemental composition [composition of dried *E. coli* is 50 wt% carbon, 20 wt% oxygen, 14 wt% nitrogen, 8 wt% hydrogen, 3 wt% phosphorus, 1 wt% each sulfur, potassium and sodium, 0.5 wt% each calcium, magnesium, chlorine and iron, and ~0.3 wt% all others (104)].

Common carbon sources include mono- and disaccharide sugars (glucose, lactose), glycerol starches, grains, molasses, corn syrup, and vegetable oils. Improvements in secondary metabolite yield are attainable by supplying a readily metabolized sugar (glucose) during the growth phase and a slowly metabolized carbon source (glycerol) during the production phase or by the slow addition of the faster metabolized sugar during the production phase.

Common nitrogen sources include corn steep liquor, meals (corn, fish, soybean), flour (cottonseed, soy, rice), extracts (non-diastatic malt, yeast), meat processing waste, amino acids, and inorganic nitrogen (free ammonia or ammonium salts). Often phosphate buffers (for secondary metabolites the limit is ~5 g/L before becoming inhibitory), insoluble carbonates (soluble carbonates will buffer at an alkaline pH) or sodium bicarbonate [144-55-8] (requires 5–10 vol% carbon dioxide in bioreactor headspace) can be useful for controlling pH swings in the range of 4–8. Other buffers such as Tris HCl [1185-53-1] can be used at the small scale but are not economical for scale up. Sometimes hard and soft metal elements (iron, copper, magnesium), vitamins (biotin [58-85-5]) and growth factors (hormones, serum) are needed, particularly for leaner medium. Initially or during the cultivation, components such as amino acids can be added to influence product–by-product levels.

The final component, oxygen [7782-44-7], is sparingly soluble in water (~10 mg/L at 25°C), with a lower solubility observed at higher temperatures. It is required for all aerobic fermentations and must be continually supplied. In cases where the culture morphology is a pellet, nutrients such as oxygen may have difficulty diffusing into the pellet core that can become starved or necrotic.

Media can contain complex and/or, chemically defined ingredients (Table 3). Complex components are "natural" ingredients, not well characterized with typical compositions available as rough percentages of carbohydrate, protein, and lipid (105). Their composition can vary according to the manufacturer (106) and the growth–harvest conditions especially for ingredients of agricultural

Table 3. **Comparison of Typical Media Components and Their Typical Initial Batch Concentrations for Various Cell Types**[a]

A. Complex media components

Nutritive Role	Example component	Single-cell bacteria	Yeast	Fungal	Animal	Insect cell	Plant
carbon	corn syrup	10–80 g/L	10–60 g/L	10–40 g/L	N/A	N/A	N/A
nitrogen	yeast extract	10–30 g/L	5–25 g/L	5–20 g/L	N/A	3 g/L	N/A
phosphate	see nitrogen	N/A	N/A	N/A	N/A	N/A	N/A
sulfur	see nitrogen	N/A	N/A	N/A	N/A	N/A	N/A
amino acids	casamino acids	5–10 g/L	5–10 g/L	5–10 g/L	N/A	N/A	0.1–0.5 g/L
	lactalbumin hydrolysate	N/A	N/A	N/A	N/A	3 g/L	N/A
growth factors, lipids	serum	N/A	N/A	N/A	0.5–10 vol%	0.5–10 vol%	N/A

B. Defined media components

Nutritive role	Example component	Single-cell bacteria	Yeast	Fungal	Animal	Insect cell	Plant
carbon	glucose	10–50 g/L	10–100 g/L	10–50 g/L	1–5 g/L	0.7 g/L	N/A
	sucrose and fructose	N/A	N/A	25–100 g/L	N/A	27 g/L	30 g/L
	organic salts (eg, sodium pyruvate, alpha ketoglutaric, fumaric, malic and succinic acids)	200 mg/L	N/A	N/A	0.1–0.4 g/L	1.2 g/L	N/A
nitrogen	ammonium sulfate	5–20 g/L	5–40 g/L	5–10 g/L	see amino acids	see amino acids	0.1–0.5 g/L
	potassium nitrate	N/A	N/A	N/A	N/A	N/A	1–3 g/L
phosphate	see buffer below	N/A	N/A	N/A	N/A	N/A	N/A
sulfur	see nitrogen and/or trace elements	N/A	N/A	N/A	N/A	N/A	N/A
amino acids	monosodium glutamate	10–30 g/L	10–30 g/L	10–30 g/L	N/A	N/A	N/A
	L-glutamine	0.1–1.5 g/L	N/A	N/A	0.6 g/L	0.6 g/L	0.1–1 g/L
	L-histidine	N/A	N/A	N/A	0.04 g/L	2.5 g/L	N/A
	other essential amino acids (up to ~15–20)	25–150 mg/L	N/A	N/A	0.015–0.15 mg/L	0.02–1.1 g/L	0.05–0.5 g/L

vitamins	vitamins such as biotin, riboflavin, choline chloride, D-Ca-Pantothenate, pyridoxal HCl, thiamine HCl, niacinamide, I- or Myo-inositol, folic acid, *p*-aminobenzoic acid	0.1–3 mg/L	0.1–150 mg/L	0.1–300 mg/L	0.4–7 mg/L	0.01–0.02 mg/L	0.5–250 mg/
other inorganic salts	sodium and/or potassium chloride	N/A	N/A	N/A	6.8 g/L	4.1 g/L	N/A
trace elements	magnesium salts	0.25–0.5 g/L	1–5 g/L	0.5–5 g/L	0.1 g/L	4–5 g/L	0.2–0.4 mg/L
	calcium salts (excluding carbonate)	0.25–1 g/L	0.25–1 g/L	0.25–1 g/L	0.2 g/L	0.75 g/L	0.15–0.6 mg/L
	ferrous salts	1–10 mg/L	1–10 mg/L	1–10 mg/L	0.1 mg/L	N/A	25 mg/L
	manganese, zinc, copper, cobalt and molybdate salts	0.02–2 mg/L	0.02–2 mg/L	0.02–2 mg/L	N/A	N/A	0.025–20 mg/L
growth factors, lipids, hormones	insulin	N/A	N/A	N/A	5 mg/L	N/A	N/A
	albumin	N/A	N/A	N/A	10 g/L	N/A	N/A
	lipid mixture	N/A	N/A	N/A	30 mg/L	N/A	N/A
	auxins, cytokinins and/or abscisic acid	N/A	N/A	N/A	N/A	N/A	0.02–175 mg/L
buffer	potassium or sodium phosphate	1–10 g/L	1–10 g/L	0.5–5 g/L	0.1 mg/L	1 g/L	0.1–0.3 g/L
	sodium bicarbonate	N/A	N/A	N/A	3.7 g/L	0.35 g/L	N/A
	calcium carbonate	N/A	N/A	1–10 g/L	N/A	N/A	N/A
antifoam	polypropylene glycol	0.1–1 mL/L	0.1 mL/L	1–5 mL/L	N/A	N/A	N/A
	pluronic F68[b]	N/A	N/A	N/A	0.1 mL/L	0.1 mL/L	N/A

[a]N/A indicates this type of defined or complex ingredient is not commonly used for this type of cell. Not all components necessarily are required to provide a complete medium. Exact concentrations depend on cell mass and product titers expected.

[b]Pluronic F-68 (block copolymer of polyoxyethylene and polyoxypropylene, average molecular weight 8400, BASF) protects animal and insect cells from damage from agitator shear and sparging.

origin. In some cases, complex ingredients are by-products from food processing. Chemically defined components are synthetic, well-characterized, pure chemicals. Although defined medium has been used successfully for a variety of processes (107), process sensitivity to any medium variations can be heightened. Although typically more expensive than complex medium, defined medium often results in a more economical isolation process. A hybrid approach is the use of semi-defined medium which has mostly defined ingredients with one (or possibly two) complex ingredients. Regardless of the types of ingredients selected (complex or defined), adequate raw material specifications are necessary and sometimes incorporate small scale use tests.

The composition of growth and production media vary considerably for the type of cell being cultivated and can contain several (2–25+) components (Table 3). Statistical experimental design can be used to screen medium components and optimize concentrations (as well as to delineate fermentation conditions). It is particularly useful when a large number of process variables (experimental factors) exist and it is suspected that two or more variables may have a synergistic effect (38,108). It permits alterations of the relative proportions of components and can be used to create a three-dimensional (3D) response surface for the most important variables using commercially available software packages for experimental design and data analysis. Compared with the "one factor at a time" approach, it reduces the number of individual experimental runs considerably and improves the chances of identifying key interactions among factors. In addition, artificial neural networks have been shown for one case of fermentation media design to reduce the number of required experimental runs even further (109).

