Introduction to Fermentation

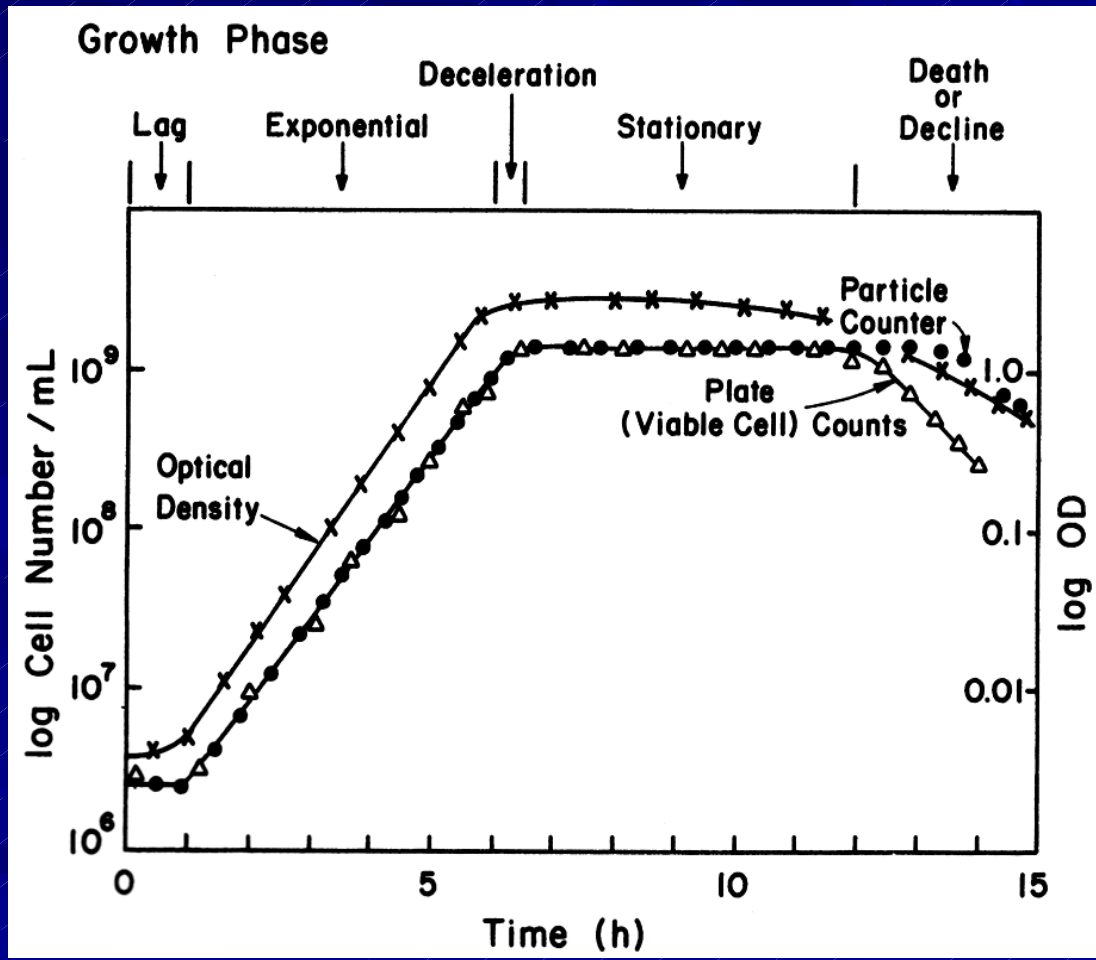
Genetically modified *Escherichia coli* have been chosen as the host organism for each of the co-proteins to be produced. Each strain of *E. coli* will contain a different gene that is responsible for producing the desired co-protein. The modified *E. coli* cells will be separately grown through the process of batch fermentation. This tutorial will introduce you to the following areas regarding batch fermentation:

- Microbial Growth Phases Associated with Batch Fermentation
  - Lag Phase
  - Exponential Phase
  - Stationary Phase
  - Death Phase
- The Stages of Batch Fermentation
  - Shake Flask
  - Seed Fermentor
  - Production Fermentor

Microbial Growth in Batch Fermentation

As the cells in a batch fermentation grow, they follow a growth curve similar to the one shown here. The growth curve contains four distinct regions known as phases. They are as follows:

1) Lag Phase
2) Exponential Phase
3) Stationary Phase
4) Death Phase



Growth curve is from Shuler p. 161.

