N. S. GOLYAK

TECHNOLOGICAL PROCESS ORGANIZATION IN BIOTECHNOLOGICAL INDUSTRY

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МИНИСТЕРСТВО ЗДРАВООХРАНЕНИЯ РЕСПУБЛИКИ БЕЛАРУСЬ
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Н. С. Голяк

ОРГАНИЗАЦИЯ ТЕХНОЛОГИЧЕСКОГО ПРОЦЕССА НА БИОТЕХНОЛОГИЧЕСКОМ ПРОИЗВОДСТВЕ

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Учебно-методическое пособие

Минск БГМУ 2021

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INTRODUCTION

The Industrial Bioprocess it is a large scale operation involving transformation of a raw material into a product.

A product in pharmaceutical biotechnology is a drug including proteins, antibiotics, amino acids and other substances.

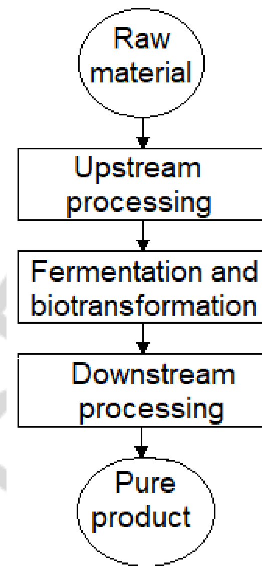

Figure 1. Principal steps of industrial bioprocess

The bioprocess typically consists of 3 steps (Fig. 1):

1. Upstream processing: preparation of the bioobject and the raw materials required for production of the desired product.

2. Fermentation and transformation: growth (fermentation) of the target bioobject in a large bioreactor with the consequent production (biotransformation) of a desired compound.

3. Downstream processing: purification of the desired compound from either the cell medium or the cell mass. Downstream processing may include filtration, centrifugation, precipitation, extraction, ultrafiltration, crystallization, chromatography, drying and packaging.

UPSTREAM PROCESSING

Expression systems for proteins of therapeutic interest include both pro- and eukaryotic cells (bacteria, yeast, fungi, plants, insect and mammalian cells) and transgenic animals.

The choice of a particular system is determined by:

– the nature and the origin of the desired protein;
– the use of the product;
– the amount needed;
– cost.

Bacteria and yeasts have been the organisms of choice for the industrial production of heterologous recombinant proteins for many years. However, certain features of these producers necessitated the use of other bioobjects like plant, insect and mammalian cells.

In principle any protein can be produced using genetically engineered organisms, but not every type of protein can be produced by every type of cell.

In the majority of cases protein is foreign to the host cells that have to produce it and posttranslation modifications of protein might be different as compared to the original product.

Correct N-linked glycosylation of therapeutically relevant proteins is significant for:

– full biological activity;
– immunogenicity;
– stability;
– targeting;
– pharmacokinetics.

Prokaryotic cells, like bacteria, are sometimes capable of producing N-linked glycoproteins. However, the N-linked structures differ from the structures found in eukaryotes. Yeast cells are able to produce recombinant proteins like albumin, and yeast has been engineered to produce glycoproteins with humanlike glycan structures including terminal sialylation. Still most products on the market and those which are currently being developed, use cell types that are as closely related to the original protein-producing cell type as possible. Therefore, human-derived proteins, especially mammalian cells, are chosen for production.

Generalized features of proteins expressed in different biological systems are listed in Table 1. However, there are exceptions from this table for specific product/expression systems.

Table 1

Generalized features of proteins of different biological origin

Protein feature	Prokaryotic bacteria	Eukaryotic yeast	Eukaryotic mammalian cells
Concentration	High	High	High
Molecular weight	Low	High	High
S-S bridges	Limitation	No limitation	No limitation
Secretion	No	Yes/no	Yes
Aggregation state	Inclusion body	Singular, native	Singular, native
Folding	Risk of misfolding	Correct folding	Correct folding
Glycosylation	Limited	Possible	Possible
Impurities: retrovirus	No	No	Possible
Impurities: pyrogen	Possible	No	No
Cost to manufacture	Low	Low	High

VIRUSES

Heterologous proteins, being the basis of viral particles and VLPs (virus-like particles), are normally expressed by transfection or transduction of cell cultures. The availability of stable packaging cell lines capable of continuously expressing a specific gene represents a step towards the scaled-up production of viral vector stocks, further used as drug delivery systems or gene therapy.

Although the productivity of some viral vector producer cell lines remain lower than expected, the generation of acceptable viral yields and the expression

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of secreted and insoluble proteins are normally favored by these stable transfected cell lines. It is essential to use the active promoter integrating into the genome which does not require an infection agent for its activation.

Product yields are strongly dependent on the chosen production strategy. Batch, fed-batch, continuous, and perfusion strategies are normally used for the production of viruses and VLPs.

The following process-related parameters strongly influence virus and VLP titers:

– multiplicity of infection (MOI);
– time of infection (TOI);
– time of harvest (TOH);
– cell concentration in infection (CCI).

Adequate MOI, defined as the number of virus per cell, determines optimal yields. The use of MOI depends on the target product, the production process, and the dimension of viral stocks. Low MOIs (0.01^{-1} virus per cell) has the advantage of requiring low concentrations of viruses. The number of viruses is normally insufficient to infect all the cells; thus, a high percentage of cells remain healthy.

A steep increase in infected cell concentration is observed as a second generation of viruses which start to affect the uninfected cell population. At the end of the bioreaction the concentration of infected cells is sufficiently high to sustain the production of viruses and/or VLPs to high levels.

The main drawback is the *protease effect*. Since the process (infection plus viral and/or protein synthesis) is slow, the bioproduct is exposed to cellular proteases for long periods of time, which may compromise the quality and quantity of the final product.

At high CCI, the change in cell's energetic state upon infection induces a significant drop in cell-specific productivity.

Another important parameter is the TOH. Delayed harvest times increase the exposure of viruses and VLPs to intracellular or extracellular proteases and induce a more pronounced cell lysis. Additionally, the release of contaminant proteins (degraded or not), host and viral DNA, cell compartments and viral or protein macrostructures to the extracellular medium will complicate the downstream processing of the product of interest. Optimal harvest time is normally between 72 and 120 hpi (hours postinfection) (40 and 70 % of cell viability).

The interplay between all the above-mentioned parameters is complex and normally requires substantial experimentation.

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BACTERIAL CELLS

The ability of cultivating bacterial strains to high cell density at a large scale has become an increasingly important technique in biotechnology. *Escherichia coli* remains one of the most attractive organisms for the production of recombinant proteins where no complex post-translational modifications (e. g. glycosylation or disulphide bond formation) are required for biological activity as its genetics and physiology are well understood. However, there are important drawbacks associated with the use of prokaryotic organisms. Among them:

1) low percentage of GC nucleotides in their genomes (compared to mammalian genes);
2) existence of rare codons (often result in low expression levels);
3) proteins, expressed as insoluble inclusion bodies in the bacterial periplasmic space.

