Discovery Research and Cell Culture

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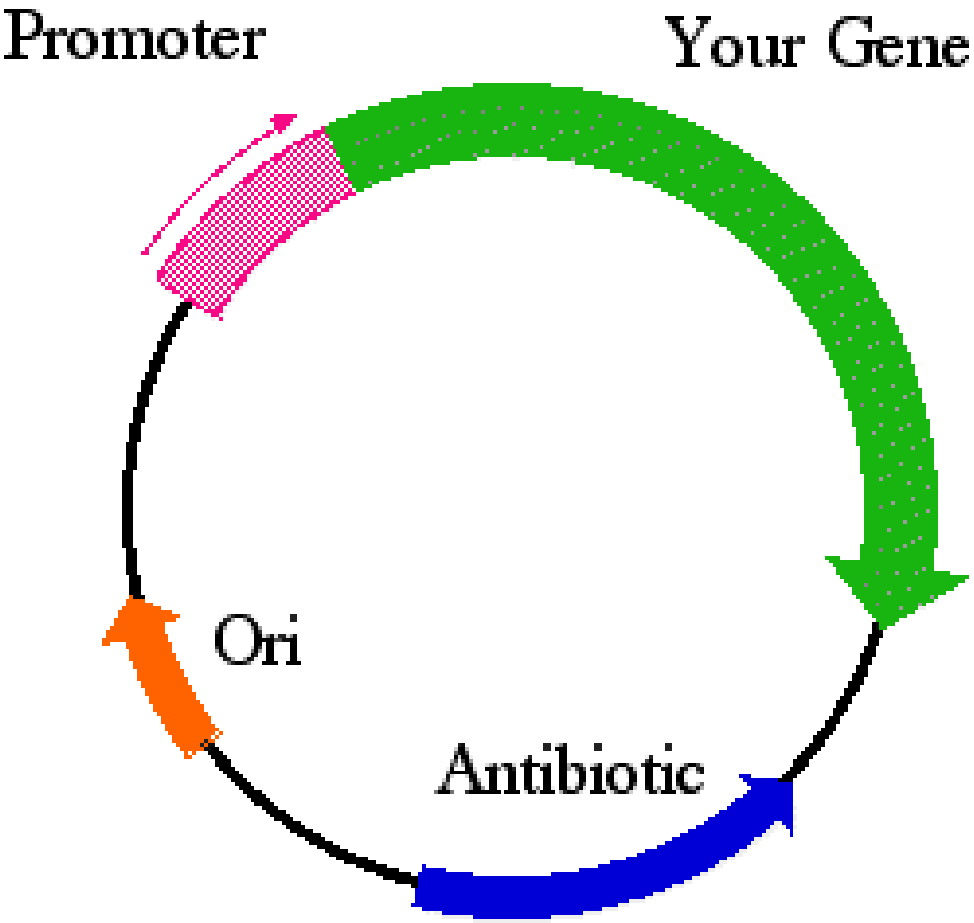
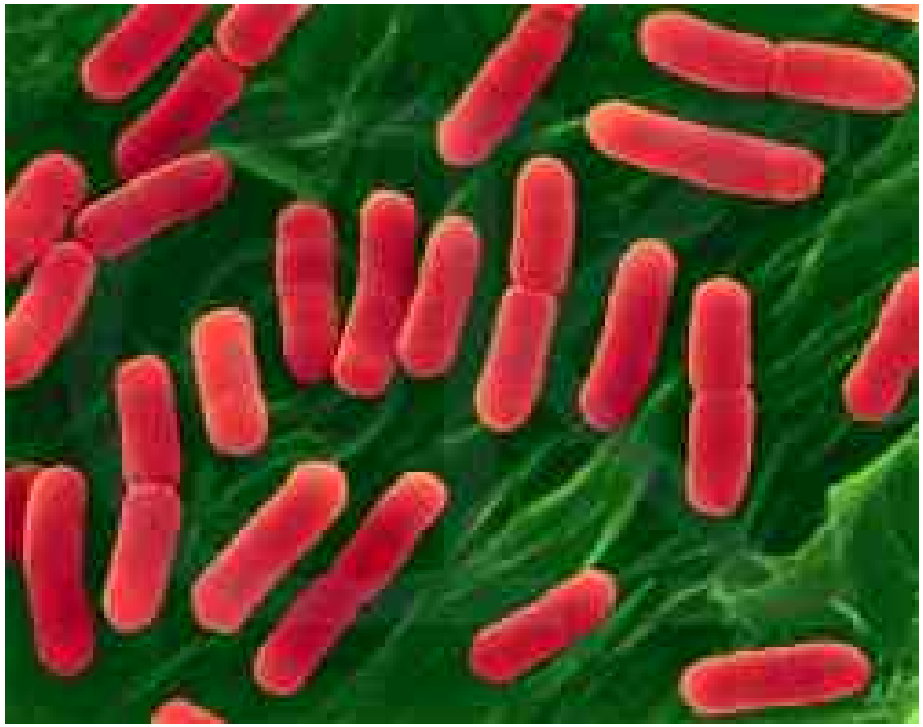
The Expression Vector: The Basis of Biotechnology Manufacturing