Microbial Growth in Batch Fermentation

Lag Phase

- The first major phase of microbial growth in a batch fermentation process
- A period of adaptation of the cells to their new environment
- Minimal increase in cell density
- May be absent in some fermentations

Shuler p. 161-162.

Microbial Growth in Batch Fermentation

Exponential Phase

- The second major phase of microbial growth in a batch fermentation process
- Also known as the logarithmic growth phase
- Cells have adjusted to their new environment
- The cells are dividing at a constant rate resulting in an exponential increase in the number of cells present. This is known as the specific growth rate and is represented mathematically by first order kinetics as the following:

$$\frac{dX}{dt} = (\mu - k_d)X$$

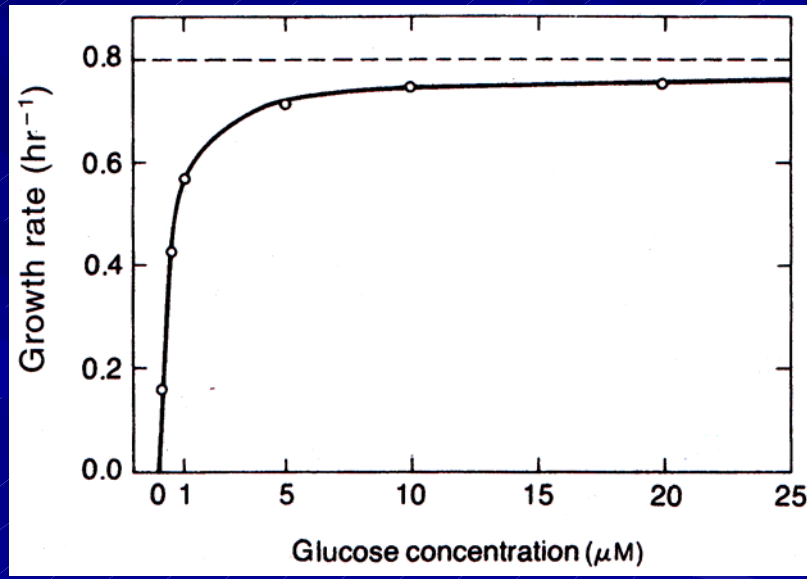
where X is the cell concentration, μ is the cell growth rate, and k_d is the cell death rate. The term $\mu - k_d$ can be referred to as μ_{net}. The cell death rate is sometimes neglected if it is considerably smaller than the cell growth rate.

Shuler p. 162-163.

Microbial Growth in Batch Fermentation

Exponential Phase (continued)

- Cell growth rate is often substrate limited, as depicted in the figure to the right.
- The growth curve is well represented by Monod batch kinetics, which is mathematically depicted on the following slide.



Shuler, p. 163.

Microbial Growth in Batch Fermentation

Exponential Phase (continued)

- Monod batch kinetics is represented mathematically in the following equation:

$$\mu = \frac{\mu_{max} S}{K_s + S}$$

where μ is the specific growth rate, μ_{max} is the maximum specific growth rate, S is the growth limiting substrate concentration, and K_S is the saturation constant which is equal to the substrate concentration that produces a specific growth rate equal to half the maximum specific growth rate. All specific growth rates account for the term $\mu - k_d$ and should be considered to be μ_{net} values.

Shuler p. 176.

Microbial Growth in Batch Fermentation

Exponential Phase (continued)

- There are other models used to determine cell growth rate that depend upon inhibition
  - Substrate Inhibition
  - Product Inhibition
  - Toxic Compounds Inhibition
- The type of inhibition causes mathematical changes in the previously presented Monod equation for batch kinetics

Shuler, p. 178-180.

Microbial Growth in Batch Fermentation

Exponential Phase (continued)

- Substrate Inhibition
  - In batch fermentation, this can occur during the initial growth phases while substrate concentrations are high
  - If this is a major problem, continuous or fed-batch fermentation methods should be considered
- Product Inhibition
  - In batch fermentation, this can occur after induction of the recombinant gene

Shuler, p. 178-180.

Microbial Growth in Batch Fermentation

Stationary Phase

- The third major phase of microbial growth in a batch fermentation process
- Occurs when the number of cells dividing and dying is in equilibrium and can be the result of the following:
  - Depletion of one or more essential growth nutrients
  - Accumulation of toxic growth associated by-products
  - Stress associated with the induction of a recombinant gene
- Primary metabolite, or growth associated, production stops
- Secondary metabolite, or non-growth associated, production may continue

Shuler p. 163-164.