Bacteria are also incapable of carrying out any post-translational modifications, which strongly influences protein stability, folding, solubility and biological activity. Additionally, bacteria cells are surrounded by a mechanically strong cell wall that may hinder recovery of any non-secreted proteins.

FUNGAL CELLS (YEASTS)

Yeast is an established industrial fermentation system which supports high-level recombinant protein production. The high-level protein production can be reached by taking care of the following factors:

– adequate copies of vector (10–100 copies per cell);
– suitable promoters;
– proper inducible system;
– targeted cellular location.

As a eukaryotic system, the *Yeast Expression System* produces mammalian-like proteins. Expression of foreign genes is achieved by integration of foreign DNA into the chromosomal DNA of the host genome. The integrated DNA is stable for many generations and all cells can produce protein. The *Yeast Expression System* requires no special handling.

Yeasts (e.g. *Saccaromyces cerevisiae* or *Pichia pastoris*) can perform some post-translational modifications similar to those of the more complex eukaryotic cells. These single-celled eukaryotic organisms grow quickly in the defined medium. They are easier and less expensive to work with than insect or mammalian cells. Besides, they are easily adapted to fermentation. Yeast expression systems are ideally suited for large-scale production of recombinant eukaryotic proteins. However, N-glycosylation of mammalian proteins in yeast seems to be very inefficient. Additionally, yeast cells are surrounded by a mechanically strong cell wall that may hinder recovery of any non-secreted proteins.

TRANSGENIC ANIMALS

Foreign genes can be introduced into animals (mice, rabbits, pigs, sheep, goats, and cows) via nuclear transfer and cloning techniques.

Desired protein can be expressed in the milk of the female offspring.

During lactation milk is collected, skimmed and further used as the starting material for purification.

Advantage. It is a rather cheap method when larger animals are used.

Disadvantages:

– long time to generate a herd of transgenic animals;

– threat to their health due to foreign genes introduction.

(An example is the expression of erythropoietin in cows)

Although protein was well expressed in milk, it caused severe health effects and the experiments were stopped.

The purification requirements for proteins from milk can be different from those derived from bacterial or mammalian cell systems.

Transgenic milk containing the recombinant protein often has also significant amounts of the nonrecombinant protein.

Separation of these closely related proteins poses a purification challenge.

The transgenic animal technology for the production of pharmaceutical proteins has progressed for the last few years.

In the US and the EU recombinant antithrombin III (ATryn ®) produced in the milk of transgenic goats is approved for application. (note: in Belarus and the Russian Federation it is not registered).

LARGE-SCALE PRODUCTION OF PROTEINS FROM RECOMBINANT MICROORGANISMS

Production of commercial products synthesized by genetically engineered microorganisms requires partnership of two kinds of experts.

– **molecular biologists**, responsible for isolating, characterizing, modifying, and creating microorganisms with recombinant DNA;

– **biochemical engineers**, responsible for genetically engineered microorganisms able to be grown in large quantities.

The fundamental **objective** of industrial fermentations is:

– to minimize costs;

– to maximize yields.

Upstream processing is deemed to commence when a single vial of the working cell bank system is taken from storage and the cells therein cultured in order to initiate the biosynthesis of a product batch.

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Recombinant biopharmaceutical production cell lines are most often initially constructed by introduction into these cells of a plasmid housing a nucleotide sequence coding for the protein of interest.

After culturing, the resultant product-producing cell line is generally aliquoted into small amounts, placed in ampoules and subsequently immersed in liquid nitrogen. Therefore, the content of all the ampoules is identical, and the cells are effectively preserved for indefinite periods in case they stored under liquid nitrogen.

This batch of cryopreserved ampoules forms a 'cell bank' system, whereby one ampoule is thawed and the cell therein cultured in order to seed, for example, a single production run. This concept is applied to both prokaryotic and eukaryotic biopharmaceutical-producing cells.

The cell bank's construction design is normally two tiered, consisting of a "master cell bank" and a "working cell bank" (Fig. 2).

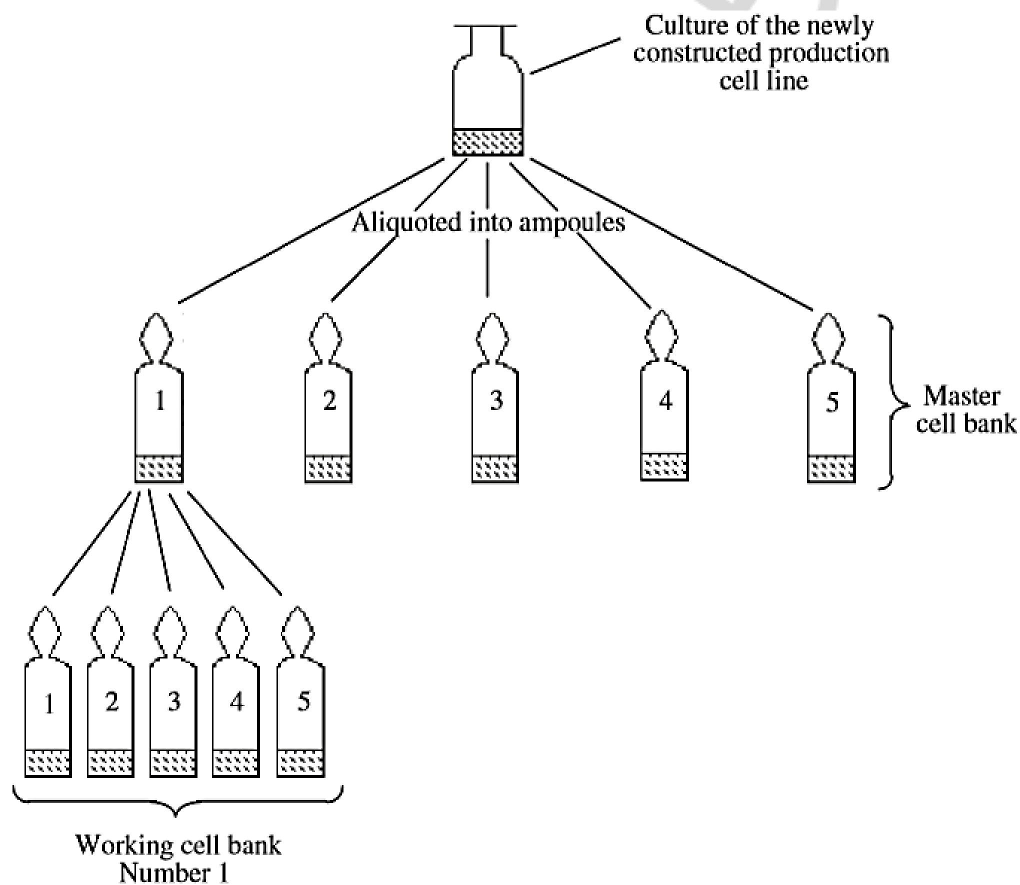

Figure 2. The master cell bank/working cell bank system [3]

First the master cell bank is constructed directly from a culture of the newly constructed production cell line. It may consist of several hundred individually stored ampoules.