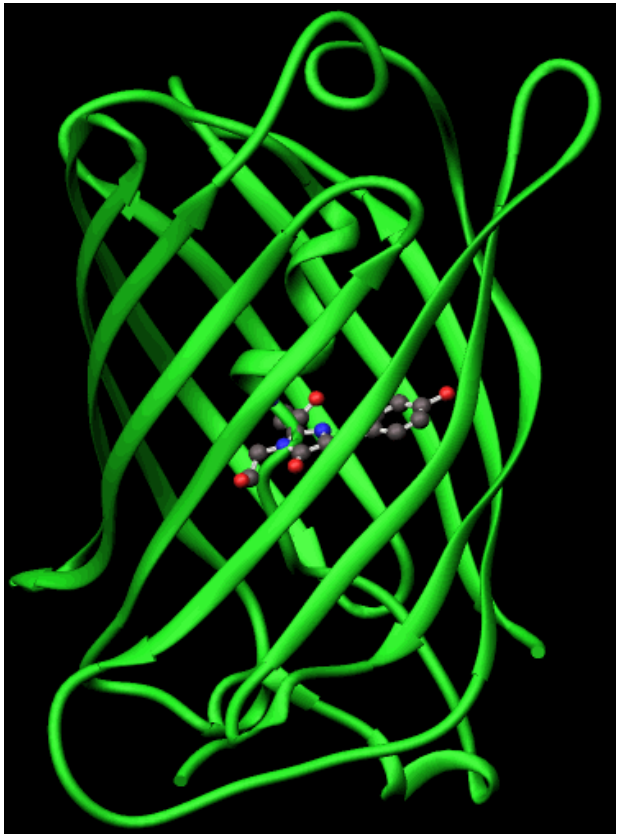


Escherichia coli – GFP

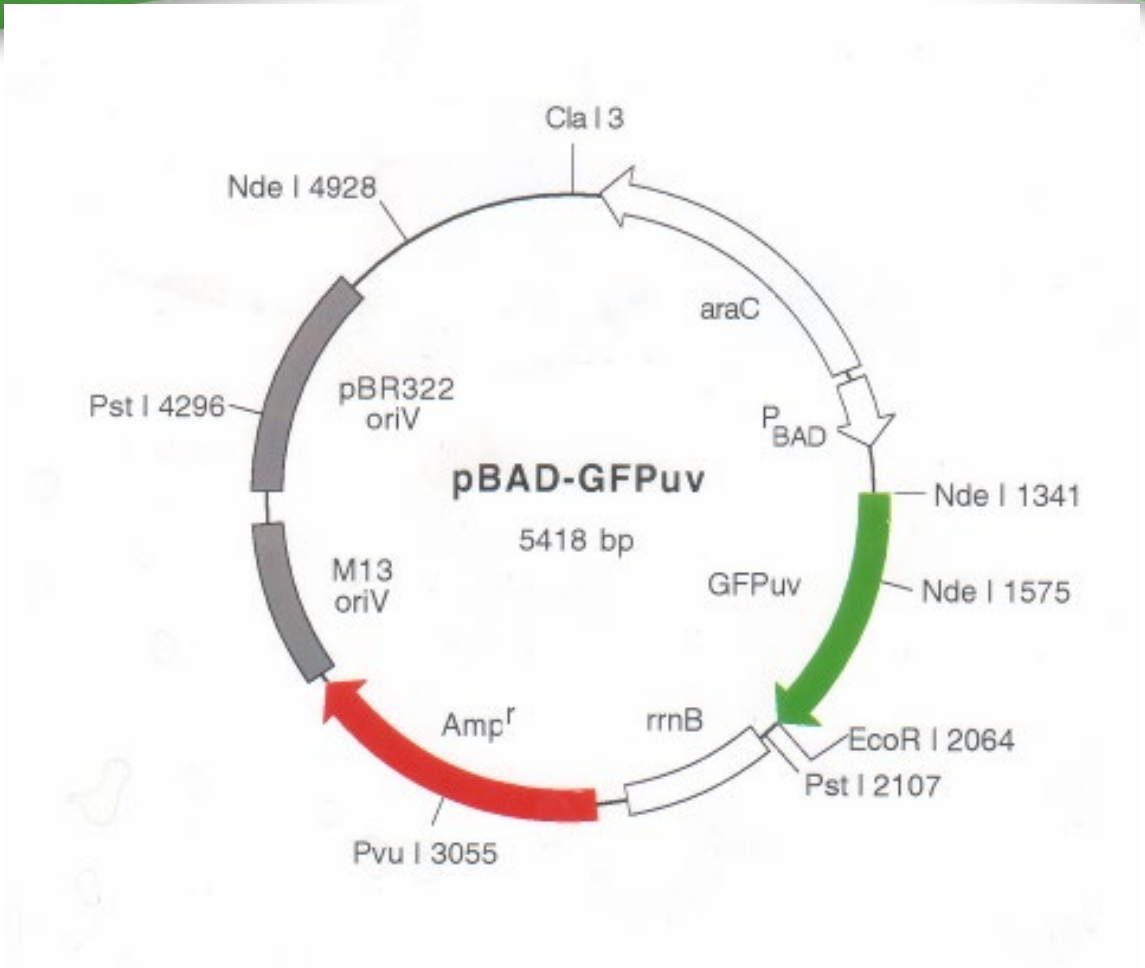
Escherichia coli



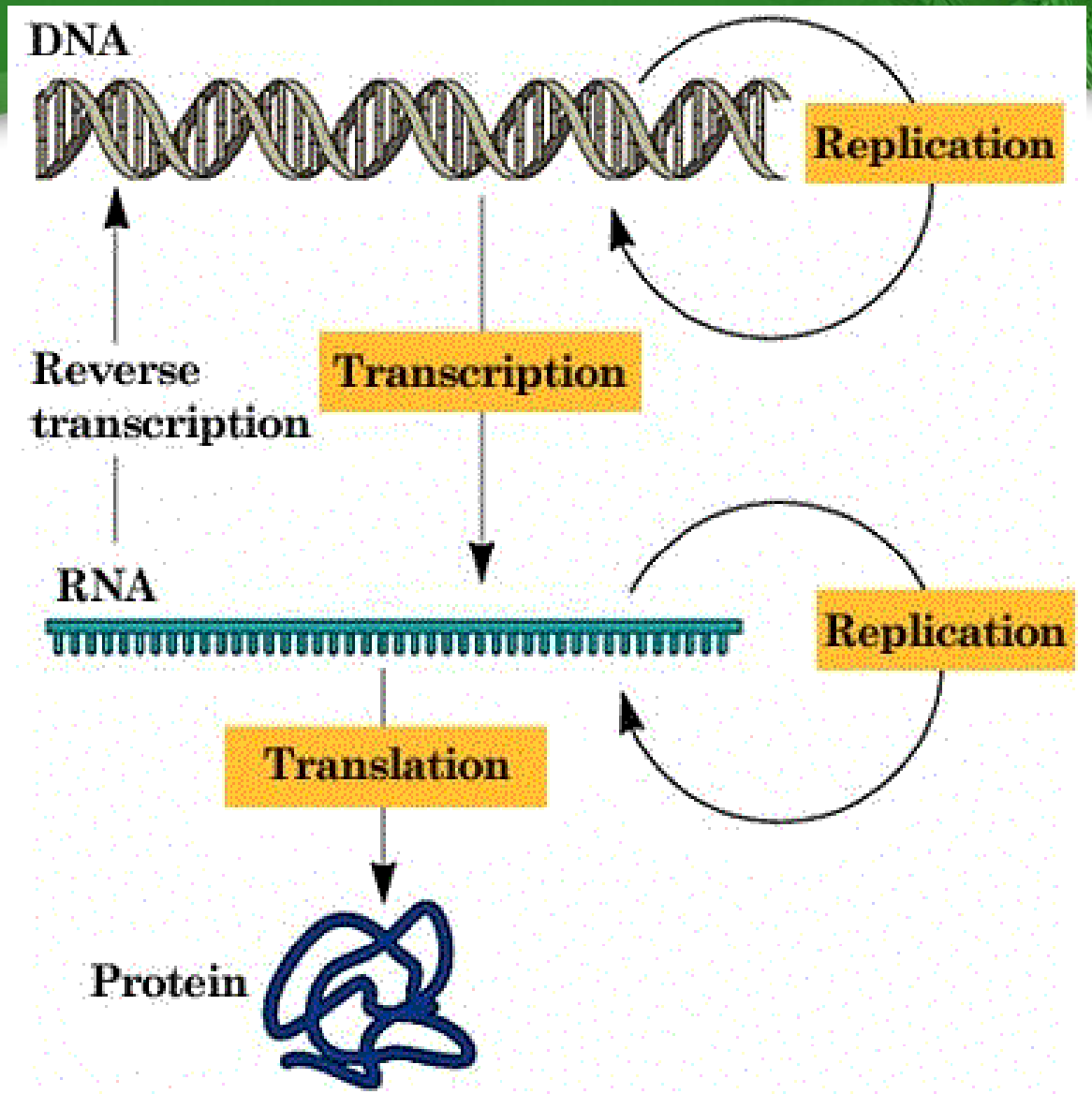
Green Fluorescent Protein



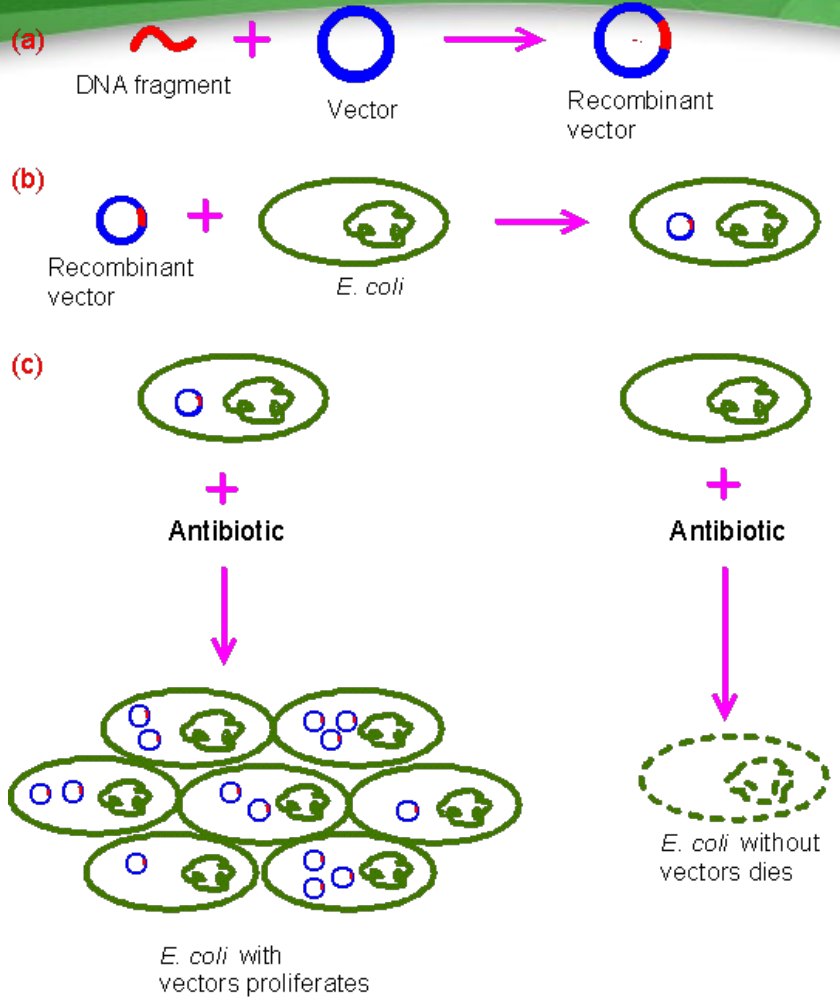
GFP Expression Vector



Central Dogma of Biology

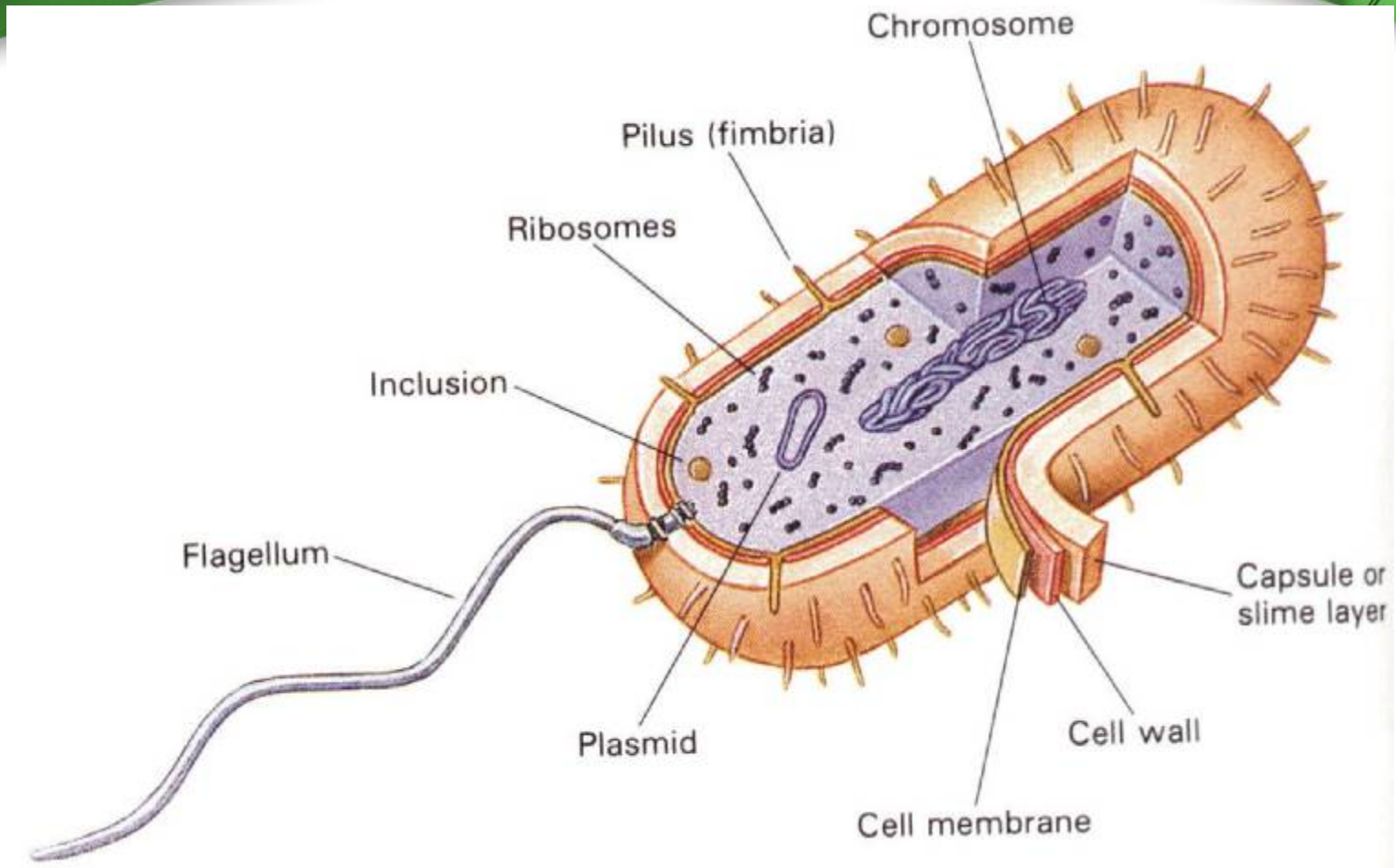


Transformation and Cloning



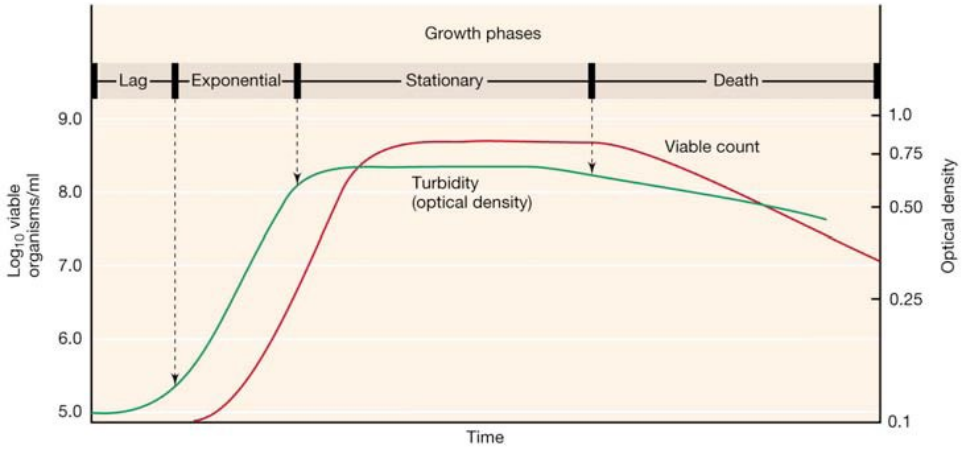
(d) **Isolation of recombinant DNA clones**