Seed stage medium is designed to minimize growth lags by promoting exponential growth without limiting nutrients. It should result in adequate growth and production in the subsequent seed or production stages and should avoid any shift to secondary metabolism or appreciable production of the product. Production medium should be optimized overall for maximum productivity that may not always correspond to maximum cell mass production. For some processes the aim is to increase cell mass to be within (often very near to) the maximum oxygen-transfer capacity of the fermentor over the course of the growth and/or production stages.

Production cultivation growth starts with all the nutrients present and continues exponentially until one substrate becomes limiting at which time linear growth occurs. The feed rate of this limiting nutrient in a concentrated solution (either as a bolus or a continuous addition) can be used to control growth and metabolism provided it is utilized as fast as it is supplied to minimize inhibition and/or accumulation (110–112). Fed-batch operation can occur either during the growth or production phases or both. For *E. coli*, restricting the growth rate via fed-batch operation to a lower than maximum level can be the preferred approach to improving cell densities (97).

During continuous culture, medium is fed to and removed from the fermentor at a specified dilution rate (ie, the volume of medium flowrate per unit fermentor volume). This technique can be used for medium optimization since step changes in medium concentrations in the inlet feed are readily implemented and effects on growth and/or production rates can be studied once steady state is

reestablished (113,114). Although most commonly used for research purposes and scarce commercially, continuous processes have been implemented industrially for yeast single cell-protein, beer, vinegar, activated sludge, and glucose isomerase (110). Cell retention devices are used in perfusion culture to remove cell mass from the outlet steam, resulting in cell accumulation in the fermenter.

6.3. Kinetics of Growth/Product Formation. Growth and production kinetics vary considerably depending on the specific cultivation conditions and the organism being cultivated. Table 4 summarizes key parameters of interest for several cell types. Figure 2 illustrates an example of the kinetics of growth, substrate utilization, product and by-product appearance as well as other fermentation parameters.

Secondary metabolite cultivations can be divided into the tropophase or growth phase and idiophase or production phase (115) with morphological differentiation often occurring during the idiophase (93). When these phases are

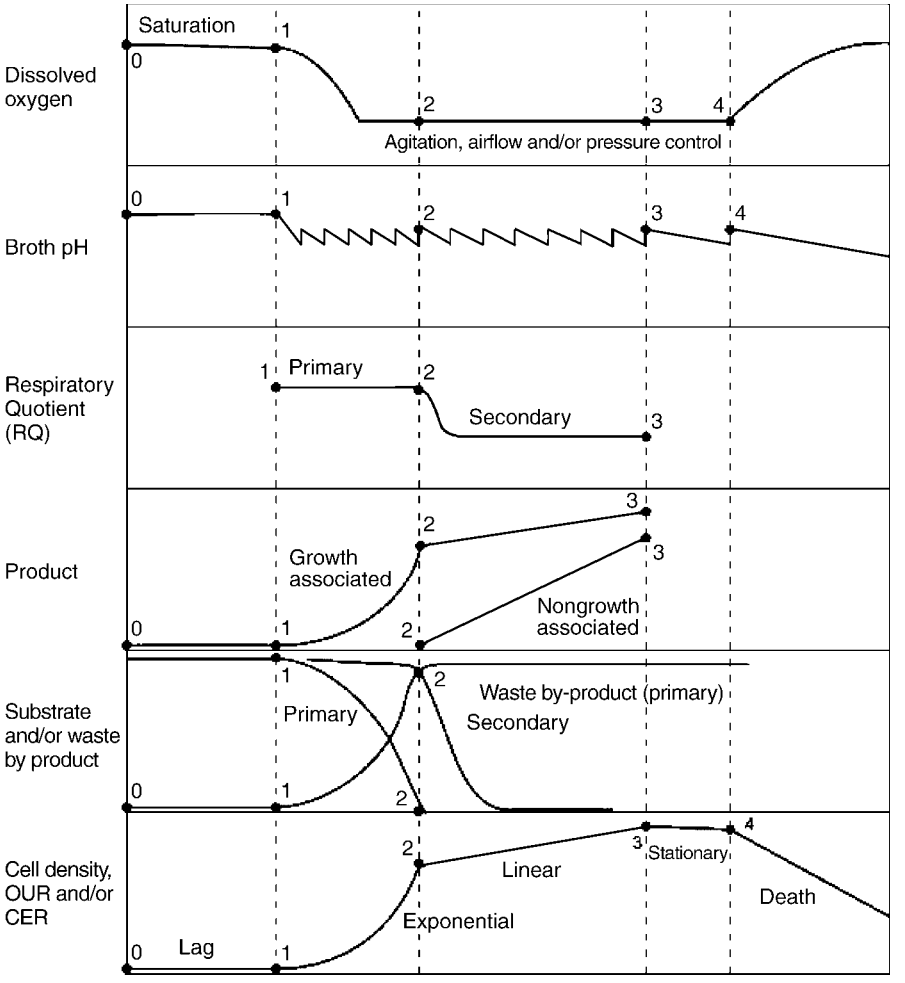

Fig. 2. Typical fermentation kinetic profiles with emphasis on secondary metabolite production.

Table 4. **Comparison of Typical Characteristics of Various Cell Cultivation Processes**

Parameter	Single-Cell bacteria	Yeast	Fungal	Hybridoma	Insect cell	Plant
growth rate (doubling time)	0.9–2.1 h^{-1} (20–45 min)	0.2–0.5 h^{-1} (90–210 min)	0.09–0.5 h^{-1} (1.5–9 h)	0.03–0.06 h^{-1} (12–24 h)	0.03–0.04 h^{-1} (12–17 h)	0.006–0.02 h^{-1} (1.5–5 days)
inoculation cell density	0.01–0.05 g/L	0.05–0.1 g/L	10^4–10^6 spores/mL 0.05–0.5 g/L	1×10^5 cells/mL	1×10^5 cells/ml	1–3 g/L
final cell density	30–100 g dcw/L	20–50 g dcw/L	10–70 g dcw/L	5×10^6 cells/mL	1.4×10^7 cells/mL	10–40 g dcw/L
size	0.2–3 μ	1–5 μ wide 5–30 μ long	4–20 μ wide up to 100 μ long	10 μ	12–20 μ	20–40 μ wide up to 100 μ long
cell morphology	Single cell	Single cell, budding, and/or clumps	Filamentous or pelleted (multicellular)	Single cell	Single cell	Single and multicellular
cell wall	Present	Present	Present	Absent	Absent	Present
inoculum transfer time	8–24 h	20–40 h	1–4 days	5–10 days	5–6 days	7 days
inoculum transfer volume	0.05–0.5 vol%	0.5–5 vol%	1.5–10 vol%	5–10 vol%	5–10 vol%	10 vol%
production cycle	1–3 days	2–5 days	7–35 days	10–30 days	7–12 days	10–30 days
growth temperature	37 °C	25–30 °C	22–28 °C	37 °C	28 °C	26–28 °C
broth pH	6.5–7.5	4.0–5.0	3.0–7.0	7.0–7.5	6.2–6.6	5–6
aeration VVM	1.0–2.0	1.0–2.0	0.5–1.0	0.1	0.1	0.1
agitation shear	Insensitive	Insensitive	Potentially sensitive	Sensitive	Sensitive	Sensitive
peak oxygen uptake rate (OUR)	50–100 mmol/L-h	25–50 mmol/L-h	10–25 mmol/L-h	1–5 mmol/L-h	1–2 mmol/L-h	1–3.5 mmol/L-h
peak specific oxygen uptake rate	0.1–0.5 g O_2/g dcw-h	0.1–.5 g O_2/g dcw-h	0.01–0.3 g O_2/g dcw-h	5 pg O_2/cell-h	5–10 pg O_2/cell-h (doubles during infection)	0.001–0.01 g O_2/g dcw-h
primary metabolite titer	1–100 g/L	1–100 g/L	1–100 g/L	N/A	N/A	0.1–4 g/L
secondary metabolite titer	N/A	N/A	0.1–20 g/L	N/A	N/A	1 g/L
recombinant protein titer	0.4–1.5 g/L	0.5–2 g/L	0.1–1 g/L	0.1–2 g/L	0.1–1 g/L	4 mg/L
primary metabolite production rate	4 g/L-h	4 g/L-h	0.1–0.6 g/L-h	N/A	N/A	1 g/L-day
secondary metabolite production rate	N/A	N/A	0.02–0.1 g/L-h	N/A	N/A	1 g/L-day
recombinant protein production rate	100–500 mg/L-day	100–500 mg L-day	10–150 mg L-day	2–10 mg L-day	2–10 mg L-day	0.2–0.5 mg L-day

distinct, the product is nongrowth associated and when, in some cases the tropophase and idiophase overlap (116,117), the product is growth associated or mixed. Secondary metabolites are produced at low growth rates and often need an induction phase resulting from a nutrient deficiency (such as carbon, nitrogen, phosphate, or sulfur) during which there are specific enzymatic changes in the cell. The culture itself is resistant to the substances it secrets primarily because antibiotic production is delayed until the rapid growth period finishes (116,118).