For simplicity each bank shown above contains only five ampoules. In reality, each bank would likely consist of several hundred ampoules.

Working cell bank number 2 will be generated from master cell bank vial number 2 only when working cell bank number 1 is exhausted and so on.

The rationale behind this master cell bank/working cell bank system is to ensure an essentially indefinite supply of the originally developed production cells for manufacturing purposes.

This is more easily understood by the following example. If only a single-tier cell bank system existed, containing 250 ampoules, and 10 ampoules were used per year to manufacture 10 batches of product, the cell bank would be exhausted after 25 years. However, if a two-tier system exists, where a single master cell bank ampoule is expanded as required to generate a further 250 ampoule working cell bank, the entire master cell bank would not be exhausted for 6250 years.

Large-scale fermentation and purification are stepwise processes (Fig. 3).

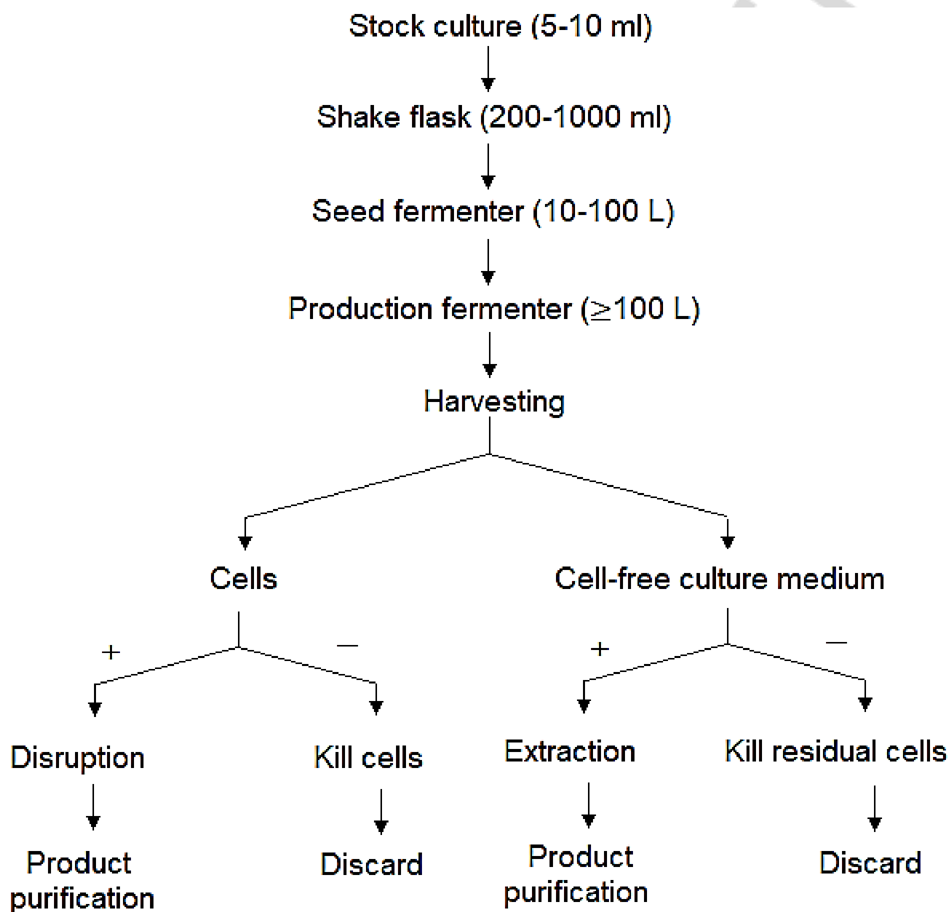

Figure 3. Generalized scheme for a large-scale fermentation process [2]

A typical procedure begins with obtaining and sterilization of the growth medium and sterilization of the fermentation equipment.

The manufacture of a batch of biopharmaceutical product begins with the removal of a single ampoule of the working cell bank. This vial is used to inoculate a small volume (5 to 10 mL) of sterile media with subsequent incubation under appropriate conditions.

The cells are grown first as a stock culture (5 to 10 mL), then in a shake flask (200 to 1,000 mL), after that in a seed fermenter (10 to 100 liters) and finally, the production fermenter (1,000 to 100,000 liters) is inoculated.

After the fermentation step is completed, the cells are separated from the culture fluid either by centrifugation or filtration.

If the product is intracellular, the cells are disrupted, cell debris is removed, and the product is recovered from the debris-free fluid. If the product is extracellular, it is purified from the cell-free culture medium.

The commercial product is usually in either the cell or cell-free fraction, but not in both. As a result, one or the other of these fractions will be processed further (+) or discarded (–).

PRINCIPLES OF MICROBIAL GROWTH

Microorganisms can be grown in batch, fed-batch, or continuous culture (Fig. 4).

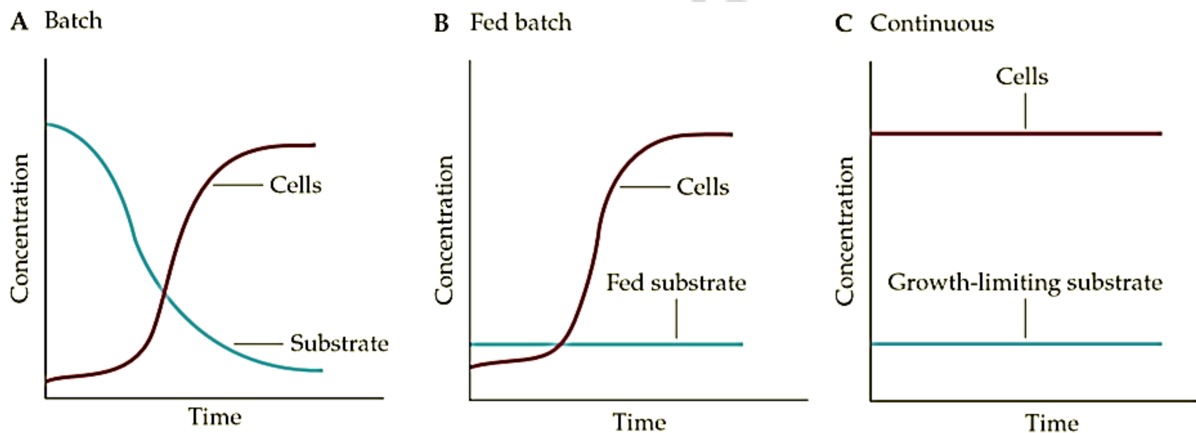

Figure 4. Schematic representation of cell and substrate concentration depending on time in batch (*A*), fed-batch (*B*), and continuous (*C*) fermentations [2]

In batch fermentation the sterile growth medium is inoculated with the appropriate microorganisms, and the fermentation proceeds without addition of fresh growth medium.