Escherichia coli

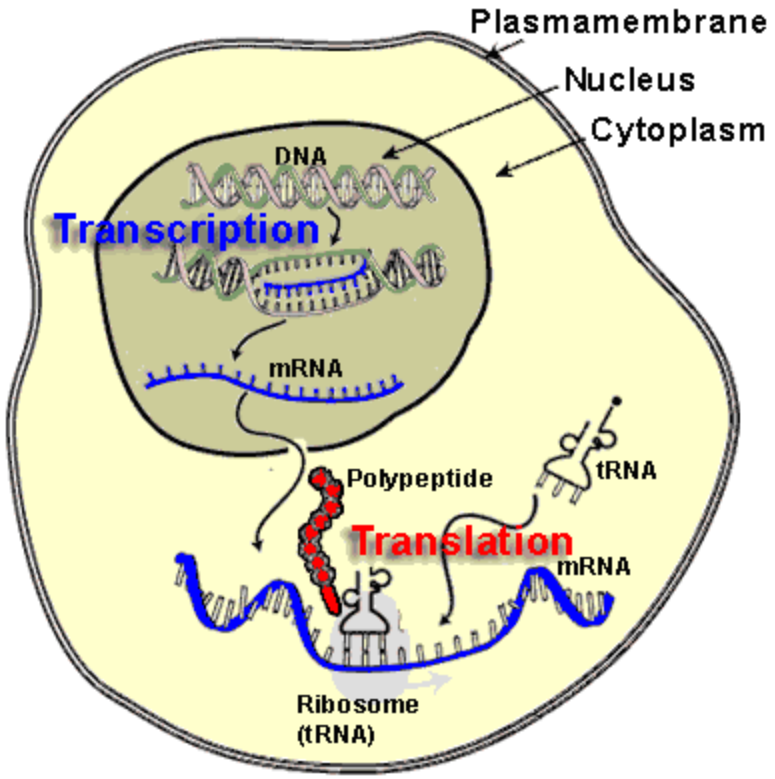


Two Stages to Production – Both Require ATP

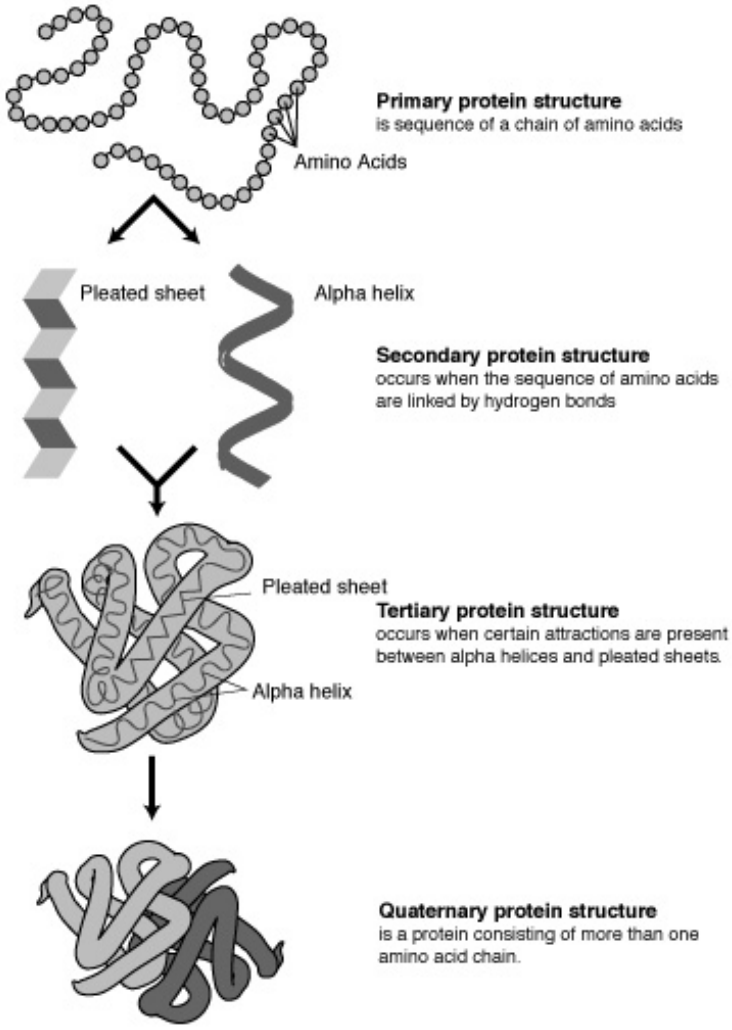
Cell Growth and Reproduction



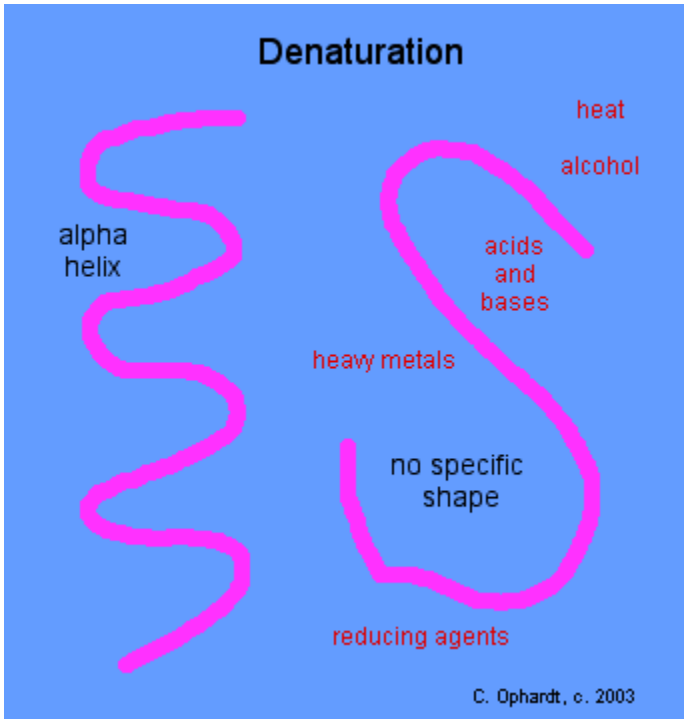
Synthesis Protein of Interest

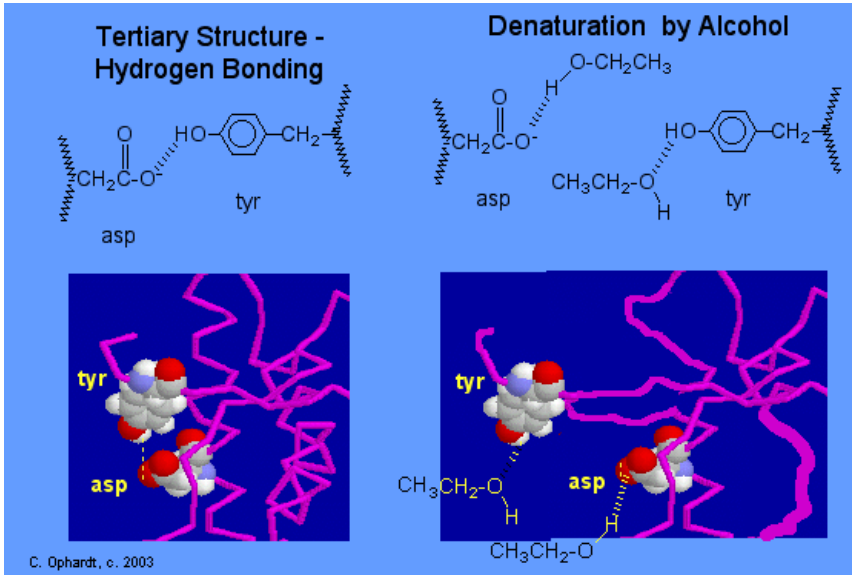


Four Levels of Organization of Protein Structure

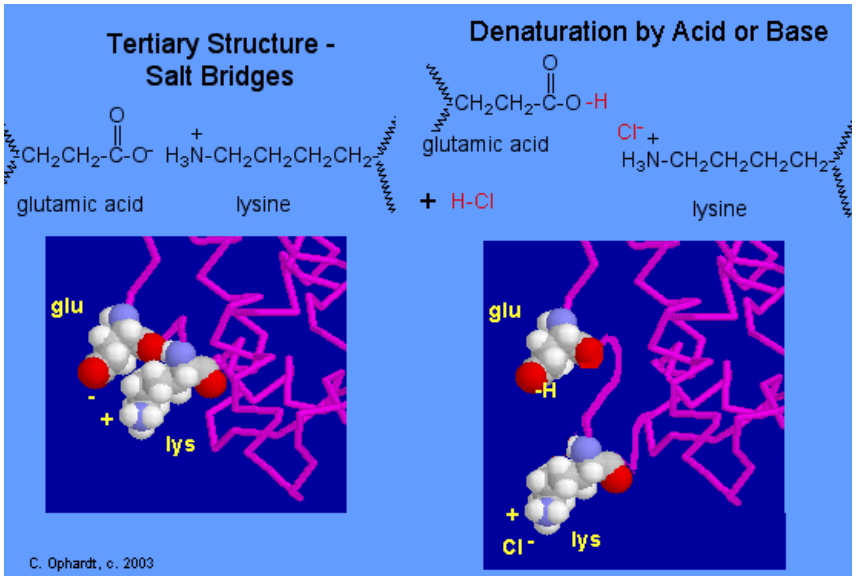


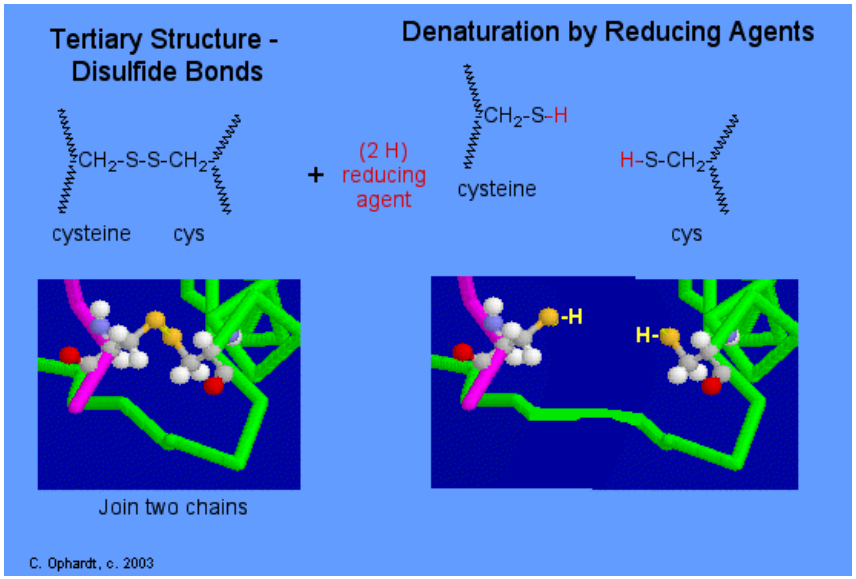
Denaturation





Denaturation





Proteins are the Machines and form the Structure of Life

Proteins are used by the body for a whole host of things, e.g. within blood (for carrying molecules and for clotting), for digestion (enzymes are proteins), for movement (actin and myosin in muscle), etc. One other major role of proteins is that of "structural proteins", i.e. those proteins that contribute to and sustain the integrity of the human structure. Collagen is a structural protein.

Proteins

- Hormones like human growth hormone and insulin
- Enzymes like lipase and protease
- Receptors for neurotransmitters, hormones, and transferrin
- Signal transduction proteins (produce cascades)
- Carrier proteins (for HDL, LDL, and iron)
- Membrane proteins (ion chanels)
- Immunoglobulins (antibodies)
- Blood Proteins: albumin, transferrin, factor VIII
- DNA Transcription Factors