Nutrients enter the metabolic pathway and are converted to intermediates by either catabolic (metabolic pathways that result in a more simple molecule than the substrate) or anabolic (metabolic pathways that result in more complex than the substrate) reactions. Metabolic pathways are physiologically and genetically controlled and regulated (119,120) and have been well studied for specific compounds (5). These kinetic limitations in the production of biological products can be reduced through process development and/or genetic manipulations.

7. Equipment

The design of fermentation equipment must consider the culture type and its characteristics as well as the potential process requirements (121). It affects culture growth, morphology, and production. Although the requirements for inoculum or seed fermentors are typically simpler than those of production fermentors, often the need for interchangeable operation necessitates the design of the more complex capabilities. Fermentation equipment used for microbial cultivation has been distinguished from bioreactor equipment used for animal cell cultivation (or mixed use) by some practitioners, with the term bioreactor implying more broad based cell host application. The description that follows is restricted to stirred tank design, although several portions apply to bubble column, airlift, packed/fluidized beds, and rotating disks that also are used industrially. A typical fermenter/bioreactor is shown in Figure 3.

Three fermentation scales generally are required: the bench or laboratory scale for process development; the pilot scale for clinical material manufacture, initial scale up, and subsequent process development; and the production scale for commercial manufacture. Often the pilot scale is within 5–10- fold of production scale volume and in some cases pilot scale equipment is used for production to delay commitment of capital if clinical outcomes are uncertain. Examples of specific vendors for fermentation equipment are listed in Table 5.

7.1. Vessels and Piping. Vessel volumes range from 1 to 200,000 L, with those >5–10 L typically constructed of stainless steel and smaller laboratory vessels made of glass. As production strains and cultivation processes improve, the required size of the fermenter vessel decreases. Although the total vessel volume is the liquid volume and the headspace volume combined, the working volume is typically 0.5–0.8 of the total volume. The working volume must be selected carefully so that (*1*) sufficient volume remains for broth expansion during heating and aeration gas hold up, (*2*) impellers and probes are adequately submerged, and (*3*) the time that the broth level is at the impeller location is minimized during fed-batch operation. The vessel aspect ratio

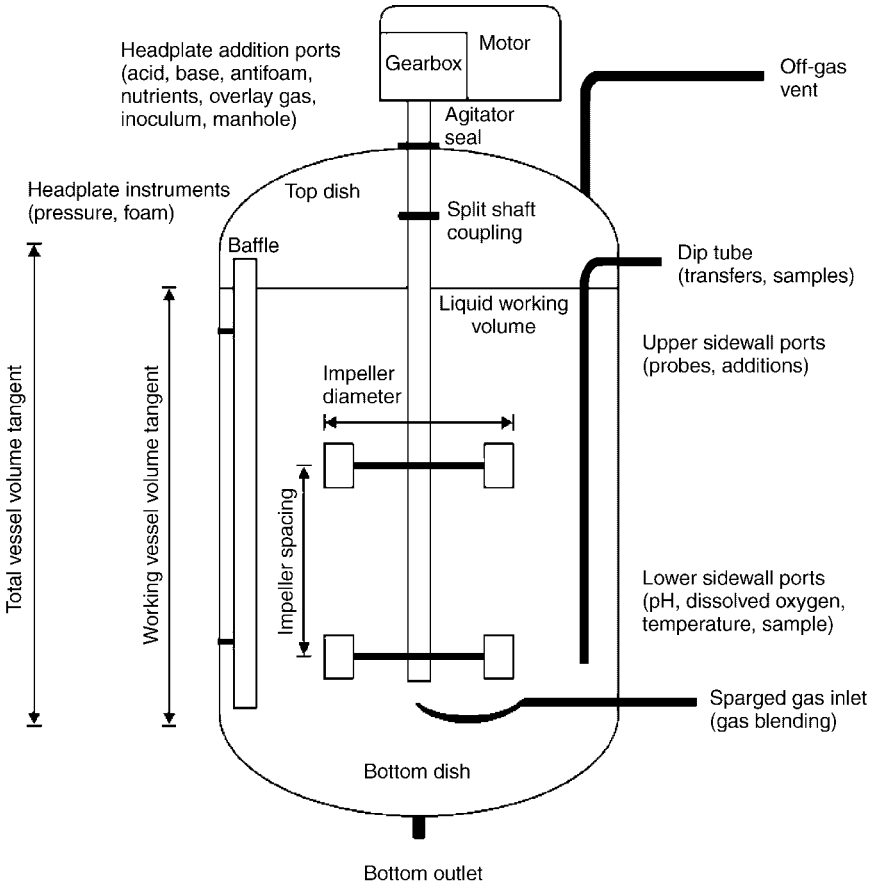

Fig. 3. Characteristics of a typical fermentation vessel.

(straight-side tangent divided by tank diameter) can range from 1:1 to 3.5:1 for stirred tanks and up to 6:1 for airlifts and bubble columns. Stainless vessels are pressure rated for at least 45 psig with jackets rated to ~100 psig. Insertion ports for instrumentation probes exist on the lower and upper sidewalls and often more than one probe of a certain type is utilized to measure gradients. Addition ports can be located above or below the liquid surface to enhance dispersion. Sampling ports can utilize a direct low volume valve with a contained sample bottle or a subsurface dip tube that requires an adequate line flush and open transfer to the sample bottle.

The vessel and its piping are constructed and installed in a sanitary fashion to facilitate sterilization and cleaning. For biologicals, high quality orbital welding and highly polished piping are required; for other processes traditional welding or flanges are adequate. Depending on the application, steam-sterilizable ball or diaphragm valves are used since vessel connections are steamed using condensate traps or bleeds to the atmosphere directly before being used for nutrient transfers. Transfer panels can be used to replace individual transfer lines for nutrients and inoculum. Although this minimizes the number of vessel

Table 5. **Examples of Fermentation Equipment and Media Suppliers**[a]

Item	Suppliers
fermenter–bioreactor and associated skids	Adaptive Biosystems (Luton, UK) Applikon (Foster City, CA) Associate Bioengineers and Consultants, Inc. (ABEC, Allentown, PA) B. Braun Biotech (Allentown, PA) Cotter Corporation (Danvers, MA) New Brunswick Scientific (Edison, NJ) Wave Biotech (Bedminster, NJ)
vessels	alloy Fab (South Plainfield, NJ) DCI (St. Cloud, MN) Lee Industries (Phillipsburg, PA) Mueller (Springfield, MO) Precision Stainless (Springfield, MO) Tolan (Rockaway, NJ)
agitators/mechanical drives	Chemineer (part of Robbins and Myers, Dayton, OH) Lightnin (Rochester, NJ)
filtration	Cuno (Meriden, CT) Domnick-Hunter (Charlotte, NC) Meissner (Camarillo, CA) Millipore (Bedford, MA) Pall (Exton, PA) Sartorious (Edgewood, NY)
on-line instrumentation	Brooks (Hatfield, PA) Endress and Hauser (Reinach, Switzerland) TBI (part of ABB, Carson City, NV) Johnson Yokogawa (Milwaukee, WI) Mettler-Toledo Ingold (Woburn, MA) Rosemount-Fisher (Chanhassen MN) Sensorex (Garden Grove, CA) Sierra (Monterey, CA) ThermoOnix (Angleton, TX) YSI (Yellow Springs, OH)
medium components	Athena ES (Baltimore, MD) Becton-Dickinson (formerly Difco, Franklin Lakes, NJ) BioSpringer (Minneapolis, MN) Bio/Whittaker/Cambrex (East Rutherford, NJ) Hyclone (Logan, UT) Invitrogen (formerly Gibco, Carlsbad, CA) Irvine Scientific (Santa Ana, CA) JRH Biosciences (Lenexa, KS) Marcor Development Corp. (Carlstadt, NJ) Quest (Hoffman Estates, IL) Serologicals Corp. (Norcross, GA)
presterilized disposables	Corning (Corning, NY) Hycone (Logan, UT) Nalge Nunc (Rochester, NY) Stedim (Concord, CA)
sterile tubing welder	Terumo (Tokyo, Japan) Wave Biotech (Bedminster, NJ)
continuous sterilizers	APV (Lake Mills, WI)
autoclaves	Fedegari (Albuzzano, Italy) Getinge-Castle (Rochester, NY) Steris (Amsco, Mentor, OH)
incubators/shakers	Barnstead (Dubuque, IA) Forma Scientific (Marrietta, OH) Hotpack (Philadelphia, PA) Kuhner (Basel, Switzerland) New Brunswick Scientific (Edison, NJ)
biosafety cabinets/laminar flow hoods	Baker (Sanford, ME) Laminaire (Rahway, NJ) NuAire (Plymouth, MN)
selected utilities	Clean steam- Steris (formerly Finn Aqua, Mentor, OH), Vaponics (Rockland, MA), Meco (New Orleans, LA) Purified water-US Filter (Lowell, MA), Millipore (Bedford, MA) Oil-free air compressors/dryers- Ingersoll-Rand (Davidson, NC), SPX Air Treatment (formerly Flair, Ocala, FL) Waste in activation- Hartel (Ft. Atkinson, WI)

[a]Information obtained from corporate websites.

entry points, it can result in misdirected flow if connections are not set correctly. Flexible hoses can be used in lieu of hard pipe, particularly at smaller scales.