In fed-batch fermentation nutrients are added periodically during the fermentation reaction; no growth medium is removed until the end of the process.

In the continuous fermentation process fresh growth medium is added continuously during fermentation, but at the same time there is also concomitant removal of an equal volume of spent medium containing suspended microorganisms.

For each type of fermentation, oxygen, an antifoaming agent, and acid or base (if required) are injected into the bioreactor. Advantages and disadvantages of different modes of bioreactor operations see in Table 2.

Table 2

Advantages and disadvantages of different modes of bioreactor operations

Reactor	Advantages	Disadvantages
Batch	Versatile, since it can be used for many different processes. Low risk of contamination. Complete conversion of substrate possible	High labor cost. Much idle time, due to cleaning and sterilization after each fermentation
Continuous	High efficiency of the reactor capacity. High productivity can be maintained for long periods of time. Automation is simple. Constant product quality	Problems with infection. Possibility of the appearance of low levels of mutant production during long operation. Inflexible since it can rarely be used for different processes without substantial retrofitting. Downstream processing has to be adjusted to the flow through the bioreactor (or holding tanks are required)
Fed-batch	Allows operating in well-monitored conditions by controlling the feed addition. Allows to obtain very high cell densities and thereby high final titres	Has the similar problems as the batch and continuous reactor, but generally the disadvantages are less pronounced with this mode of operation

The pattern of microbial cell growth in a batch fermenter see in Fig. 5.

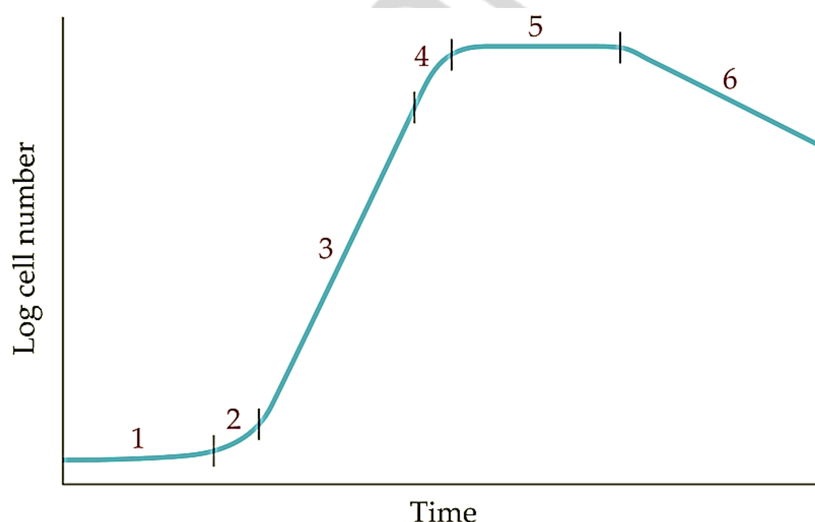

Figure 5. The pattern of microbial cell growth in a batch fermenter [2]

Six phases of the growth cycle are: 1) lag (latent phase); 2) acceleration; 3) log (exponential); 4) deceleration; 5) stationary; 6) death.

Lag phase. During this phase the cells are adapting to the conditions in the bioreactor but do not grow yet.

Acceleration phase. Following the lag phase, the brief period when the rate of cell growth increases until the log-phase growth is attained is called the acceleration phase.

Exponential growth phase. During this phase cells grow in a more or less constant doubling time for a fixed period. The mammalian cell doubling time is cell-type dependent and usually varies between 20 and 40 h. Plotting the natural logarithm of cell number against time produces a straight line. Therefore, the exponential growth phase is also called the log phase. The growth phase will be affected by growth conditions including temperature, pH, oxygen pressure, and external forces (stirring and baffles) inserted into the bioreactor. Furthermore, the growth rate is affected by the supply of the sufficient nutrients, buildup of waste nutrients, etc.

Stationary phase. In the stationary phase the growth rate of the cells slows down due to nutrients depletion and/or building up of toxic waste products. During this phase, constant cell numbers are found due to equal cell growth and cell death.

Deceleration phase. Because of the large cell population at the end of the log phase, the substrate may be so rapidly assimilated that the deceleration phase is short lived and not observable.

Death phase. Cells die because of nutrients depletion and/or presence of high concentrations of toxic products.

MAXIMIZING FERMENTATION EFFICIENCY

Regardless of the type of the fermentation process used to grow cells, it is necessary to monitor and control the below culture parameters:

– **dissolved oxygen concentration;**
– **pH;**
– **temperature;**
– **mixing degree.**

Changes in any parameters can have a dramatic effect on the yield of cells and the stability of the protein product.

High-Density Cell Cultures. High cell density is absolutely necessary for high productivity. Dry weight of *E. coli* cells is approximately 20 to 25 % of the wet weight.

Cultivation Systems. Cells can be cultivated in vessels containing appropriate liquid growth medium. Production-scale cultivation is commonly performed in fermenters, used for bacterial and fungal cells, or bioreactors, used for mammalian and insect cells.

The design of the bioreactor is important. It should ensure adequate sterility and provide appropriate levels of genetically engineered microorganisms. The reactor should also include probes that permit the accurate and continuous

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on-line monitoring of as many critical reaction parameters as possible, so that adjustments can be made rapidly and easily throughout the course of the fermentation reaction.

Bioreactor systems can be classified into four different types, including: stirred tank (Fig. 6), airlift bioreactor (Fig. 7), fixed bed bioreactor (Fig. 8), membrane bioreactors (Fig. 9).