- Actin and myosin
- Hemoglobin
- Structural proteins like collagen, elastin, spectrin

Animation of Signal Transduction Pathway involving Multiple Proteins

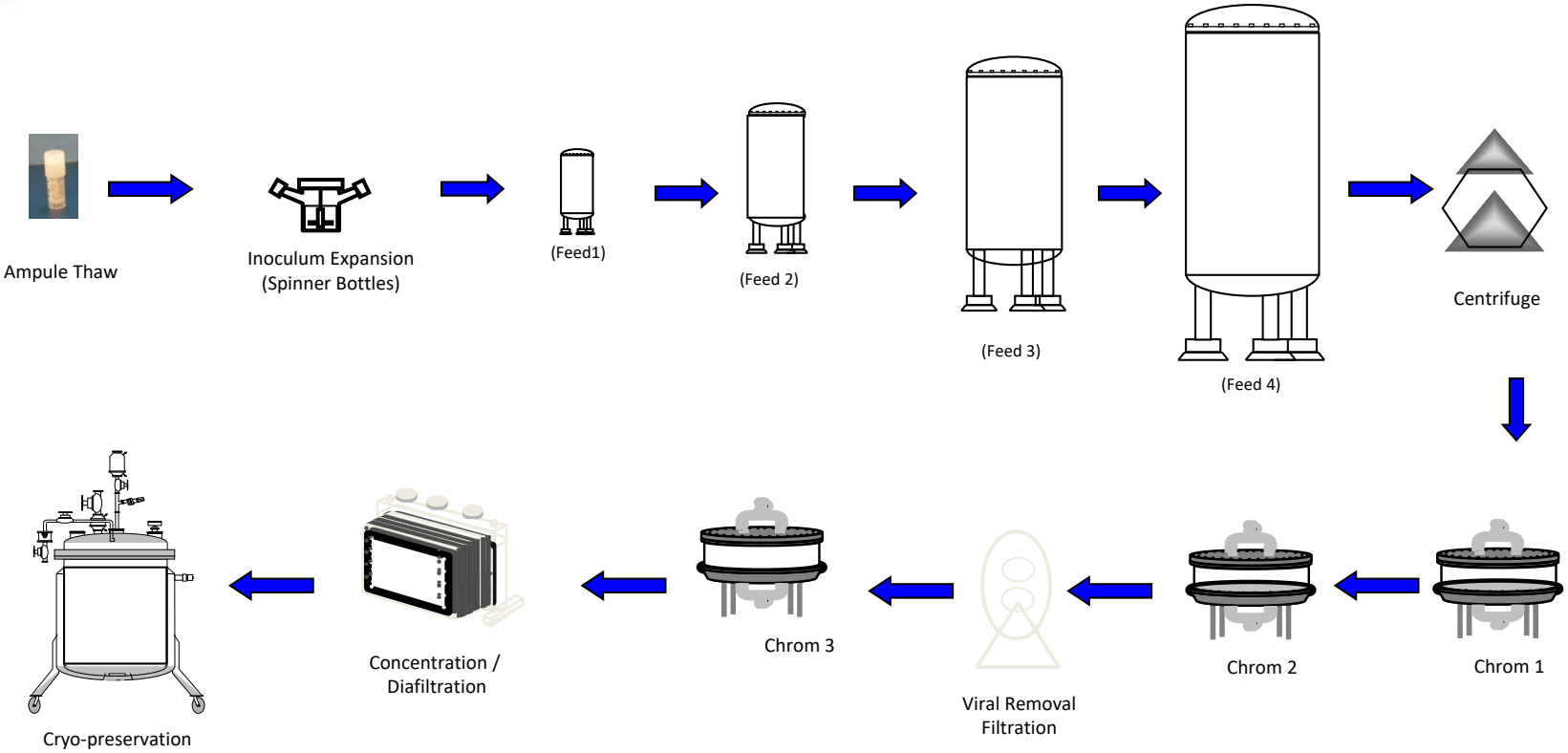
http://www.learner.org/courses/biology/archive/animations/hires/a_cancer1_h.html

Best Site for PROTEIN Research

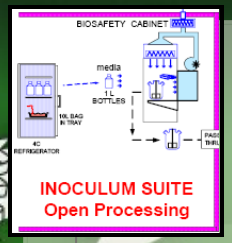
www.drugbank.ca

NBC²

Typical Production Process Flow



Upstream/Downstream Manufacturing Overview



Working Cell Bank

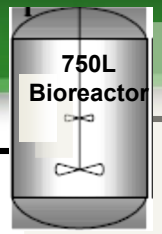
Media Prep

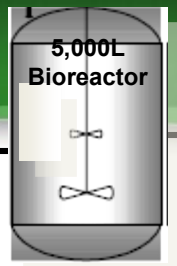
Large Scale Bioreactor

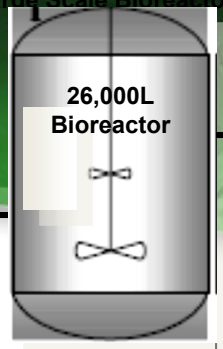
1 day

Seed Bioreactors

Sub-Culture | Sub-Culture | Sub-Culture | Sub-Culture | Sub-Culture







Centrifuge

Depth Filtration

Collection

Inoculum
24 days

Fermentation
31 days

Harvest/Recovery

Harvest Collection Tank 1,500L

Filter

Chromatography Skid

Column

Eluate Hold Tank 8,000L

Anion Exchange Chromatography (QXL)

Chromatography Skid

Column

Eluate Hold Tank 20,000L

Viral Inactivation

Hydrophobic Interaction Chromatography (HIC)

Purification

Filter

Chromatography Skid

Column

Protein A Chromatography

Eluate Hold Tank 6,000L

Viral Filtering

Post-viral Hold Vessel 3,000L

Filter

Chromatography Skid

Column

Anion Exchange Chromatography (QFF - Fast Flow)