Spargers can be open pipe (wide opening to create large bubbles), ring (perforated and curved to create small bubbles), or frit (sintered metal to create fine bubbles). Careful placement of the sparger under the agitator is key to breaking

up and/or dispersing bubbles and maximizing gas–liquid oxygen mass transfer. Volumetric air flowrates can vary from 0.01 to 2.0 VVM (equivalent vessel liquid volumes of gas flowrate per minute). Superficial velocities (the volumetric air flowrate divided by the vessel cross-sectional area) need to be maintained at levels of ~5–10 cm/s to prevent gas flooding of the agitator. Gas blending of nitrogen, high oxygen-content air and/or carbon dioxide is used to control pH for bicarbonate-buffered media or to improve mass transfer. (Oxygen-enriched air used at the large scale can be costly and present safety hazards if not carefully implemented.) When bubbles need to be minimized to avoid cell damage during some sensitive animal cell cultivations, surface aeration across the liquid surface is effected by use of a gas overlay applied to the vessel headspace. Mass transfer rates for surface aeration are notably lower ($0.5\ h^{-1}$) compared with those obtainable for gas bubble aeration ($2.5–500\ h^{-1}$), making surface aeration less suitable for most animal cell cultivations >300 L and for active microbial cultures >0.5 L in volume.

Depending on the vessel volume, fermentor agitators consist of at least one and often multiple impellrs spaced appropriately so each works at its maximum effectiveness. Four baffles, equally spaced around the vessel, can be used to promote mixing and avoid vortexes. Common designs are the Rushton (high shear, radial flow) and the hydrofoil (low shear, axial flow) impellers. A split shaft holds the impellers with one portion connected to the mechanical gear drive through a double mechanical seal lubricated with pressurized steam or condensate. This design permits the sterile lubricating fluid to leak into the vessel rather than medium/broth to leak out of the vessel as the seal ages. Magnetic drives are one alternative that avoids an agitator seal but these are usually limited to smaller and/or lower power input vessels. Agitators can be mounted on the top or bottom of the fermentor vessel. Although bottom mounted agitators liberate space on the vessel headplate and often result in shorter shafts, both the agitator seal and the split shaft connection are submerged in broth. At some point as the fermentor size increases (perhaps >100,000–200,000 L) mechanical agitation becomes less economical relative to pneumatic agitation used in airlift/bubble columns.

Vessel heating and cooling can be accomplished using a straight, dimple, or half-pipe coil jacket for smaller vessels (less than ~20,000 L) and internal coils (which can act as baffles) for larger vessels. Cooling can be accomplished through direct application of steam or cooling water to the jacket or indirect application through heat exchangers using an external closed recirculating loop (for vessels less than ~1000 L in volume). Leaks of nonsterile cooling water from the jacket or coils through hairline cracks are one potential source of broth contamination.

Vessel and piping design must consider the postrun cleaning of the vessel and its piping. To prepare for this operation, the operator must disassemble and reassemble connections to form cleaning agent flow paths. It is common to start with a low liquid level in the tank that is recirculated using an external pump through each flow path and back to the vessel interior through spray balls. The pressure and flowrate design of the sprayballs must permit cleaning agent flow velocities sufficient to remove soils upon contact and result in total coverage of all product-contact surfaces.

7.2. Sterilization. The purpose of sterilization is to irreversibly inactivate indigenous fauna present in medium ingredients and sparger air that would compete with and potentially outgrow the culture of interest, thus reducing productivity. Bacterial endospores (vegetative dormant cells with several protective membrane layers and a hard covering) are 1 millionfold more resistant to moist heat than larger mold exospores or conidiospores (primarily seed-like reproductive structures). The kinetics of death by sterilization are exponential and are based on the kill kinetics of bacterial spores, specifically *Bacillus stearothermophilus*. Sterilization by moist heat is most common for vessels and equipment; sterilization by γ-irradiation is common for disposable plastic cultureware items.

All equipment, components and additions must be sterilized prior to inoculation with the pure production culture. During vessel sterilization, live steam is injected into the fermentor and the fermentor jacket. The key is to remove air from the system (either the fermentor or autoclave) so that all areas are exposed to moist heat and to avoid vacuum by adding filtered sterile air to the system during the cool down phase. The total heat stress of the medium is the combination of time–temperature profile during the vessel heat up, sterilization temperature hold and cool down periods. This stress is quantified as F_o, the rate of killing organisms, referenced to *B. stearothermophilus*, for moist steam sterilizations (122). The value of R_o tracks the destruction of heat labile media components, referenced to vitamin B_{12}, so it can be minimized. Typically, the rate of media component destruction is less sensitive to temperature than death rate of *B. stearothermophilus*.

Media sterilization effects must be considered carefully whenever components are added/removed or concentrations appreciably altered during medium development. The order of component addition presterilization can affect the sterilized medium especially if one component dramatically alters the pH, potentially affecting the solubility of subsequent components. Recirculation of medium through a high shear in-line mixer serves to break up and wet clumps to assure contact with steam. Certain media components (eg, glucose and inorganic–organic nitrogen) are sterilized separately to avoid the formation of growth inhibitory toxic by-products, known as the browning or Maillard reaction. Carbohydrates (eg, glucose) sterilized too long can turn brown, carmelize and also form toxic by-products. Other components (eg, calcium and iron cations) form an inorganic precipitate in the presence of higher phosphate concentrations and thus require separate sterilization of a concentrated trace element solution or addition of a chelating agent such as ethylenediaminetetraacetic acid [60-00-4] (EDTA) presterilization.

Batch sterilization is conducted by sterilizing the vessel and the medium *in situ* at a temperature of at least 121°C (typically 122–125°C depending on medium composition and temperatures reached at the hardest to sterilize locations) for at least 15 min (typically at least 30–45 min) at a corresponding pressure to maintain saturated steam conditions (typically at least 16 psig). The medium is agitated for temperature uniformity.

During continuous sterilization, medium is sterilized prior to its entering the presterilized empty fermentor using high temperature short time (HTST) continuous sterilization. In this procedure, widely used in the food industry,

medium is heated via heat exchangers, held in an adiabatic (constant temperature) loop for the required residence time to achieve sterility, cooled in at least two heat exchanger stages first by preheating the incoming cold medium and second by cooling water, and then distributed to the fermentor. Continuous sterilization is conducted at a higher hold temperature of 135–150°C and at a shorter hold time of 4–15 min for which the rate of spore inactivation is much higher (F_o increases 10-fold with each 10° rise in temperature over a range of 120–150°C). The breakdown of heat sensitive medium components is minimized since R_o is less affected by the higher hold temperature than F_o(R_o increases about threefold with each 10° rise in temperature over a range of 120–150°C). Overall, continuous sterilization is more energy efficient for the large scale since it avoids inefficient heating and cooling of a large vessel (greater than ~20,000 L) filled with liquid.

Sterile filtration is effective for heat labile liquid medium components. A 0.22-μ hydrophilic absolute filter, sized smaller than the contaminating microorganisms, is used. Postuse integrity testing can be performed, typically after the filter has been flushed thoroughly with water, to measure bubble point and/or diffusion flow rate to demonstrate that the filter was not compromised during operations. Sometimes preuse integrity testing is conducted or two sterile filters are installed in series to minimize risk.

Sparger air filtration is accomplished using 0.2-μ hydrophobic (absolute) membrane or depth filters (glass wool has been phased out in favor of polymeric hydrophobic membrane materials). The filter itself can be sterilized *in situ* and is typically used in a stainless steel housing. A low pressure drop across this filter and its housing is most desirable especially for larger vessels. Similarly to liquid sterile filters, integrity testing can be conducted with the filter prewet with an alcohol–water solution (the specific composition of which greatly affects the bubble point and diffusion rate measurement values).

During the cultivation, positive pressure is maintained on the fermentor using sparger and/or overlay air and fermenter back-pressure. Periodically, a small sample is removed aseptically from the fermenter and cultured in a nutrient-rich medium and at temperatures to favor contaminants as a test for the sterility of prepared medium or culture purity of inoculated broth. Analysis of these samples can be key to investigating contamination events and minimizing their occurrence.