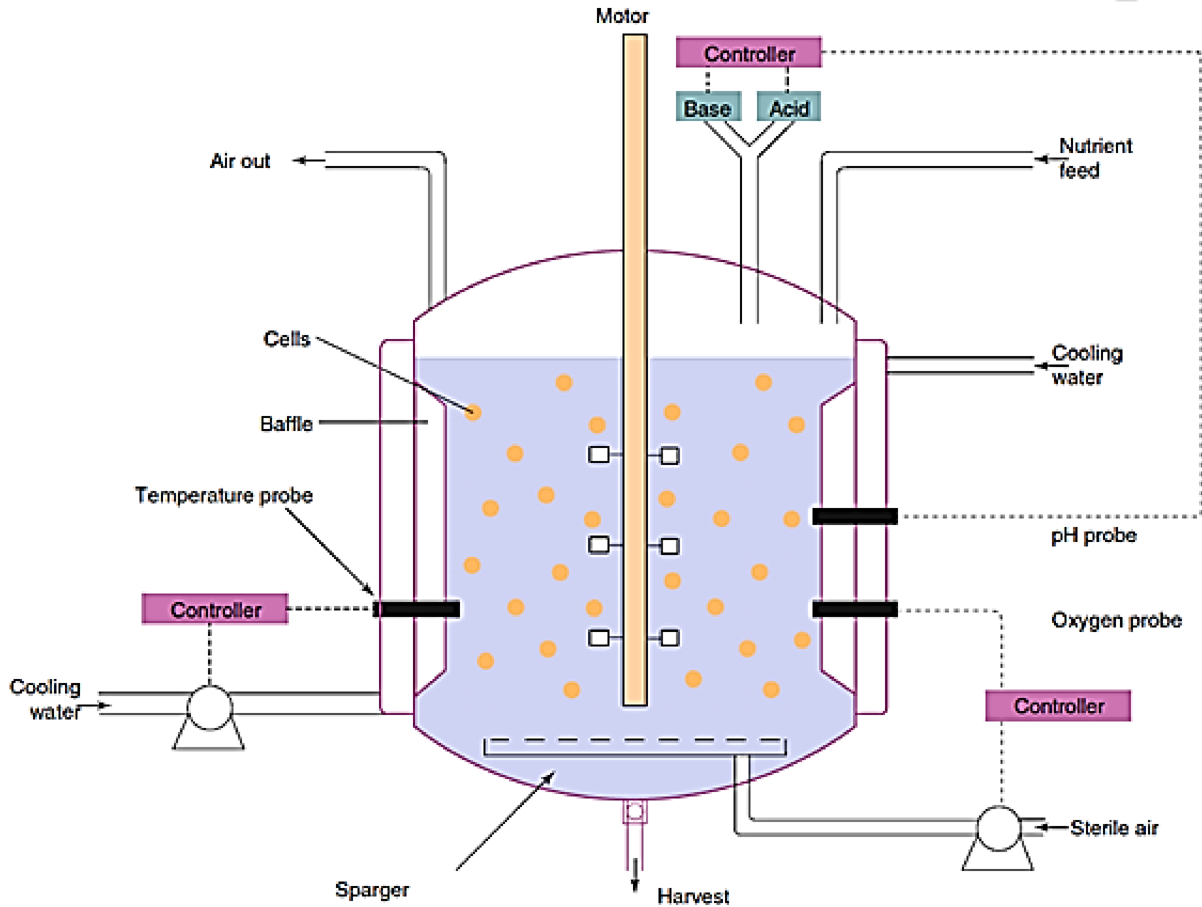

Figure 6. Stirred tank [1]

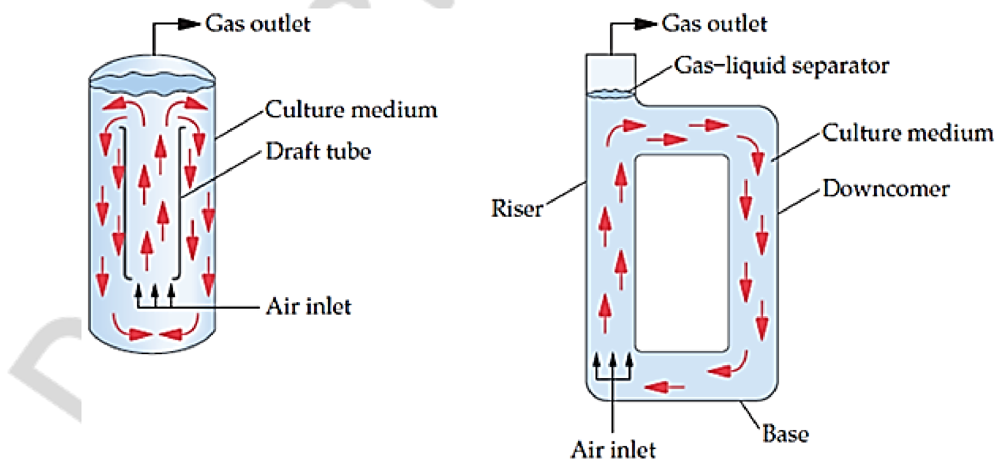

Figure 7. Airlift bioreactor [2]

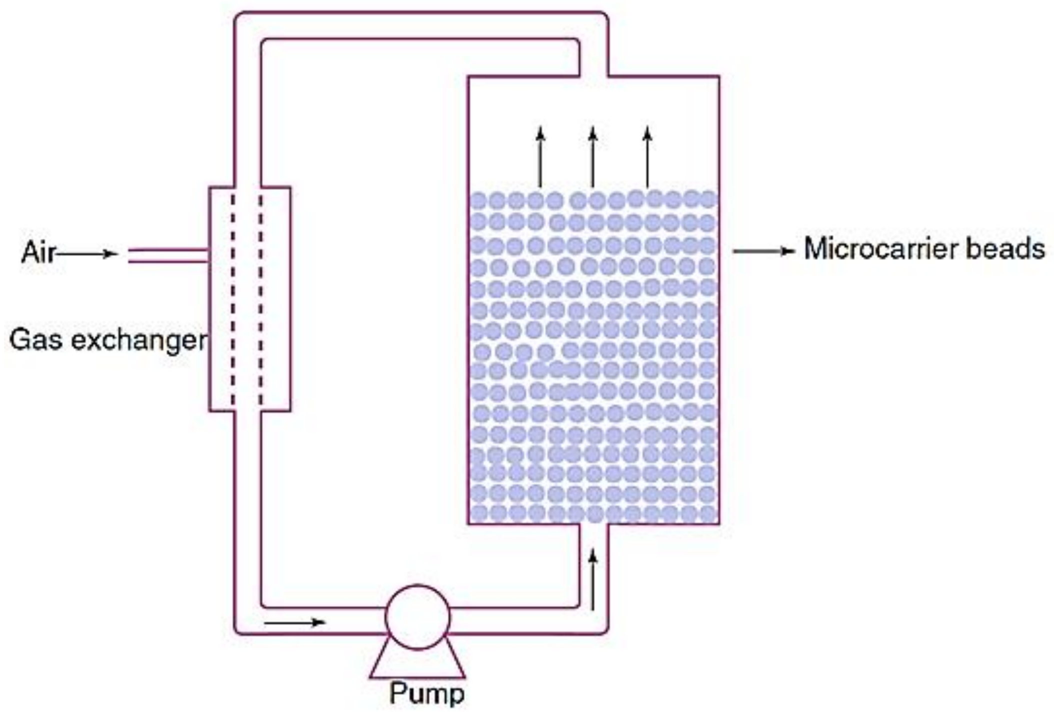

Figure 8. Fixed bed bioreactor [1]