Microbial Growth in Batch Fermentation

Death Phase

- The fourth major phase of microbial growth in a batch fermentation process
- Also known as the decline phase
- The rate of cells dying is greater than the rate of cells dividing
- Similar to Exponential phase, it is represented mathematically by first order kinetics as the following:

$$\frac{dX}{dt} = -k_d X$$

Shuler p. 164-165.

Microbial Growth in Batch Fermentation

There are a two main methods primarily used to establish a growth curve. Both of which are represented on the previously shown growth curve.

- Viable Cell Count
  - Initially lower curve representing the number of cells that are actually viable
  - Determined by plating a sample from the culture
- Optical Density
  - Initially higher curve representing the number of cells that are both viable and non-viable
  - Determined by taking an optical measurement using a spectrophotometer

Shuler, p. 161.

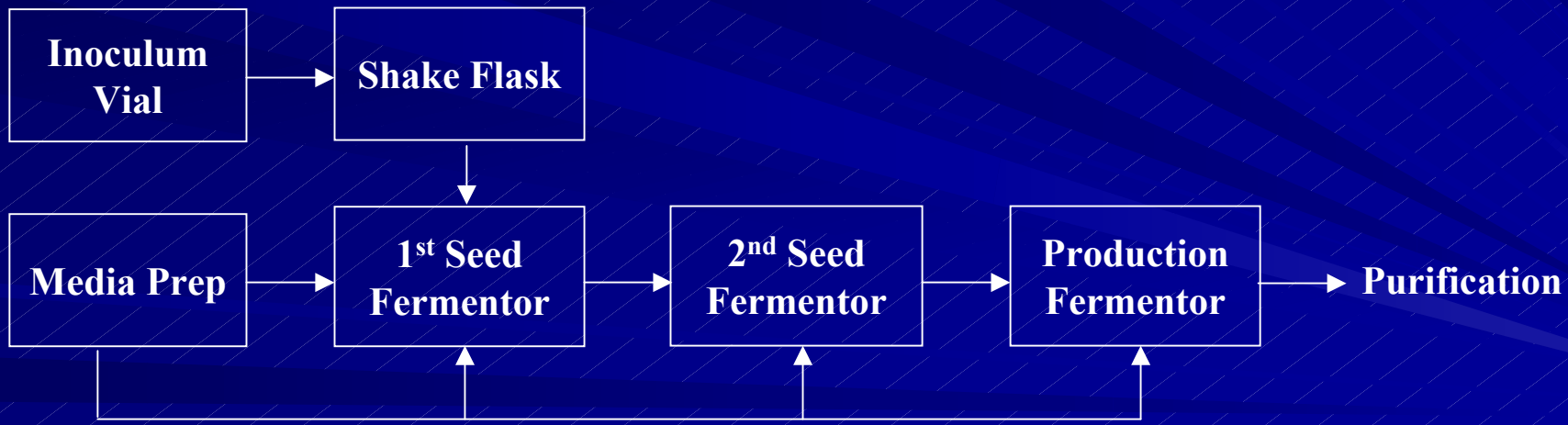
Microbial Growth in Batch Fermentation

Measuring the optical density with a spectrophotometer is a quick and easy way to to develop a growth curve. One takes a sample of the fermentation broth and measures the absorbance at a particular wavelength in the spectrophotometer. For *E. coli* cells in a typically LB medium, the wavelength used in 600 nm. The measured value can be compared to previous measurements made in conjunction with cell plating or cell counting. The negative side of using the optical density is that both viable and non-viable cells absorb this wavelength. As a result, the values taken are not representative of only viable cells.

Spectrophotometer pictured above is a copyright of Perkin Elmer

Batch Fermentation

Now that you understand how microbial cells grow in a batch process, it is time to see how a general biotechnology fermentation process works. An example, of a fermentation process is represented in the block flow diagram shown below. The different blocks depicted are described in detail in the following slides.



Batch Fermentation

First, a frozen vial containing a few milliliters of one recombinant *E. coli* strain is taken out of a freezer and thawed. This vial is sometimes referred to as an inoculum vial and its' contents is known as an inoculum.

After thawing, the inoculum is transferred in a sterile manner to a shake flask containing growth media. This process is known as inoculation. For *E. coli*, the initial pH of the media is typically around 7 and is controlled by using a buffering agent in the media. A picture of a shake flask is depicted to the right. The volume of media in the shake flask is usually on the order of magnitude of hundreds of milliliters.