7.3. Instrumentation and Control Strategies. Instrumentation and control are critical to the successful monitoring of the fermentation environment surrounding the culture. On-line or continuous measurements can be used for process control. On-line *in situ* sensors need to be reliable, accurate, readily calibrated, low maintenance, steam-sterilizable, and low in drift, especially for long cycle processes. Off-line measurements from in-process broth samples, typically taken and analyzed manually, are delayed in time. The key in development is to link off-line changes to on-line changes to reduce the amount of required sampling and to devise processes able to be monitored fully and effectively, both saving manpower and minimizing process variations. Typical examples of on-line and off-line measurements are shown in Table 6 with key measurements described below and in the literature (123).

Often ignored as a key variable in biological systems, temperature is required to be controlled tightly, typically within ± 0.5°C from the set point

Table 6. **Examples of Typical On-Line (Continuous) and Off-Line (from Broth Samples) Measurements Obtainable During Fermentation**

Parameter	Instrument	On-line, off-line or both
dissolved oxygen	electrodeblood gas analyzer	on-lineoff-line
carbon dioxide	sensorblood gas analyzer	on-lineoff-line
broth pH	electrode	both
conductivity	sensor	both
osmolarity, ionic strength	osmometer	off-line
oxidation reduction (redox) potential	electrode	on-line
cell density	optical density probe (turbidity)	on-line
	fluorescence probe (NAD/NADH level)	on-line
	capacitance probe	on-line
	spectrophotometer	off-line
	centrifuge (packed cell volume)	off-line
	microwave/oven (dry cell weight)	off-line
	hemocytometer	off-line
	automated cell counter	off-line
cell viability	trypan blue dye exclusionfluorescence dye exclusionviable plate countslactate dehydrogenase activity	off-line off-lineoff-lineoff-line
protein concentration	BCA assay	off-line
morphology	image analysisautomated cell sorter	off-lineoff-line
viscosity	viscometer	off-line
off-gas composition (oxygen, carbon dioxide, nitrogen, solvent vapor)	mass spectrometer	on-line
foam	capacitance probe	on-line
agitation speed	shaft tachometer	on-line
power	watt meter, strain gauge	on-line
temperature	RTD (thermocouples)	on-line
pressure	sensor	on-line
level	differential pressure cell, load cell	on-line
liquid flow rate	mass flow meter, loss in weight via scale/load cell	on-line
air flow rate	mass flow meter	on-line
heat transfer rate	jacket RTD and mass flow meter	on-line
substrate/product/by-product/ impurity concentrations	near infrared probefilter probe (cell free sampling)	on-lineon-line
	chromatography	off-line
	ELISA	off-line
	SDS page	off-line
sterility/culture purity	gram stain	off-line
	culture plate growth	off-line
endotoxin	limulus amoebocytes lysate (LAL)	off-line
foreign DNA	polymerase chain reaction (PCR)	off-line
plasmid retention	polymerase chain reaction (PCR)	off-line
glycosylation	chromatography mass spectrometry	off-line

with the exact amount of tolerance dependent on the specific culture. A platinum resistance thermometer device (RTD) is commonly used with a backup RTD or an analog gauge. Tight levels of control can be harder to achieve in very large scale and viscous fermentations due to mixing and heat transfer limitations. Heat must be removed efficiently otherwise temperature rises in the fermentor due to biological heat evolution from metabolism and mechanical heat evolution from agitation.

Medium pH is initially adjusted prior and/or poststerilization. Once the culture is inoculated, control is accomplished within 0.2 pH units by automatic addition of acid or base, commonly in the pH range of 4–8. Often pH electrodes are prone to fouling and can drift particularly during long fermentations. Readjustments can be made using a representative sample promptly measured off-line using a laboratory pH meter. In the case of animal cell culture medium, phenol red is used as a visual indicator of media pH.

Concentrated and dilute acids and bases are used to control pH depending on the tolerance of the organism to localized pH excursions, vessel mixing patterns, and the acceptability of broth dilution. Acids used include sulfuric acid [7664-93-9], phosphoric acid [7664-38-2] (not usually used for secondary metabolites since it influences phosphate regulation) or carbon dioxide (for bicarbonate buffered media). Hydrochloric acid [7647-01-0] is rarely used since it is corrosive to stainless steel. The broth pH rises when nitrogen sources such as proteins or amino acids are metabolized and ammonium ions are released. Bases used include sodium hydroxide [1310-73-2], ammonium hydroxide [1336-21-6] or gaseous ammonia. The broth pH falls when carbon sources such as glucose are metabolized and organic acids are excreted. (It is important to note that organic acids can become inhibitory even if the pH controlled so their formation may need to be minimized by the selection and feeding of carbon substrates.) Broth pH also falls when carbon dioxide accumulates in the broth.

Dissolved oxygen (DO) is measured using steam-sterilizable electrodes in which the oxygen molecules diffuse through the probe's gas-permeable membrane. The more expensive polarographic type probe generally is preferred over the galvanic type. Electrode outputs are calibrated to zero in the presence of nitrogen (or when the circuit is open) and to their maximum value just prior to inoculation at the initial fermentor back-pressure setting. As with pH probes, DO probes are prone to fouling, drift and other errors and they can give difficult to interpret readings in fermentations containing higher amounts of oil or silicone-based antifoams since oxygen has a greater solubility in these media components (124).

Above a critical level (typically 10% of air saturation), cell growth rate is independent of DO concentration. Reduction below this level results in lowered cell growth rate of an amount directly proportional to the dissolved oxygen level. These Monod kinetics dictate that DO be controlled prior to reaching this critical level.

In addition to gas blending using oxygen-enriched air, this control can be accomplished using fermentor back-pressure, air flowrate or agitation rate. Raising back-pressure increases the DO according to Henry's law but also increases dissolved carbon dioxide levels that may not be tolerable for some cultures. Raising air flowrate increases DO by increasing the gas-liquid mass transfer

coefficient through increasing the number of bubbles and gas entrainment (hold-up) in the broth. For some cultures, aeration rates may need to remain low to ensure that sufficient dissolved carbon dioxide remains in the medium to promote growth and/or production. Finally, raising agitation has the greatest effect on DO since it increases the gas–liquid mass transfer coefficient primarily by decreasing bubble size. At the same time, it also raises the impeller shear that can alter culture morphology and possibly damage cells.

Several novel on-line probes exist to measure quantities such as broth optical density, capacitance and dissolved carbon dioxide. Filter "probes" contain a membrane filter through which a cell-free sample can be obtained directly and then routed to an on-line high performance liquid chromatography (HPLC) system or other instrument for analysis. The degree of success for implementation of novel probes is scattered owing to challenges in cost, calibration, drift and interpretation of results limiting their becoming as commonplace as pH and DO probes.

Foaming is influenced by the medium composition, secretion of surface active agents, cell lysis and gas sparging (125). Foam character varies from a stable (beer head) foam with small bubbles to unstable (bubble bath) foams with larger bubbles. Although antifoam is initially present in the medium at a concentration of 0.5–5 mL/L to prevent foaming during mixing and sterilization, additional quantities can be added mid-cycle up to ~20 mL/L depending on whether the antifoam becomes sequestered or consumed by the organism. The presence of foam is measured by a capacitance-based foam probe located in the headspace of the vessel. Antifoams (typically nonionic surfactants) then can be added based on this signal or prophylactically at set intervals. Example defoamers include silicone oils, polypropylene glycol [25322-69-4] and vegetable oils. Drawbacks of antifoams are that they accumulate at the gas–liquid interface and thus reduce gas–liquid mass transfer rates. Their presence also can adversely affect isolation performance by reducing clarity of initial broth solid-liquid separation steps or by carrying through initial isolation steps, and then adversely affecting the crude product crystallization.

In addition to adding antifoam, foam can be reduced by lowering the superficial air velocity or increasing the fermentor back-pressure. Top driven mechanical foam breakers can be installed but these rotational devices can require an additional seal on fermenter if they are not placed on the impeller shaft. Foam (as well as impeller shear) can be minimized by avoiding operation with fermentor broth level right at the impeller level.

Nutrient feeding during fed-batch cultivation to increase cell mass can involve the control of volumetric feed flow rates to the fermentor based on measurements of cell density either off-line, online using an *in situ* optical density probe, or estimated via material balances (126,127). Control for other purposes can be done directly based on on-line measurements of nutrient concentration or indirectly via monitoring of the loss in weight or level of the nutrient feeding vessel or the gain in weight or level of the broth itself.

Aerated (gassed) power draw per unit volume typically ranges from 1 to 4 kW/m^3 for microbial fermentation although some microbial processes are well above and some animal cell processes are well below this range. Power can be indirectly and qualitatively measured using watt transducers or other means to quantify the electrical output from the variable frequency drive

(VFD), which does not account for various motor and gearbox mechanical losses between the VFD and the agitator shaft. Power can be measured directly (and more quantitatively) using a transducer located directly on the agitator shaft inside the vessel. The drawback is that it has been difficult to develop steam-sterilizable and sanitary devices for this application.