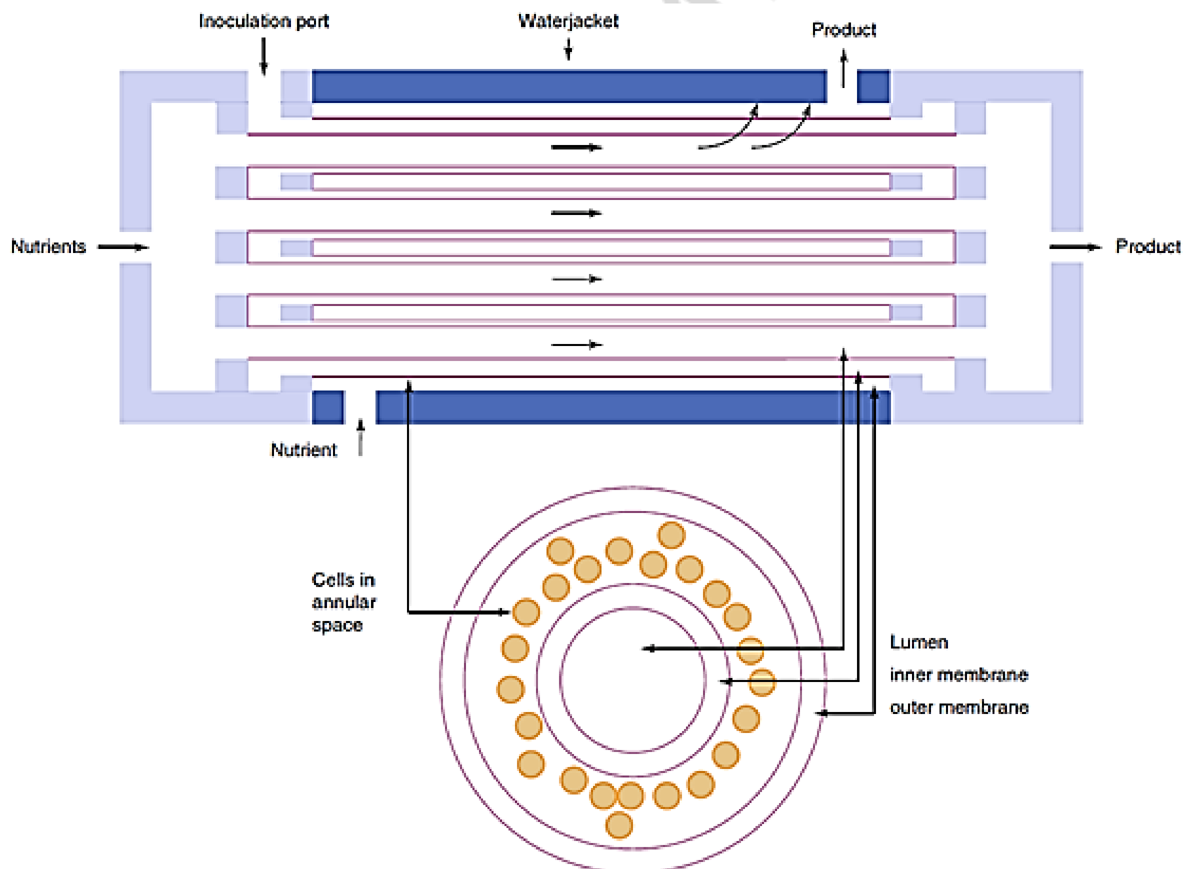

Figure 9. Membrane bioreactor [1]

SINGLE-USE SYSTEMS

In the last decade the development of single-use production systems was boosted.

Their advantages are as follows:

– Cost-effective manufacturing technology. Clean-in-place (CIP) and steam-in-place (SIP) systems can be removed.

– Increasing number of batches.

– Flexibility in facility design.

– Speedup implementation and time to bring them to the market.

– Reduction in water and wastewater costs.

– Reduction in validation costs.

The disadvantages may include:

– Increased number of the operational expenses.

– Increased storage location for single-use bags and tubing.

– Increased dependence of the company from one supplier of single-use systems.

CULTURING MICROORGANISMS

Media needs to be:

– cost effective;

– of high quality;

– easily available.

Sources of nutrition.

Carbon: sugarcane molasses, beet molasses, vegetable oil, starch, cereal grains, glucose, sucrose, lactose, malt, hydrocarbons.

Nitrogen: corn steep liquor, urea, ammonium salts, nitrate, peanut granules, yeast extract etc.

Growth factors: vitamins and amino acids are added when MO cannot synthesize them:

– Trace elements: Zn, Mo, Mn, Cu, Co required for metabolism.

– Inducers, precursors, repressors. For example streptomycin is induced by yeast extract.

– Antifoams: sunflower oil, olive oil to prevent foaming.

– Water: dissolves chemicals.

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MAMMALIAN CELL CULTURING

Table 3

Major components of growth media for mammalian cell structures

Type of nutrient	Example(s)
Sugars	Glucose, lactose, sucrose, maltose, dextrins
Fat	Fatty acids, triglycerides
Water (high quality, sterilized)	Water for injection
Amino acids	Glutamine
Electrolytes	Calcium, sodium, potassium, phosphate
Vitamins	Ascorbic acid, -tocopherol, thiamine, riboflavine, folic acid, pyridoxine
Serum (feat calf serum, synthetic serum)	Albumin, transferrin
Trace minerals	Iron, manganese, copper, cobalt, zinc
Hormones	Growth factors

Many of these ingredients are pre-blended either as concentrate or as homogeneous mixtures of powders.

To prepare the final medium, components are dissolved in purified water prior to the filtration.

The final medium is filtrated through 0.2 μm filters. But to prevent possible mycoplasma contamination 0.1 μm filters are required.

Some supplements, especially fetal calf serum, contribute considerably to the presence of contaminating proteins and may seriously complicate purification procedures.

Moreover, the composition of serum is variable. It depends on the:
– individual animal;
– season;
– suppliers’ treatment, etc.

The use of serum may introduce adventitious material such as viruses, mycoplasma, bacteria, and fungi into the culture system.

Examples of animal cells commonly used to produce proteins of clinical interest are the following:
– Chinese hamster ovary cells (CHO);
– immortalized human embryonic retinal cells (PER.C6 ® cells);
– baby hamster kidney cells (BHK);
– lymphoblastoid tumor cells (interferon production);
– melanoma cells (plasminogen activator);
– hybridized tumor cells (monoclonal antibodies).

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DOWNSTREAM PROCESSING

Fermentation takes place in an aqueous medium required for microorganisms. The fermenter may contain up to 95 % water and much effort has to be put to concentrate the product. There is a correlation between the concentration of a product in the broth and its price in the market place. The more diluted a product, the higher is its cost.