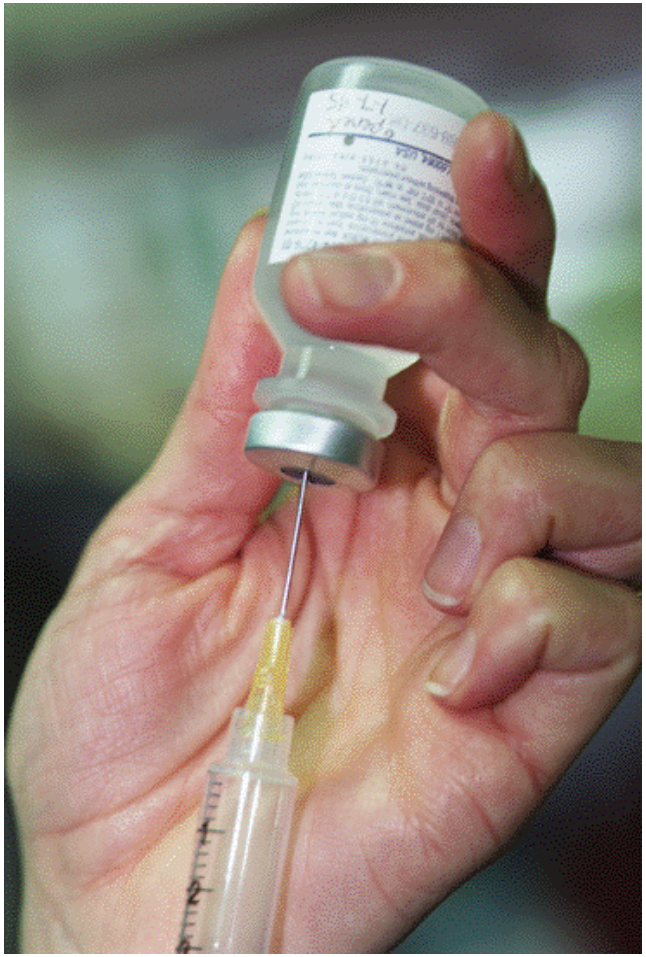
Eluate Hold Tank 5,000L

Ultra Filtration Diafiltration

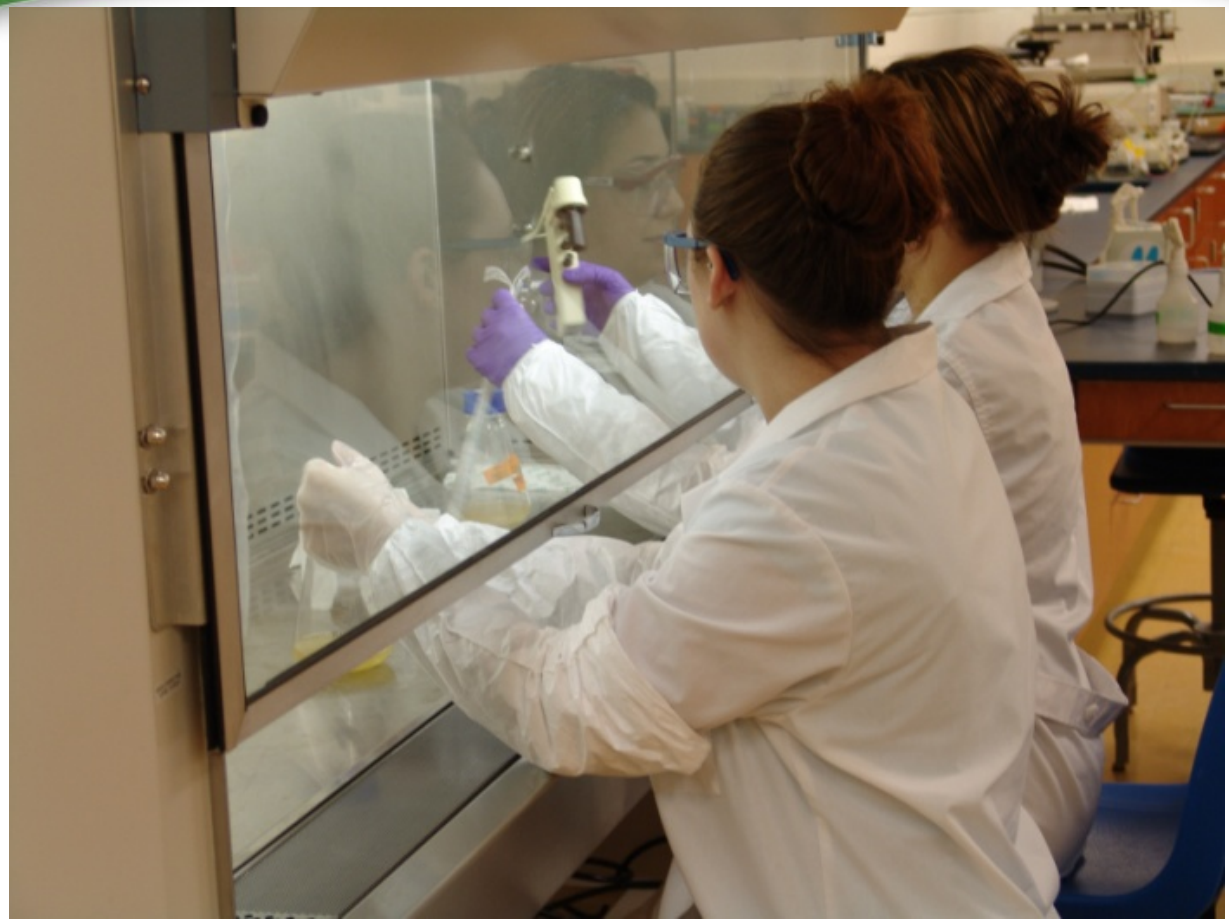
Bulk Fill

8 days

Biopharmaceutical Proteins are Parenteral



Use Aseptic Technique in Clean Rooms



Shake Flask Inoculation using BSC Class 100 (5)

Media Preparation for Cell Growth and Protein Expression

Feeding → *Doubling of Cells and Synthesis of Protein*

Media and Feeds Support each Stage

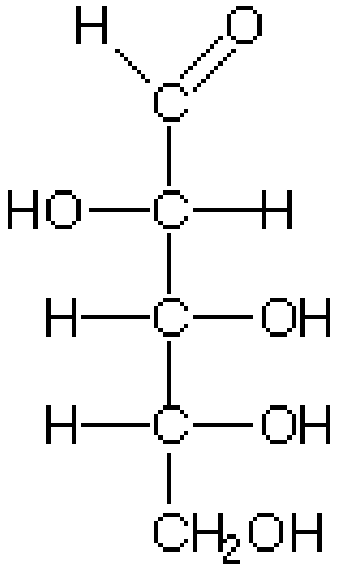
- *E. coli* media requires some chemicals and non-defined components (hydrolyzed protein and yeast extract) to grow a batch and an inducer to produce the protein of interest. This is the cheapest medium.
- CHO cells require complex medium containing all 20 amino acids, fatty acids, and carbohydrates. Growth media requires 10% fetal bovine serum (FBS) but can be weaned to a serum-free medium. Most expensive medium.
- *Pichia pastoris* requires chemicals and non-defined components (hydrolyzed protein, yeast extract and yeast nitrogen base) to grow a fed-batch and an inducer (methanol) to express large quantities of the protein of interest.

Escherichia coli (Prokaryot)Media

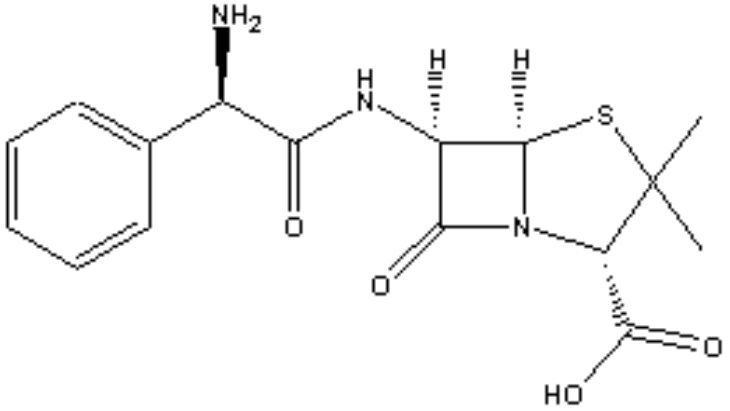
LB Broth with Arabinose

NaCl

Yeast Extract



Arabinose – The Inducer



Tryptone or Peptone

Yeast Extract

The main components of yeast extract are:

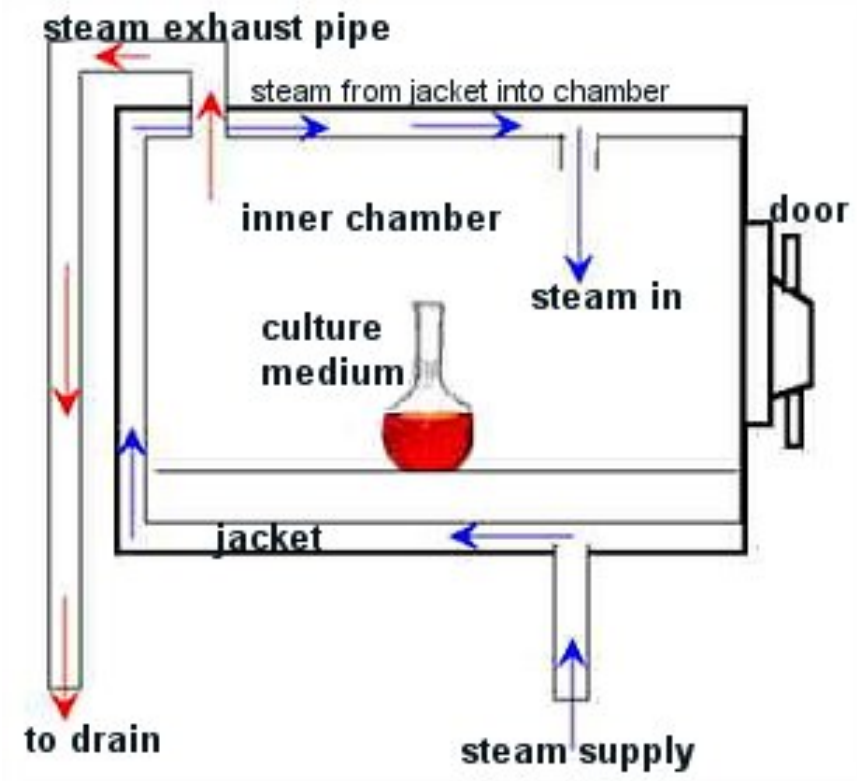
- total nitrogen content : 8 to 12 %, corresponding to a protein content of 50 to 75 %
- amino nitrogen content : 3.0 to 5.2 %
- total carbohydrate content : 4 to 13 %
- lipid content : none or very little.