Fermentor vent off-gas is directed to a take-off valve located between vessel and its back-pressure control valve to assure adequate flow to the mass spectrometer unit. Inlet (reference) gas to the fermentor also is measured. The analysis results of the difference between the inlet and outlet stream compositions are combined with on-line measurements of air flowrate, liquid volume, back-pressure, and dissolved oxygen to calculate the oxygen consumed (oxygen uptake rate, OUR), carbon dioxide produced (carbon dioxide evolution rate, CER) and the volumetric gas–liquid oxygen mass transfer coefficient (K_La; 128). These uptake rates generally can be used to follow cell growth profiles on-line. The respiratory quotient (RQ), the ratio of the CER to the OUR, can be used to follow the carbon source consumption on-line and thus avoids manual sample analysis. RQ values scattered above/below 1 are common initially due to noise from low OUR and CER measurements, values $\sim$1 are common for glucose (and other hexoses), values $\sim$0.7 are common for glycerol and values $\sim$0.5 are common for oil. Oxygen consumption rises (and RQ falls) for substrates like glycerol and oil with lower oxygen to carbon atom ratios since the organism must consume additional oxygen to fully metabolize them. Mass spectrometer vent gas analysis can be used to perform mass balances for carbon and oxygen for cell cultivation processes.

Off-line measurements are initially valuable to improve the understanding of changes in on-line measurements. Often several techniques are available for the same quantity (Table 6) and selection of the appropriate method must consider sources of error relative to process development requirements. Implementation of robotics for the preparation and analysis of off-line samples has been used successfully to increase throughput, provide off-hour support and improve reproducibility.

7.4. Automation and Sequencing. Application of automation to fermentation varies depending on both the process complexity and the end user philosophy (129). Automation in the most basic sense consists of air actuated on/off valves and control valves for utilities and process additions. An additional level of sequencing uses recipes of several steps with intermittent operator inputs to confirm that any required manual preparations are complete. Fermentor operations such as sterilization-in-place (SIP) or clean-in-place (CIP) can be controlled automatically using sequencing after the initial manual setup. Vessel-to-vessel transfers of product, inoculum, nutrient media and/or buffers via hard-piped or removable connections also may be automated. In one example of extensive automation and sequencing applied to both fermentation and isolation stages, "lights out" production (130) was implemented for automation of sterilization, cleaning, transfers of raw materials and media feeds, sampling and harvesting. Only selected process phases occurred during off hours with an impressively high level of reliability as to not require night shift personnel.

Key issues affecting the complexity, maintainability, and reliability of an automated design include the number of automated valves as well as the number of limit (proximity) switches to indicate proper automatic valve opening and/or

closing or proper transfer pipe connection. Implications of a power outage or network disruption must be evaluated to determine the ability to continue an in-process fermentation after such an interruption. Finally, for highly automated and sequenced systems, the process must be extensively and reliably defined since it can be challenging to detect errors and to change programming quickly.

As more sensors become available for the continuous measurement of more variables, larger amounts of historical batch data are accumulated. Methods for data collection and archiving for both on-line and off-line fermentation data, operator changes and batch alarms should be determined based on the ease of retrieval and analysis desired both for historical postbatch analysis and for real time in-process trending for trouble shooting purposes. Approaches such as storage of data based on its difference by a designated amount from prior values have successfully minimized these volumes.

Fermentation data then can be used to improve the control and in-process parameter estimation for complicated bioprocesses (131). Modeling of cellular metabolism using both stoichiometric and kinetic models has been enhanced by greater availability of genetic information, improved analytical techniques and more powerful computational platforms (132). Although the history and future prospects of mathematical modelling and analysis in biochemical engineering are extensive, the focus now is on applying modelling, analysis and computational techniques to experimental data to analyze and design metabolism (104). Limitations of modeling may be complemented by artificial intelligence (131).

Several computer programs are available commercially for integrated process design and simulation: SuperPro (Biopro) Designer (Intelligen, Scotch Plains, N.J., 133), Process Evaluator (Icarus Corp., Rockville, Md.), Batch Plus Bioprocess Simulator (Aspen Technology, Cambridge, Mass.), and Batch Process Technology (West Lafayette, Ind.). Knowledge-based systems or artificial intelligence include artifical neural networks, fuzzy logic, and expert systems.

Often one or more types of artificial intelligence are combined together as well as artificial intelligence being combined with modelling (131). Artificial neural networks (a highly interconnected network of simple processing units that process information by activating and inhibiting the connections) are able to model highly nonlinear multivariable processes such as complex microbial pathways (134). Fuzzy logic permits partial degrees of truth to deal with subjective reasoning and data uncertainty (131). Finally, knowledge-based expert systems consist of a database of rules and frame sets related to culture conditions and resulting outcomes (G2, Gensym, Cambridge, Mass., 135–137). They use available process information to make decisions in a human fashion and are able to ferret out inaccurate data and adapt to incomplete data. Interestingly, newer versions of the G2 fermentation expert (NeurOn-Line) incorporate neural networks, fuzzy logic, and rule-based reasoning.

8. Inoculum Development and Scale-Up

8.1. Inoculum Development. Inoculum development passes through several stages, starting from a frozen vial containing a few milliliters and moving

to the production fermentation volume containing tens of thousands of liters. Actively growing cultures in mid-exponential growth are preferred for transfer to the next stage that serves to minimize the lag phase in the subsequent stage before growth is noticeable. Inoculum quality (eg, viability, morphology) can impact production performance although this impact can be difficult to quantify directly (138). Although the elapsed cultivation time is a common indicator of when the culture should be transferred to the next stage, it is not always a reliable one. Quantitative measures of transfer times can be based on cell mass, glucose depletion, oxygen uptake rate, or other on-line or off-line measurements.

Since the microorganism can be the most valuable part of the entire process, the culture storage method must assure longevity, specifically high cell viability. Industrially improved high producing strains are proprietary and usually only lower producing strains are deposited in publically accessible cell banks as part of patent requirements. The source material, master and working seed banks are frozen to $-70^{\circ}C$ (microbial cells, spores or mycelia) or to $-196^{\circ}C$ (animal and insect cells) in a high viscosity cryoprotective agent such as 10–25 wt% glycerol to minimize ice crystal damage. Alternatively, lyophilization can be used to freeze-dry spores by removal of water from the frozen culture by sublimation under a vacuum. After preparation, vials are tested for culture purity and viability. Production testing potentially also includes use testing for both the seed and production stages, isolation evaluation to determine if the impurity profile has changed, culture purity testing and viability measurements. Any variation in seed stock cultures must be minimized including variations of productivity among isolated cell colonies.

Seed trains can vary tremendously depending on the culture. For a traditional fungal seed train, a starter culture (1–4 mL in volume) is inoculated into a 250–500-mL first stage seed flask containing 50–100-mL medium and then transferred into multiple 2–4-L second stage seed flasks each with 500–1000-L medium. Flasks are incubated on a rotary shaker at 200–400 rpm with an appropriate "throw" (wideness of the rotational circle) so that medium mixes and aerates but is not violently splashing. Erlenmeyer flasks often are used that are sometimes baffled for improved oxygen transfer if this does not result in increased foaming. Alternatively, wider bottomed Fernbach flasks or lower liquid volumes are used to raise oxygen transfer by an increase in the gas–liquid surface area per unit volume of liquid. Multiple second seed stage flasks then are pooled and used to inoculate the first fermentor seed stage (100–500 L). A second fermentor seed stage can be added for large production volume fermentations.

Transfer times, cell concentrations, and inoculum volume percentages depend on the seed stage itself, the culture growth requirements and/or the facility staffing constraints. Generally, 1–4 days per stage is optimal with a minimum of 20 h desirable so that adequate time exists to obtain initial culture purity results prior to transfer. If a seed stage shorter than 20 h exists then it may be possible to alter the pre- and poststages to omit it from the process. Seed morphology must be consistent as it can influence production culture morphology and productivity.

8.2. Scale-Up. For scale-up to production fermentors, there are many process dependent factors to consider (32,139). Key variables must be selected to be maintained constant upon scale-up since if all variables were held constant

the production fermentor design would be overconstrained. Geometric similarity (the same tank diameter, working volume, and tank volume) typically is maintained.

Oxygen transfer can change radically upon scale-up since surface aeration becomes less of an influence as the surface area at broth level to broth volume ratio decreases and mass transfer rates requirements increase. Superficial velocity (gas flowrate divided by fermenter cross-sectional area) increases upon scale-up for the same air flowrate to vessel volume ratio (VVM), which can increase foaming. Vessel jacket and piping heat-transfer requirements can be estimated by using the linear correlation of heat evolution to oxygen consumption during culture growth with a slope of ~0.11 kcal/mmol oxygen consumed (140).

The design and operation of the agitator is critical to providing adequate mixing to disperse additions and minimize gradients (such as pH, dissolved oxygen, carbon dioxide, nutrient/substrate, and temperature). Mixing tends to become less uniform and takes longer with increases in scale. Agitator shear cannot be permitted to damage cells and it must be considered that both shear and tip speed increase upon scale up for the same agitator speed. Similarly vibration increases with scale up due to larger impeller, agitator shafts, gearboxes, and motors.