Apart from this there are many other problems in downstream processing. The product may be intracellular and the cells have to be disrupted to release the product. The fermenter fluid may be complex containing compounds resembling the product, which prevents its purification (for pharmaceutical products up to 99 % purity is required). These problems govern the approach used to separate the product. Usually the following steps are required:

– cell disruption (only in case of intracellular products);
– clarification (separation of the cells and cell debris from the liquid);
– concentration of the product stream;
– purification (often requires multiple steps);
– product formulation (giving the product a suitable form).

CELL DISRUPTION

Some products have to be released by cell disruption. Cell walls can be disrupted in several ways. These methods form two main groups: mechanical and non-mechanical. Non-mechanical methods are used on a small scale. Among them the most often applied are:

– drying (freeze drying, vacuum drying);
– osmotic shock (a change of ionic strength of the solution causing the cells to swell and burst);
– temperature shock;
– chemolysis (addition of surface active chemicals, solvents, antibiotics or enzymes to degrade the cell walls).

For large applications mechanical methods are used:

– ultrasonic disrupters;
– bead mills;
– homogenisers.

Prior to product concentration and purification cells or cell debris are removed. Clarification yields a clear liquid containing the dissolved product. Two major techniques are available: centrifugation and filtration. Large particles can be removed from the liquid by sedimentation but in biotechnology this is only used for large agglomerates.

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Filtration/Centrifugation are the most frequently used methods. However, the cost effectiveness and efficiency of such methods highly depend on the physical nature of the particulate material and the product.

CENTRIFUGATION

In many cases, total biomass can be easily separated from the medium by centrifugation. Larger and/or denser particles sediment more rapidly in the centrifugal field and thus form a pellet on the wall or the rotor floor faster than smaller or lighter particles, which tend to remain in the supernatant.

Continuous centrifuges are more efficient at the process scale. Here the feed stream is loaded into the centrifuge at a constant rate and the supernatant (containing the target protein) is continuously discharged while the waste pellet is either scraped intermittently from the centrifuge walls or removed continuously by a scrolling plate.

For influenza vaccines, continuous centrifugation is already for decades the workhorse to purify influenza viruses on an industrial scale.

FILTRATION

Centrifuges are effective in case of larger particles removal but for very small particles (known as “fines”) and colloids, filtration is preferred. However, filters are easily clogged by larger particles, so the format of a clarification module depends on the volume. Fermenter offloads of up to 3000 l often feature two depth filters in series to clarify the fermentation broth, whereas reactors with greater volumes usually employ a continuous centrifuge to remove the largest particles, followed by depth filtration and membrane filtration in series to remove smaller particles and fines. Filtration is any process in which a liquid feed stream moves over or through a selectively permeable medium so that only certain components of the feed emerge in the permeate or filtrate, while contaminants are retained in the retentate.

Microfiltration is the coarsest type of filtration in DSP, using media with pore sizes in the range 0.1–10 μm. This is suitable for removing suspended particulates but not for molecules separation (Table 4).

As a mechanical process, filtration requires a driving force across the filter medium, which in case of microfiltration is usually provided by a pressure differential. Microfiltration is often used for clarification. As it can be carried out at relatively low temperatures and pressures, and also because it requires no phase changes or chemical additives (therefore resulting in minimal denaturation of labile target proteins), it is suitable for application throughout the process.

Table 4

Different forms of filtration used in downstream processing

Method	Pore size	Retained	Applications
Microfiltration	100 nm – 10 µm	Cells, cell debris	Clarification, sterile filtration (fill and finish)
Ultrafiltration	10–100 nm (M_r 10^3–10^6)	Fine particles, viruses, large proteins	Clarification, virus clearance, size fractionation of proteins, concentration, diafiltration
Nanofiltration	1–10 nm ($M_r < 10^3$)	Nucleic acids, viruses, proteins	Purification of proteins, virus clearance
Reverse osmosis	0.1–1 nm ($Mr < 10^3$)	Salts, sugars	Water purification

Filter media can be divided into two major types: surface filters and depth filters (Fig. 10). Surface filters are essentially thin membranes containing capillary like pores. Particles or molecules that are too big to pass through the pores are retained on the membrane surface, that is, filtration is absolute at a certain particle — size cut — off. In contrast, depth filters have a bed of filter medium rather than a thin membrane, and particles are trapped into the interstices, which describe a convoluted path from one side of the filter to the other.

To increase the surface area available for filtration without increasing the footprint, depth filter pads are often supplied in a lenticular format comprising multiple filter pads pre-assembled inside a plastic casing. The materials used to construct depth filters include cellulose fibers, inorganic filter aids such as diatomaceous earth, resin binders, and synthetic polymers.

The filtration mechanism of membrane filters (Fig. 10, *a*) is absolute, with particles above a certain size rejected at the surface and smaller particles allowed to pass through to the permeate, whereas that of depth filters (Fig. 10, *b*) is not absolute, with particles becoming trapped into the internal matrix but only some getting through [3].

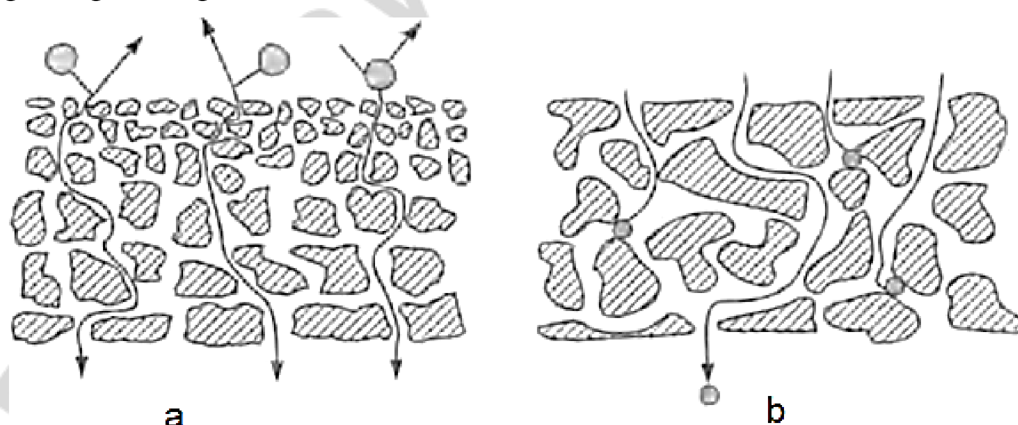

Figure 10. Comparison of membrane (*a*) and depth (*b*) filters

There are two main configurations of filter devices in downstream processing (Fig. 11). In dead-end filtration (also known as normal-flow filtration) the feed stream is perpendicular to the filter device, which is usually a membrane or pad. The filter device effectively blocks the feed, which must be forced through it under pressure. Because this configuration inevitably leads to the rapid build — up of retentate on the filter surface, it is used when the retentate load in the feed stream is expected to be low (e. g., pre-filters for chromatography devices, virus filters, and also sterile filters for product filling).