Shake flask photo above is a copyright of Kimax Kimble USA

Batch Fermentation

After inoculation, the shake flask is placed in an incubator shaker so the cells can grow and reproduce. The shaker is operated at a constant temperature, which is around 37 °C for *E. coli*. The shake flask holders in the shaker are attached to an orbital plate that rotates horizontally at a programmable rate. This shaking motion has two purposes:

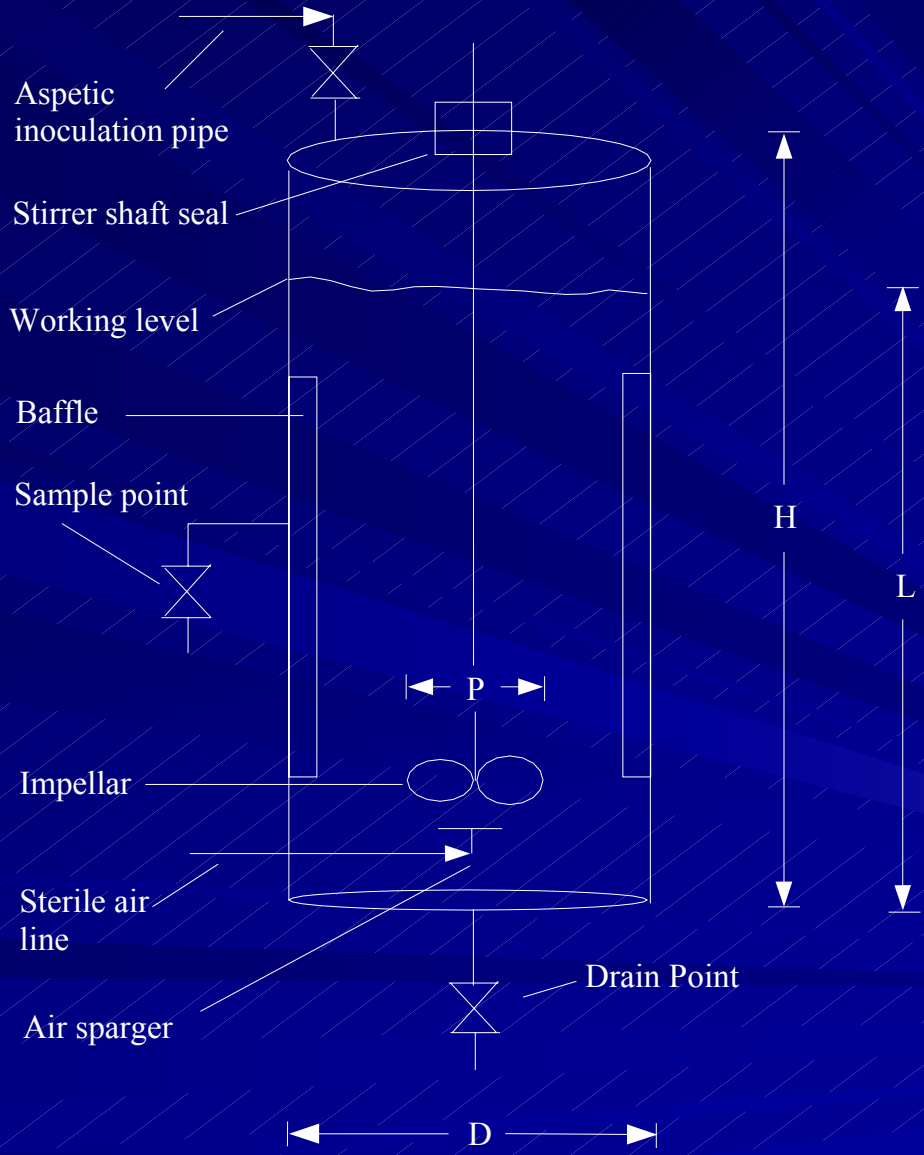
- Keep the cells and the nutrients in the growth media homogeneous
- Increases the rate of oxygen uptake by the media for the aerobic *E. coli* cells. The cells are grown to a particular density near the end of their exponential phase and used to inoculate a small fermentor known as a seed fermentor.