9. Isolation

Product concentrations in the broth can be as low as 0.1 wt% for some processes (such as therapeutic proteins and vitamins), but can be as high as 5–15 wt% for organic acids and solvents (such as lactic acid and ethanol). Postfermentation the first isolation step is the cell harvest, a liquid–solid separation. Commonly, unit operations of centrifugation and filtration (with flocculation used to a lesser extent) are used to separate the cells from the broth. The nature of harvested fermentation broth (eg, level of antifoam added, residual quantities of medium components) influences the harvest conditions.

For primary or secondary metabolites, if the product is excreted, the broth can be extracted either before or after cell separation, the extract concentrated, then a crude crystallization performed. Since some hydrophobic secondary metabolites are attached to the outer cell wall, acid, or base adjustment may be used to release the product prior to extraction if necessary. For excreted protein products, cells are separated from the product-containing broth and discarded. Ultrafiltration can be used to concentrate the protein of interest and discard lower molecular weight contaminants.

For protein products that accumulate intracellularly, cells must be separated then lysed by a homogenizer (or by chemical means) in such a fashion as to release the product without becoming overly disintegrated. This step then is followed by centrifugation or microfiltration to remove cell debris. Ammonium sulfate or polyethylene glycol precipitation followed by low pressure column chromatography (such as ion exchange, affinity, and/or size exclusion) then might be used to eliminate contaminants. Any inclusion bodies present can be easily separated from lysed cells by ultracentrifugation, dissolved in a strong denaturing solution, and then renatured (refolded into their tertiary form) usually through dilution by adding high volumes of buffer.

10. Utilities

10.1. Product Contact. Product contact utilities are those that directly mix with the process fluid, environment, or equipment. Since they are utilized continuously, back up units usually are installed to provide redundancy for planned and unplanned maintenance outages.

Required water quality is influenced by both process and regulatory factors. Process (city) wateroften is used for medium make up for larger scale lower cost products if trace element composition is not a medium issue and/or has been quantified. Although least expensive, it can be subject to seasonal variations, the magnitude of which depend on the municipal water company practices and the water source (well, reservoir, surface run-off). Deionized or USP (United States Pharmacopeia) water is more expensive to use at the large scale but assures a consistently low amount of trace elements (so that known amounts can be added back to the medium). It can be useful for process development and for fermentation products requiring higher water quality. The most expensive but highest quality water is low bioburden and low endotoxin water-for-injection (WFI), which is made by condensation of clean steam. WFI is most suitable for animal cells which can be sensitive to endotoxin content but is used for many other microbially sourced biological products as well.

Saturated steam in product contact normally is in the range of 30–45 psig, which provides sufficient dynamic pressure to attain sterilizing temperatures of $>121^{\circ}C$. Normal industrial steam supply, plant steam, might have small amounts of corrosion-preventative additives carried over from the powerhouse boilers. It can be filtered prior to entering the process since plant steam transfer piping typically is carbon steel. In some cases, plant steam can be fortuitously low in endotoxins. Higher quality (high resistivity, low endotoxin) clean steam is produced by heating purified water via sanitary heat exchangers using plant steam. Clean steam is used for animal cell culture as well as other biologics products.

Compressed oil-free air is required for sparging and surface aeration at a supply pressure of at least 30 psig or equivalent to/slightly higher than the product contact steam pressure. This air typically passes through a holding tank (to maintain consistent pressure and supply air during momentary outages), a desiccant dryer (to remove moisture down to a dew point at or below $-40^{\circ}F$) and several filters prior to entering the vessel. Enormous quantities of air are needed for commercial scale aeration creating capital and operating expenses due to the required compressor size, redundancy, and energy consumption.

HVAC-supplied air contacts equipment during open transfers (when the product is exposed directly to the environment) and surrounds equipment during closed transfers. It can be a major cost factor for therapeutic proteins and vaccines, both operational in addition to capital, depending on the level of cleanliness required. In some facilities, 100% once through air (no air recirculation) is implemented with heat recovery either avoided or implemented using heat exchangers to avoid any direct contact of inlet and outlet steams. Separate air handling systems may be used for areas needing additional segregation such as inoculum preparation, live virus, or β-lactam antibiotic suites. Typically, 10–40 room air changes per hour are utilized depending on level of cleanliness

anticipated to attain the appropriate air quality required for the processing step being executed in the area. Tight humidity limits may be implemented depending on the hygroscopic nature of raw materials/product and the level of cleanliness required.

Inlet and outlet terminal HEPA filtration, room air changes and laminar air flow all are used to attain viable and nonviable particulate reduction. Although normal air might have ~500,000–1,000,000 nonviable and 3–100 viable particles/ft^3, classified areas for cleaner processing have anywhere from 100,000 down to 100 nonviable particles/ft^3 and 2.5 down to <0.1 viable particles/ft^3. It is usually desirable to minimize open transfers in the developed process and to ensure that the fermentor is a closed system with no leaks and sterilization of all in-process connections.

Room air supply rates and returns are balanced so that air pressurization forms containment barriers. The lowest absolute pressure is used for those areas with operations that are most likely to release contaminants. A higher pressure airlock bridge acts as a barrier between two rooms of lower pressure, often with controls on the doors on each entrance to prevent them from opening simultaneously. Pressurization, typically ~0.03 in water or higher, is used to minimize cross-contamination among different process suites, between clean and less-clean areas, and between live culture/virus and inactivated culture/virus areas.

10.2. Nonproduct Contact. Although nonproduct contact utilities often are assumed to be less critical, their reliability and the presence of back up equipment can dramatically influence facility operation. The nonproduct contact utilities of cooling water, steam, and power are major cost factors for antibiotics and industrial enzymes.

Cooling water might be supplied by a cooling tower or a water–glycol chiller depending on process loads. Greater cooling challenges exist with highly viscous mycelial cultures or metabolically active high cell density *E. coli* cultures. Cooling water can pass through a holding tank that also feeds the chiller. Return cooling water temperatures must be assured to be within reasonable limits to avoid spikes in the cooling water supply. Supply pressures of 45–60 psig are useful to provide reasonable flowrates into vessel jackets. Cooling water can be an expensive utility for the cooling of large production fermenters poststerilization. The use of high temperature short time (HTST) sterilization minimizes the cooling required since fermenters are sterilized empty and often can be allowed to cool down unaided. Up to 75–80% of the heat is recovered from the hot sterilized medium to preheat incoming cool nonsterile medium prior to HTST sterilization.

Plant steam is used for vessel heating, typically at a supply pressure of 60 psig to insure that the jacket temperature can reach 135–150°C for prompt heat up.

Filtered dry uninterrupted instrument air, at a supply pressure of over 80 psig (and dew point at or below −40°F), is key to reliable automation. If supply is interrupted, automatic valves move to their failure states, typically designed to remove positive pressure from the fermentor. An instrument air hold tank assures a steady supply pressure plus offers some buffer capacity in case of a temporary outage.

The major amount of electrical energy is required to power the agitator drive to facilitate mixing and oxygen transfer, the compressors for sparger air

supply, and the chillers/cooling tower for process cooling. Minor amounts are required for the control system components. Back-up of control panels with an uninterruptible power source can minimize the impact of power blips; back up of instrument inputs with a second power supply can provide added reliability in case of failure.

For fermentation processes requiring a high efficiency of biological waste destruction, all product contact streams must be treated using a HTST biowaste sterilization system prior to disposal. Depending on the facility design and operation, the flows to the biowaste system may become limiting during certain peak periods.

11. Regulatory Aspects

11.1. Product Quality. Product quality is determined by purity as well as by titer and product characteristics. In addition to the desired compound, similar molecules may be produced during the cultivation. Structural analogues, differing by as little as a single methyl or hydroxyl group, are found in secondary metabolite fermentations; heterogeneity in glycosylation (glycoforms) or minor differences in amino acid sequence can emerge during protein production. Glycosylation differences affect parameters such as protein solubility, stability, and biological clearance.

Culture purity, contamination or the presence of foreign cells is influenced mainly by equipment design (eg, size of pockets or deadlegs, crevices), standard operating procedures, training, preventative maintenance (eg, instrument calibration, gasket/o-ring replacement) and validation. Some fermentation processes are self-protected and enjoy repeatedly low levels of contamination. These cultivations include (*1*) processes conducted at low (15°C)/high (50°C) temperatures or low pHs (<4.0), (*2*) anaerobic processes, (*3*) processes with less nutritious medium components, or (*4*) secondary metabolite cultures that secrete broad spectrum potent antibiotics.