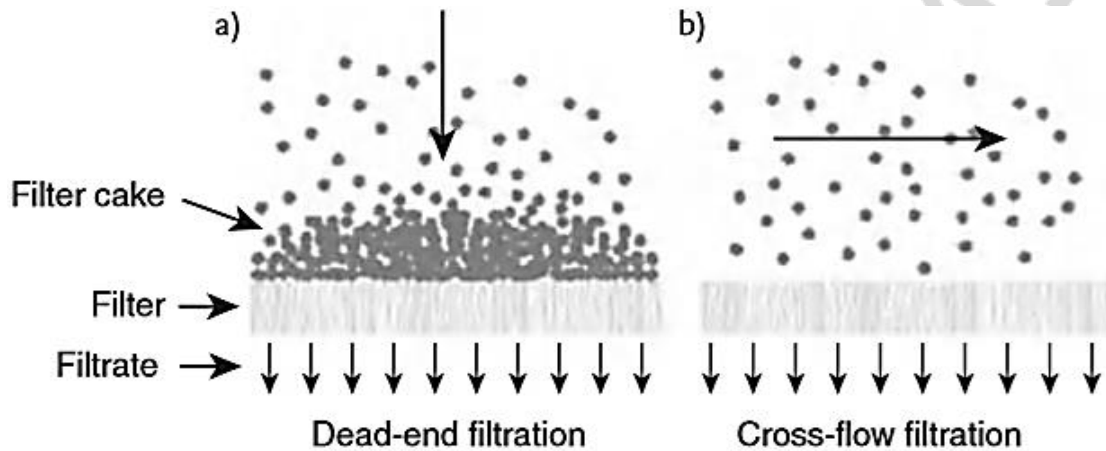

Figure 11. Comparison of (*a*) dead-end (normal flow) and (*b*) tangential (cross-flow) filtration. In each panel, the large arrow shows the direction of feed flow and the small arrows show the direction of permeate accumulation [3]

The preferred configuration for clarification is tangential-flow filtration (also known as cross-flow filtration), where the feed flow is parallel to the filter medium and thus perpendicular to the flow of permeate. This allows retained species to be swept along the filter surface and out of the device, helping to maintain high flux levels even with large amounts of retentate.

PRECIPITATION

The solubility of a particular protein depends on the physicochemical environment (e. g. pH, ionic species, and ionic strength of the solution). A slow continuous increase of the ionic strength will drive proteins out of solution. This phenomenon is known as “salting out”. A wide variety of agents, with different “salting-out” potencies are available. Chaotropic series with increasing “salting-out” effects of negatively (I) and positively (II) charged molecules are given below:

I. SCN^-, I^-, ClO_4^-, NO_3^-, Br^-, Cl^-, CH_3COO^-, PO_4^{3-}, SO_4^{2-}

II. Ba^{2+}, Ca^{2+}, Mg^{2+}, Li^+, Cs^+, Na^+, K^+, Rb^+, NH_4^+

Ammonium sulfate is highly soluble in cold aqueous solutions and is frequently used in “salting-out” purification.

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CHROMATOGRAPHY

Chromatography is often used for final purification of proteins and other pharmaceutical substances. There are the following types of chromatography:

– Adsorption Chromatography;
– Ion-Exchange Chromatography;
– Affinity Chromatography;
– Immunoaffinity Chromatography;
– Hydrophobic Interaction Chromatography;
– Gel-Permeation Chromatography;
– Expanded Beds.

Downstream protein purification protocols usually have at least from two to three chromatography steps.

For pharmaceutical applications product purity is mostly ≥ 99 %.

REVIEW OF THE COVERED ISSUES

1. How many experts are required for a commercial product production?
2. List the responsibilities of molecular biologists and biochemical engineers.
3. What is the fundamental objective of industrial fermentation?
4. Describe the industrial bioprocess.
5. What substances must a pharmaceutical biotechnology product include?
6. What expression systems are recommended for therapeutic protein production?
7. What criteria is the choice of a particular system determined by?
8. How are virus and virus-like particles normally expressed?
9. What parameters influence virus and virus-like particles titers?
10. What are the advantages and disadvantages of bacterial cells as the expression systems?
11. What are the advantages and disadvantages of fungal cells as the expression systems?
12. What are the advantages and disadvantages of transgenic animals as the expression systems?
13. What is protein concentration in different biological cells?
14. Is it possible to form S-S bridges in prokaryotic bacteria?
15. Is the glycosylation process possible in prokaryotic bacteria?
16. What does MOI mean?
17. What does TOI mean?
18. Describe the general scheme of a large scale fermentation process.
19. What are the principles of microbial growth?
20. Describe batch fermentation.

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21. Describe fed-batch fermentation.
22. Describe continuous fermentation.
23. List the phases of microbial cells growth in a batch fermenter.
24. What parameters should be controlled during fermentation?
25. What is the best density of cell culture for *E. coli*?
26. What types of bioreactors do you know?
27. What are the advantages and disadvantages of single use systems?
28. What are the requirements to media in case of microorganisms culturing?
29. What types of nutrients are required for mammalian cell culturing?
30. What steps does the downstream processing include?

FILL THE GAPS

1. The fundamental objective of industrial fermentations is to … costs and … yields.

2. The following process-related parameters strongly influence virus and VLP titers:

– multiplicity of infection;
– …
– …
– cell concentration in infection.

3. Generalized features of proteins of different biological origin.

Protein feature	Prokaryotic bacteria	Eukaryotic yeast	Eukaryotic mammalian cells
Molecular weight	…	High	…
Folding	Risk of misfolding	…	…
Glycosylation	…	…	Possible
Cost to manufacture	…	…	…

4. Examples of animal cells commonly used to produce proteins of clinical interest are as follows:

– Chinese hamster ovary cells (CHO);
– immortalized human embryonic retinal cells (PER.C6 ® cells);
– … (BHK);
– lymphoblastoid tumor cells (interferon production);
– … (plasminogen activator);
– hybridized tumor cells (monoclonal antibodies).

5. Bioreactor systems can be classified into four different types:

– …
– …

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– fixed bed;
– membrane bioreactors.

6. Media needs to be:
– cost effective;
– of high …;
– easily … .

7. Downstream processing may include:
– extraction;
– ultrafiltration;
– crystallization;
– …
– drying;
– … .

Choose the correct variant from the table below

Protein feature	Prokaryotic bacteria	Eukaryotic yeast	Eukaryotic mammalian cells
Concentration	Low / High	Low / High	Low / High
S-S bridges	Limitation / No limitation	Limitation / No limitation	Limitation / No limitation
Secretion	Yes / No	Yes / No	Yes / No
Impurities: pyrogen	Possible / No	Possible / No	Possible / No

Is the statement True or False:

1. In batch fermentation the sterile fresh growth medium is added. True/False.
2. In fed-batch fermentation the sterile fresh growth medium is added. True/False.
3. In continuous fermentation the sterile fresh growth medium is added. True/False.

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