Click here to see how yeast extract is made:

http://www.eurasyp.org/public.levure.extrait.screen

Sterilizing Media/Solutions
Goal: To remove microbial contamination (bioburden)

Autoclave

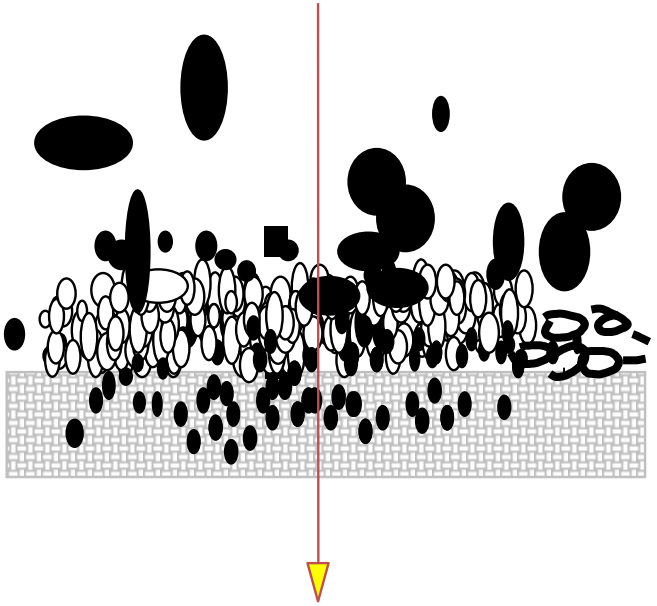


Sterile Filtration (.22u pores remove bacteria)

Sterilization by Filtration at .22u Normal Flow Filtration : (NFF)

- Build up of retained components on filter surface and within filter matrix.
- NFF is robust and easy-to-use

Normal Flow (NFF)

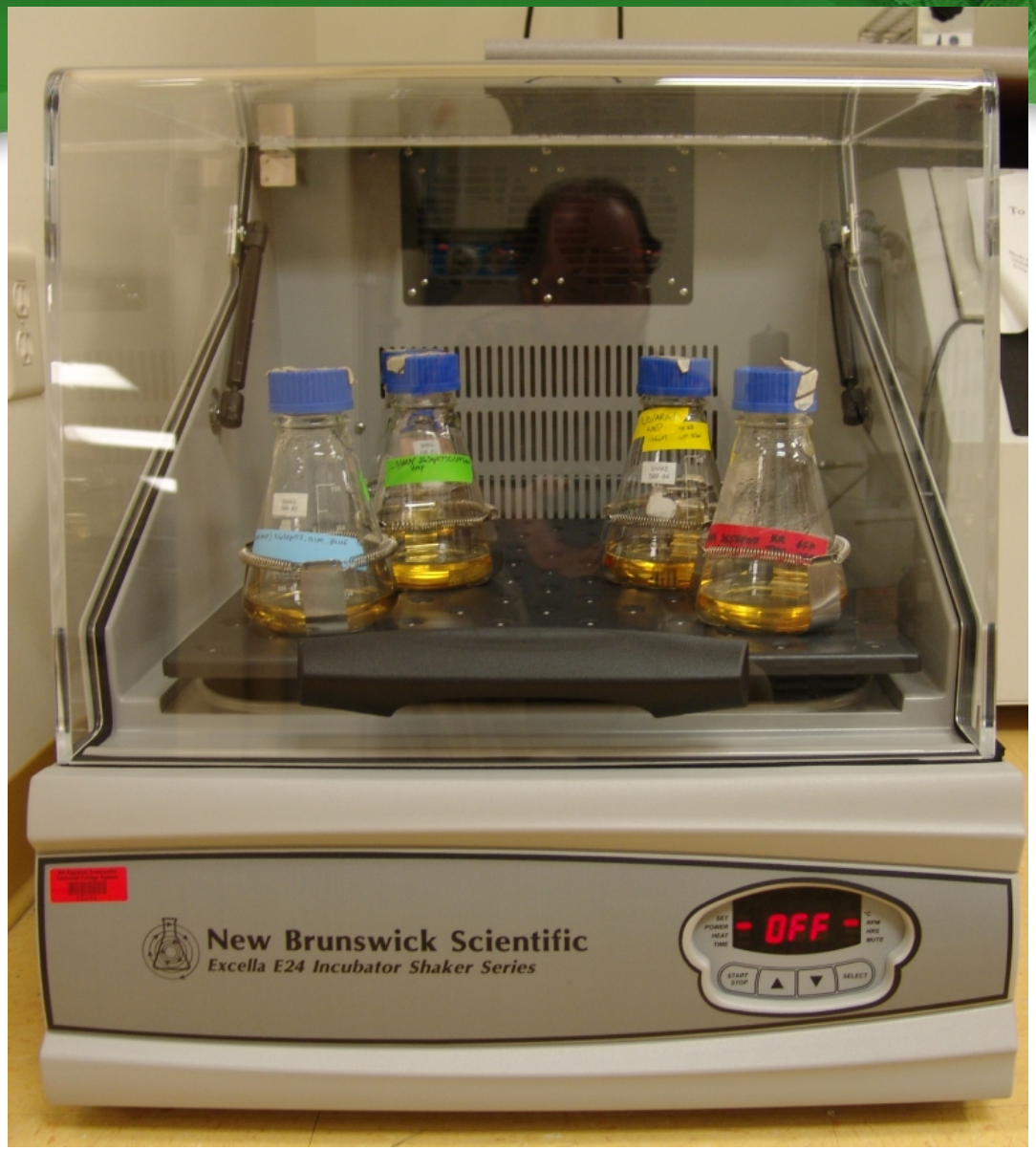


Spinner Flasks

Placed in a CO_2 incubator to provide a controlled environment for CHO cell scale-up

- Temperature: 37°C
- CO_2: 5%
- pH: 7.2
- Agitation via Magnetic Stir Plate: 75 rpm

Shake Flasks in Shaking Incubator



A disposable WAVE bioreactor

Upstream Processing Equipment

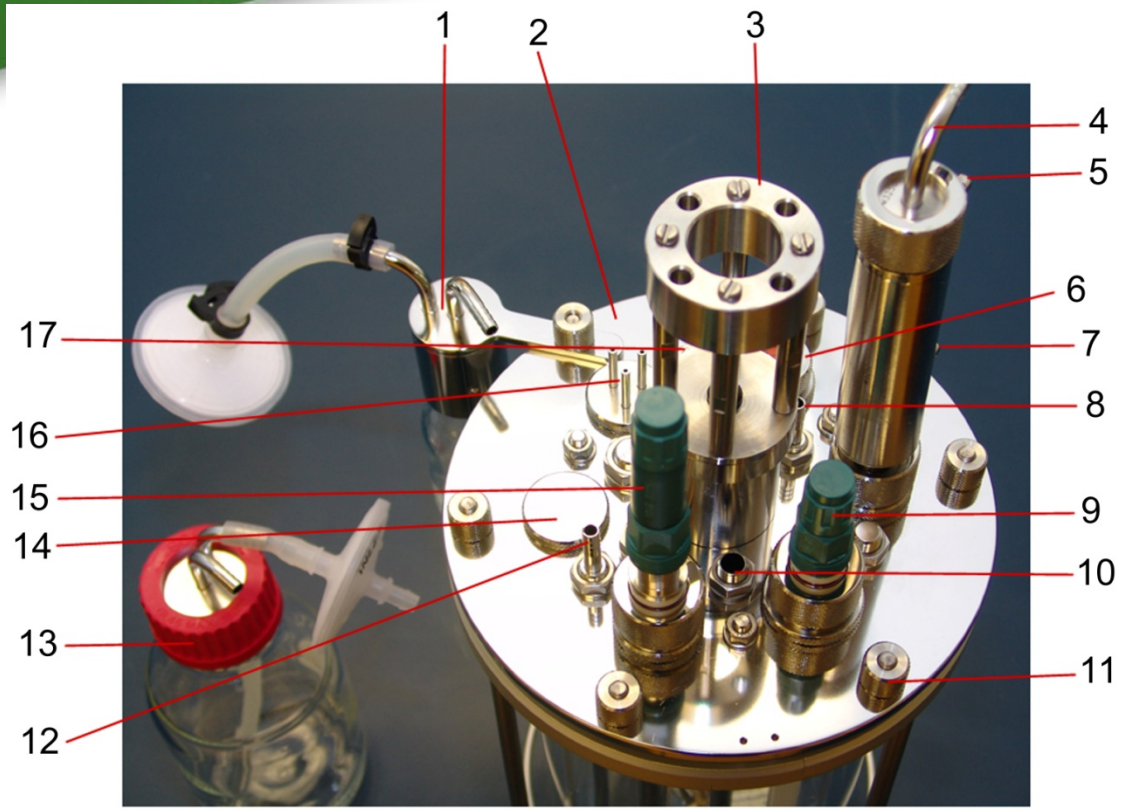
Lab-Scale Bioreactor
3 liters

Large-Scale Bioreactor
25,000 liters

Types of Bioreactors

The Top of a 20,000 liter Commercial-Scale Bioreactor (Process-Controlled)

Topside Head plate



1. Sample bottle assembly
2. Head plate assembly
3. Stirrer motor mount
4. Condenser air outlet
5. Condenser water outlet (from)
6. Inoculation port
7. Condenser water inlet (to)
8. CO_2 overlay port
9. pH probe
10. Thermowell port
11. Mill fastener
12. Sparger
13. Feed bottle
14. Blind stopper
15. DO probe
16. 3 Feed ports
17. Harvest tube

Monitoring Growth

- The importance – The growth rate (u) and doubling time (T_d) help to determine when to feed, when to harvest and such.
- The assays for cell growth and reproduction – live cell counts, optical density (OD) readings, and WCW measurements give you the data needed to determine the growth rate and doubling time.