High contamination rates often are problematic depending on the product application in roughly increasing levels of process tolerance: biologicals (therapeutic proteins, vaccines), secondary metabolites (antibiotics), enzymes, specialty chemicals, bulk chemicals, food, and waste treatment. Quality implications generally follow this order as well although each regulatory application is unique. Contamination rates can vary from <1% to >20% with actual rates dependent on the degree that the facility can afford to delay production to investigate incidents and implement corrective action.

The presence of bacteriophage (bacterial viruses), actinophage (actinomycetes viruses) or mycoplasma (tissue culture prokaryotic infectious agents lacking a cell wall) can comprise culture performance. Bacteriophages, entering through the compressed air supply, raw materials, or the culture itself, are exceedingly difficult to eradicate and infection can shut down a fermentation facility (65).

Contaminants from animal-sourced components are another potential quality problem. Bovine spongiform encephalopathy (BSE), also known as “mad cow” disease, is a chronic degenerative nervous disease, resulting in the accumulation of an abnormal protein isoform (prion protein) that is resistant to protease degra-

dation and heat denaturation. (Many researchers believe that the prion protein might be the actual infectious agent.) Validation studies to demonstrate removal or inactivation of prions are difficult to interpret so the presence of animal-sourced components from unacceptable herds is avoided in raw materials for some fermentation products. Restrictions and guidelines for the use of animal-derived components in pharmaceutical processing are being implemented even for isolated well-characterized compounds. As a consequence, fermentation media development is focussed on replacing nutrients with nonanimal (usually vegetable) sourced materials or on assuring raw materials come from certified BSE-free herds (141). How far back in the fermentation process seed train and vial preparation it is required to implement these replacements is product dependent.

Endotoxins or lipopolysaccharides are cell wall components of gram negative bacteria (eg, *E. coli* host cell debris), which are pyrogenic (can cause fever in mammals) and must be reduced for injectable products. Host cell or foreign DNA must be decreased to at least <10 ng/dose. Beta-lactam antibiotics often are avoided as selection pressure agents in seed–production fermentors due to segregation issues. Although typically antibiotics are not desirable to use on a production scale due to their cost and associated regulatory issues, usually they can be used for early culture laboratory development and possibly early seed stages.

11.2. Good Manufacturing Practice (GMP) and Validation. The Food and Drug Administration (FDA) has jurisdiction over those types of fermentation products destined for food or pharmaceutical applications in the United States. The Center for Drug Evaluation and Research (CDER) examines products that are defined by analytical specifications. These molecules generally can be purified, characterized, and identified using accurate and reproducible methodology. Examples include small molecules, well-characterized proteins, and most recently all therapeutic proteins such as antibodies. The Center for Biologics Evaluation and Research (CBER) examines products that are defined by analytical specifications as well as the individual manufacturing steps of the process and facility. These molecules are not readily characterized and prime examples are vaccines.

Selection of dedicated versus multiuse facility design determines future processing flexibility and is dependent on the product regulatory requirements. Dedicated operation permits one product only and is typically found in a manufacturing area. Multiuse operation permits running various products simultaneously in multiple segregated suites within a single building. Each suite can be used for more than one product on a campaign basis with not more than one product in the same suite at one time. Although this approach is attractive for research and development as well as contract manufacturing organizations, it requires extensive documented product changeover efforts to demonstrate that all traces from the prior product were removed. During initial facility planning, care is taken to provide unidirectional flows of people, clean–dirty equipment, raw materials, product and waste so processing areas remain clean and the potential for mixups is minimal.

Fermentations are conducted in closed systems with the product not exposed to the immediate environment during processing. Everything entering

the fermentation system is either filtered or autoclaved with all connections being aseptic. Pressure leak tests and/or microbial ingress–egress tests are performed on vessels, container closures and aseptic connections such as sterile tubing welds. Any open system transfers are conducted under a biosafety cabinet or laminar flow hood.

Depending on the regulatory requirements surrounding the product itself and its stage of development, process equipment, utilities, and the facility itself frequently undergo validation or qualification in which a preapproved comprehensive plan is executed to demonstrate that the equipment performs as it was intended to perform. It is tested against both its specifications and its range of intended uses with test methods and acceptance criteria documented and agreed upon in advance of the initiation of testing.

For fermentation equipment validation (142) each piece of equipment in product contact undergoes installation and operational qualification with several pieces undergoing performance qualification or load pattern tests (eg, autoclaves, glasswasher), plus SIP and CIP testing. Although there is some overlap among the various testing divisions, general distinctions may be made. The installation qualification (IQ) focuses on the equipment installation by verifying utility supply information, agreement with vendor–user specifications and the purchase order itself, and calibration and loop checks. The operational qualification (OQ) checks the overall system performance function through function and alarm testing, confirming that key findings are incorporated in operational SOPs. Performance qualification and other testing demonstrates that the equipment can be utilized for a specific process or group of related processes. Materials that are to be sterilized or cleaned are directly tested under fractional (reduced) conditions to verify that proposed loading of the equipment is appropriate. For sterilization validation, thermocouples and spore strips or suspensions (containing biological indicators such as *B. stearothermophilus*) are used to monitor temperatures achieved and confirm the inactivation of a worst-case indicator organism. For cleaning validation, the glassware or vessel product contact surface is physically swabbed and a rinsewater sample analyzed for product or cleaning agent residues.

After the initial equipment, utility and facility qualification effort, ongoing testing and continuing validation are required on a regular basis. Specifically, the quality of key product utilities such as water and steam might be tested daily or weekly and the efficacy of an autoclave's sterilization load pattern might be tested annually. Any changes to the validated systems must be evaluated to assure that there was no impact on the previously executed testing before they are implemented. If an impact is suspected, additional testing may be needed postinstallation and prior to use for GMP processing.

Computer validation is used to assure that automated systems have controlled specifications, design and qualification according to published standards such as GAMP (Good Automation Manufacturing Practice). A system life cycle approach can be introduced to break down the necessary stages into specification, design, coding, testing, operation, maintenance, change control, and retirement. Recent FDA regulations, known as 21CFR Part 11, address the requirements for electronic records and signatures (143).

The aim of process validation is to provide documentation that the manufacturing process consistently meets the predetermined quality parameters

(eg, CPPs—critical process parameters and CQAs—critical quality attributes) for the product. Its purpose is to minimize unexpected lot failures by validation of the operating ranges for critical operating or process parameters, process consistency and clearance of a potential contaminant (eg, adventitious agent, host cell foreign DNA).

11.3. Safety Considerations. Fermentation containment levels, established by the National Institute of Health for the United States, contemplate the potential health risk to workers based on prior experiences with the microorganism and any known pathology. There are similar but not always analogous standards in Europe and Japan. Containment levels span from G(I)LSP (Good (Industrial) Large Scale Practice) for GRAS (Generally Recognized As Safe) cultures such as Bakers' yeast to biosafety level 1, 2, etc (BSL-1, BSL-2). Increasing levels of containment are devised to reduce the likelihood of the release of live organisms from aerosol generation in off-gas or during operations such as sampling and waste disposal. These requirements dictate the installation of systems such as contained 0.2-μ vent line filters and sampling devices which minimize loss of culture outside the fermenter and reduce operator exposure.

Review of cultures to establish their containment levels typically is done by an internal institutional biosafety committee using externally available database resources. This committee also reviews all recombinant DNA host–vector plans prior to their execution to assure regulatory compliance.

11.4. Environmental. Fermentation medium components rarely are toxic to the environment and usually have few environmentally adverse consequences and processing conditions than synthetic alternatives. However, fermented broths can have appreciable aquatic toxicity, both to organisms in municipal sewage treatment facilities and to freshwater–saltwater species in local waterways. Often, some aquatic toxicity is observed if the active product component is an antiparasitic or an antiinfective, compounds screened to kill other life forms. Releases of large broth quantities to the environment can raise the biochemical oxygen demand (BOD) in the receiving water body unless there is a large dilution factor. Broth waste disposal concerns have been addressed most successfully by using fermenter waste as an animal feed additive or a crop fertilizer when applicable.

Fermentor off-gas can have strong odors during media SIP, waste broth heat inactivation and during the cultivation itself. Process parameters as well as vent line "stack" locations can greatly impact the degree of objectionable odor. Alternatively, off-gas can be treated through scrubbing or other means.

12. Summary

The major tenet of fermentation is to consider the needs of the microorganism itself. Several notable microbiologists have stated this reality, perhaps as a reminder to their engineer counterparts:

Kei Arima: "Microorganisms will never betray our needs if we create rational, sensitive and reliable screening and assay methods, since microorganisms are extremely excellent chemists" (91).

Jackson W. Foster: "Never underestimate the power of the microbe" (31).

D. Hockenhull: "Once a fermentation has started it can be made worse not better" (138).

David Perlman: "If you take care of your microbial friends, they will take care of your future" (31).

Consequently, it is critical for those engaged in any significant aspect of biochemical processing to adopt a multidisciplinary approach to problem-solving so that the most efficient, economical and robust processes may be developed.

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BETH JUNKER
Merck Research Laboratories