Incubator shaker photo above is a copyright of New Brunswick Scientific

Batch Fermentation

A schematic of a fermentor is shown on the following slide. It is representative of both a seed and a production fermentor. The *E. coli* cells are supplied with filtered oxygen through the sparger located at the bottom of the fermentor. The agitator is used to keep the mixture of cells and growth media inside the fermentors relatively homogeneous. It also increases oxygen mass transfer by decreasing the size of the oxygen bubbles. The fermentor is operated at a constant growth temperature to achieve the required growth rate. Since cells liberate heat during growth, a constant temperature is maintained using either cooling jackets surrounding the fermentors, coils inside the fermentor, or a combination of both. In addition, the cells secrete acids as they metabolize, which decrease the pH level within the fermentor. As a result, a base is usually added to the fermentor whenever the pH drops below its optimum value.

Batch Fermentation

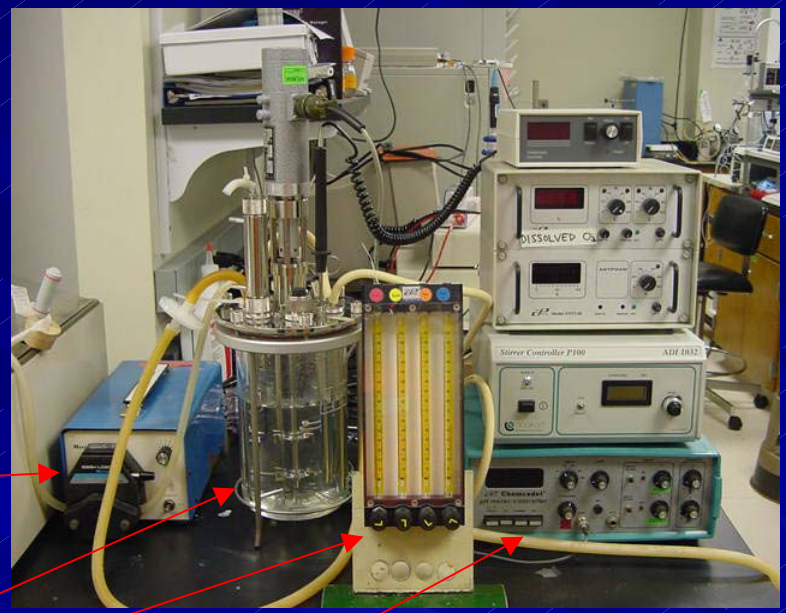


Fermentor schematic is adapted from Stanbury, p. 168.

Batch Fermentation

Once the cells are transferred to the seed fermentor, they are grown to a particular density near the end of their exponential phase. The picture presented to the right is of a 2.2 L glass laboratory scale seed fermentor. The devices associated with the fermentor and their function are listed from left to right:

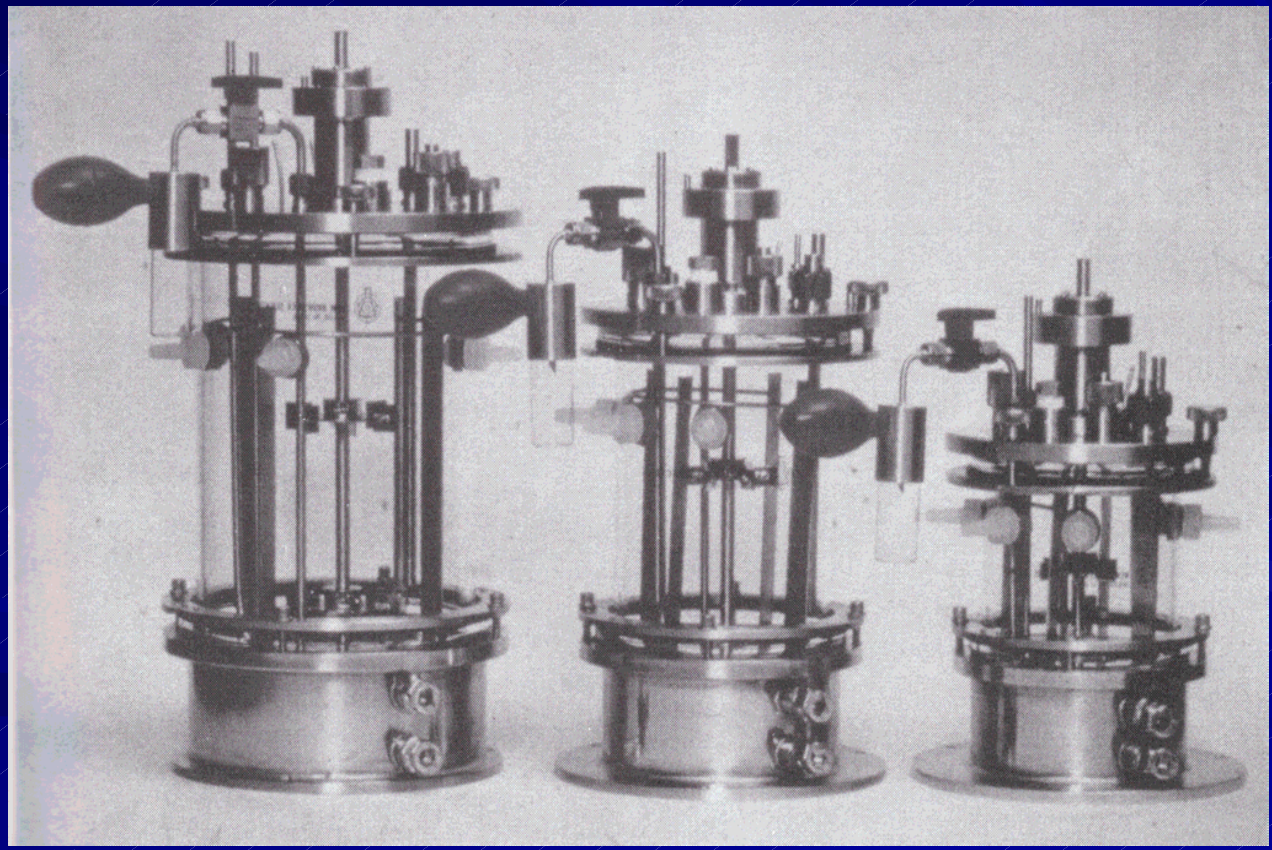
- Peristaltic Pump for pH control through base addition
- Fermentor for cell growth
- Valves for oxygen flow rate
- Electronic devices for pH and dissolved oxygen measurements and controls for agitator speed



Fermentor and associated equipment in the photo above is a copyright of Applikon, Cole Parmer, and Chemcadet.

Batch Fermentation

This picture shows several different sized laboratory scale fermentors.



Fermentors picture is from Stanbury, p. 173.

Batch Fermentation

After the cells reach the required optical density in the seed fermentor, the cells can either be used to inoculate several increasingly larger seed fermentors until the required volume and density is reached, or the cells can be transferred directly to the production fermentor to where they will eventually synthesize the co-protein. Typically, genetically engineered E. coli cells are grown to a particular volume and density through a series of increasingly sized fermentors. This group of seed fermentors is sometimes referred to as a seed train.

Batch Fermentation

After the cells reach their required volume and density, they are transferred to the production fermentor where they are grown to a particular density. The density in which they are grown to depends upon the desired product being growth or non-growth associated. For growth associated, the cells are grown to their mid to late exponential phase. At this point, a chemical is added that induces the cells to begin over-expressing the gene responsible for the recombinant protein. The over-expression of the particular gene and the depletion of nutrients eventually cause the cells to enter their stationary growth phase. At this point, the cells are no longer capable of producing appreciable amounts of the desired protein and the fermentation is ended.

14 L Fermentor photo above is a copyright of New Brunswick Scientific

Fermentation Conclusion

Now that the fermentation process is over, the fermentation broth containing the cells and the extracellular media is removed from the production fermentor. This is called harvesting and that completes the upstream process of fermentation. After the cells are harvested, the recombinant protein needs to be separated from the cells that produced them. This is accomplished through the downstream process of purification.

Fermentation Conclusion

The following is a list of references that can further explain the topics discussed in this tutorial:

- Bailey, J. E. and D. F. Ollis, *Biochemical Engineering Fundamentals*, 2nd ed., McGraw-Hill Book Co., New York, 1986.
- Brown, T. A., *Gene Cloning and DNA analysis*, 4th ed., Blackwell Science Ltd, Oxford, 2001.
- Shuler, M. L. and F. Kargi. *Bioprocess Engineering Basic Concepts*, 2nd ed., Prentice Hall, Upper Saddle River, NJ, 2002.
- Stanbury, P. F., A. Whitaker, and S. J. Hall, *Principles of Fermentation Technology*, 2nd ed., Butterworth Heinemann, Oxford, 2000.

This concludes the upstream biotechnology process known as fermentation and brings us to the end of the fermentation tutuorial. Please proceed to the Purification Tutorial for information regarding downstream processing.