Growth Rate and Doubling Time Calculations

Growth Rate

$u = (\ln OD_2 - \ln OD_1)/T_2 - T_1$

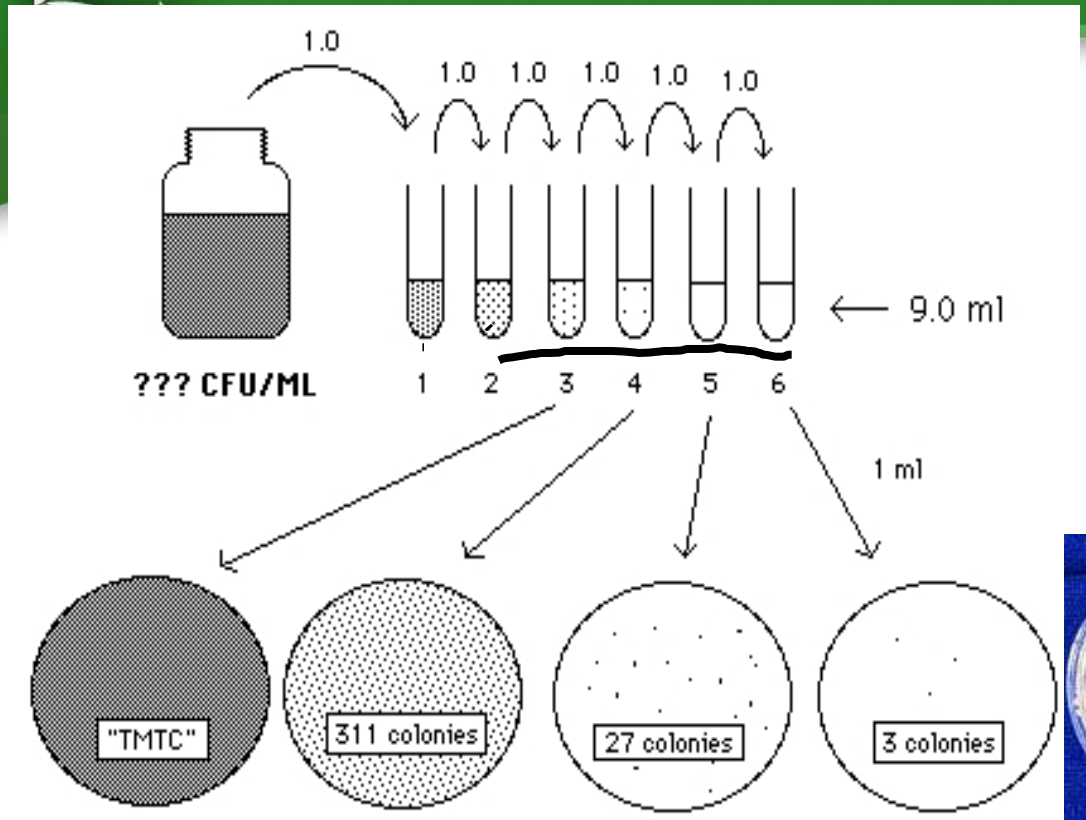
Or

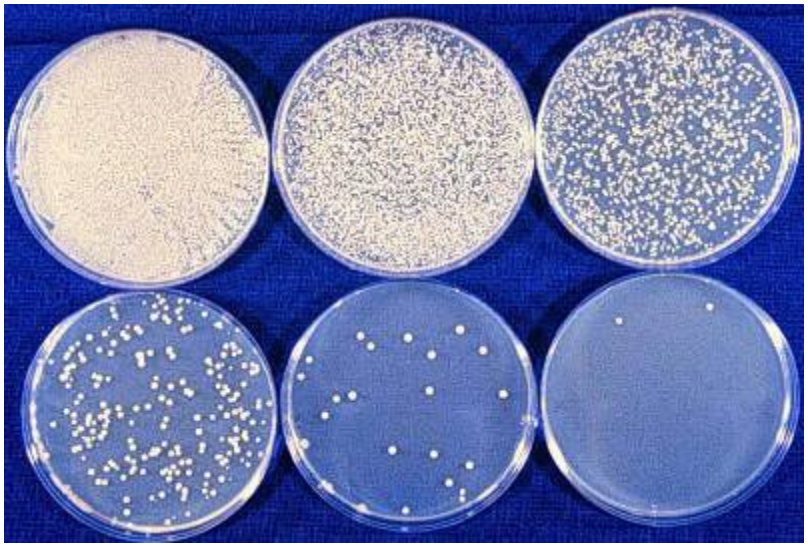
$u = (\ln X_2 - \ln X_1)/T_2 - T_1$ (where X=live cell count)

Doubling Time

$T_d = \ln 2/u$

Live Cell Count: Spread Plate Method





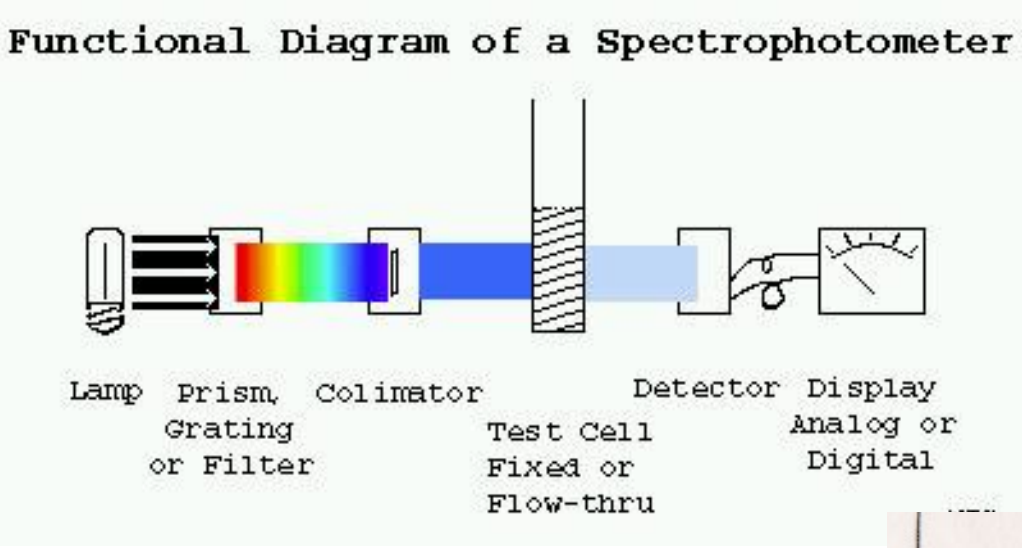
Carefully prepared spread plates for viable cell counts

Prepare serial dilutions and pipet onto the agar plate

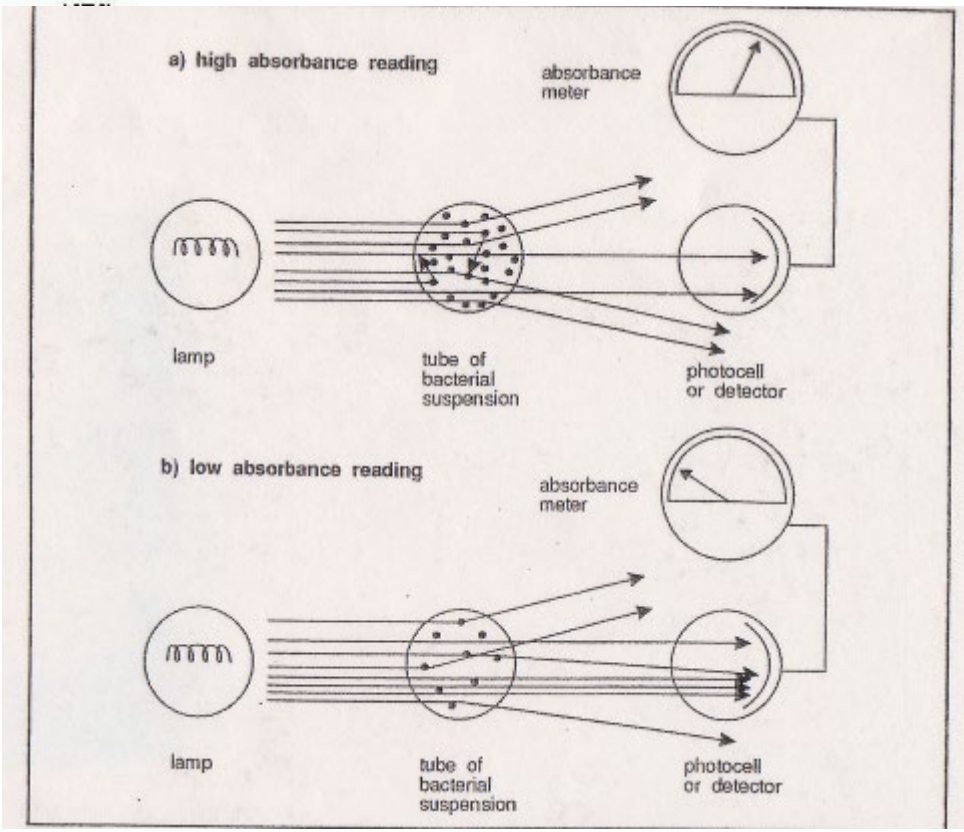
Flame sterilize the spreader

Spread the cells onto the agar plate

Optical Density (OD) Measurements using a Spectrophotometer



Spectrophometers measure Optical Density. Knowing the OD or, one can determine the growth rate (u) and from the growth rate, the doubling time (T_d) of the fed-batch culture can be determined.



Monitoring The Product (Protein)

A uv-visible spectrophometer may be set at 280nm to determine the concentration of protein in the media.

Monitoring Growth Conditions

- pH – Often drops as cells grow and divide, if the culture doesn't get enough oxygen so that glucose is broken down by glycolysis into lactic acid which crosses the cell membrane enters the media and creates an acid environment. If there is plenty of oxygen, glucose is broken down into pyruvic acid which enters the mitochondria producing H_20, CO_2, and energy (ATP and heat).
- Analyate analysis - Glucose concentration measurements using an analyate analyzer such as a Biolyzer or a Nova, allows us to determine when glucose has been used up and therefore when to start feeding methanol for protein production or to determine when lactate is being produced, a sign of anerobic respiration
- Temperature – Each cell type needs different temperature; *Pichia pastoris* require 30 degrees C.
- Dissolved Oxygen – Oxygen is needed to accept protons from the NADH hydrogen atoms in the mitochondrial electron transport chain.

Process Control

- Head Plate
  - Stirrer motor mount (motor controls the agitation rate)
  - Condenser (outlet)
  - Inoculation port (pump seed reactor contents here)
  - pH probe (measures pH of media; negative feedback loop adds acid or base)
  - Thermo well port (put temperature probe in here to measure temperature; negative feedback loop cools or heats)
  - Sparger (bubbles gasses into media)
  - Feed bottle (to add glucose, acid or base, methanol)
  - DO probe (measures dissolved oxygen in the media; negative feedback loops control agitation, air and oxygen)
  - Harvest port (for harvesting batch)
  - Impeller (like a propeller – moves fluid and propelled by motor)
- Computer Controller

Parameters Monitored (most Process-Control via feedback loops)

- pH (via addition of base or acid)
- Temperature (via jackets that heat or cool)
- Oxygen (via sparging air or oxygen and agitation)
- Rate of Agitation (via need for air or oxygen)
- Carbon Dioxide (via sparging)
- Feed (via addition of appropriate nutrients)
- OD (via spectrophotometer)

Characteristics of Microbial and Mammalian Cell Culture

Parameter	Microbial Cell Culture	Mammalian Cell Culture
Growth Rate (u)	Doubling Time = minutes to hours	Doubling Time = days
Temperature (T)	Great diversity: -0 to +100 degrees (plus/minus 1) C often by cooling	Most: 37 to 42 degrees C Control to plus or minus .1 degree C
pH	Great diversity: pH 2-10 (*Pichia* = pH 5.8) Control by adding acid or base	Narrow range: pH 6.8 to 7.2 (CHO = pH 7.2) Control by sparging CO^2
Dissolved Oxygen (DO)	Air or Oxygen sparged Robust cell walls allow rigorous sparging	Air sparged Extremely shear sensitive use sintered sparger
Agitation Rate	Agitation rates can be >800rpm Use Rushton impeller	Agitation rates <150rpm Use maine impeller
Foam	Foam probe, anti-foam agent required	No foam probe or anti-foam required

Temperature Control for Mammalian Cell Culture

For mammalian cell culture heating is more critical than cooling due to slow metabolic rates (doubling time) .

Temperature Probe

Heating Blanket on single wall vessel

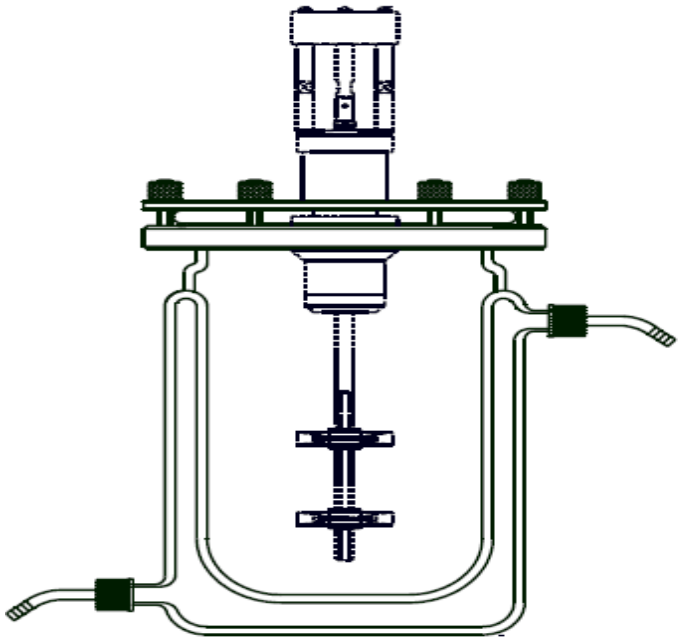
Jacketed Vessel for Heating or Cooling

HEATING

- City water
- Heater with recirculation loop
- Used to heat bioreactors for mammalian cell culture at 37 degrees Centigrade

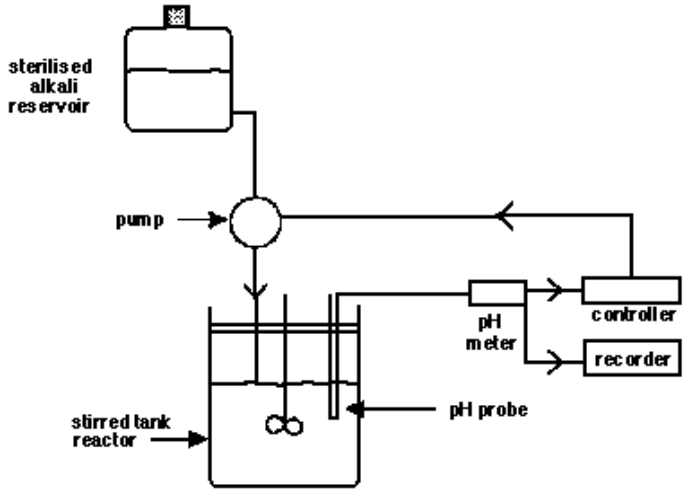
COOLING

- City water
- Chiller with recirculation loop
- Used to cool fermenters for microbial cell culture, e.g. *Pichia pastoris* or *Escherichia coli*

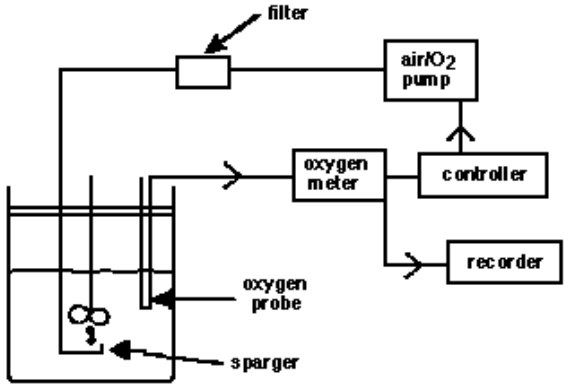


Process-Control Loops

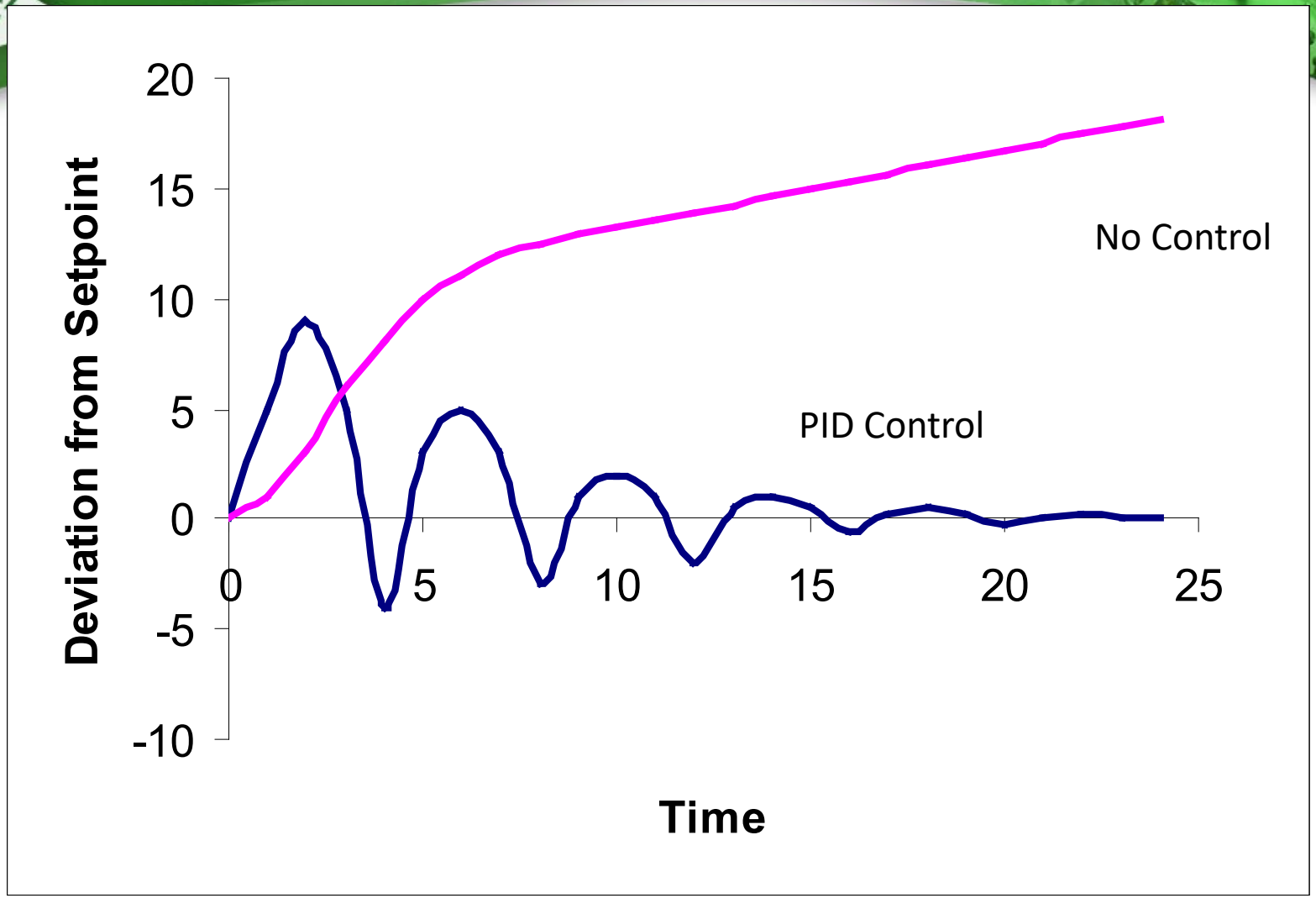
pH Process-Control Loop



DO Process-Control Loop



PID Control


Deviation from Setpoint
20
15
10
5
0
-5
-10
0
5
10
15
20
25
Time
No Control
PID Control