Discovery Research and Cell Culture
Discovery Research and Cell Culture
The Expression Vector: The Basis of Biotechnology Manufacturing
*Escherichia coli* – GFP

*Escherichia coli*

Green Fluorescent Protein
GFP Expression Vector
Central Dogma of Biology

- DNA Replication
- Reverse transcription
- Transcription
- RNA Replication
- Translation
- Protein
Transformation and Cloning

(a) DNA fragment + Vector → Recombinant vector

(b) Recombinant vector + E. coli → E. coli

(c) E. coli + Antibiotic → E. coli with vectors proliferates
    E. coli without vectors dies

(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

- **Primary protein structure** is the sequence of a chain of amino acids.
- **Secondary protein structure** occurs when the sequence of amino acids are linked by hydrogen bonds.
- **Tertiary protein structure** occurs when certain attractions are present between alpha helices and pleated sheets.
- **Quaternary protein structure** is a protein consisting of more than one amino acid chain.
Denaturation

- Heat
- Alcohol
- Acids and bases
- Heavy metals
- No specific shape
- Reducing agents

Tertiary Structure - Hydrogen Bonding

Denaturation by Alcohol

C. Ophardt, c. 2003
Denaturation

Tertiary Structure - Salt Bridges

Denaturation by Acid or Base

Tertiary Structure - Disulfide Bonds

Denaturation by Reducing Agents

Join two chains

C. Ophardt, c. 2003
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 channels)
- 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
Typical Production Process Flow

Ampule Thaw → Inoculum Expansion (Spinner Bottles) → (Feed1) → (Feed 2) → (Feed 3) → (Feed 4) → Centrifuge

Cryo-preservation → Concentration / Diafiltration → Chrom 3

Viral Removal Filtration → Chrom 2 → Chrom 1
Upstream/Downstream Manufacturing Overview

Inoculum Preparation
- **24 days**

Fermentation Process
- **31 days**

**Media Prep**

**Working Cell Bank**

**Sub-Culture**

**Seed Bioreactors**
- 150L Bioreactor
- 750L Bioreactor
- 5,000L Bioreactor
- 26,000L Bioreactor

**Large Scale Bioreactor**

**Wave Bag**

**Centrifuge**

**Depth Filtration**

**Collection**

**Inoculum Suite**

- **Open Processing**

**Sub-Culture**

**Sub-Culture**

**Sub-Culture**

**Sub-Culture**

**Wave Bag**

**Seed Bioreactors**
- 150L Bioreactor
- 750L Bioreactor
- 5,000L Bioreactor
- 26,000L Bioreactor

**Filtration**

**Collection**

**Harvest/Recovery**

**Harvest**

**Collection Tank**
- 1,500L

**Filter**

**Chromatography Skid**

**Anion Exchange Chromatography (QXL)**

**Column**

**Eluate Hold Tank**
- 8,000L

**Hydrophobic Interaction Chromatography (HIC)**

**Column**

**Eluate Hold Tank**
- 20,000L

**Protein A Chromatography**

**Column**

**Eluate Hold Tank**
- 6,000L

**Post-viral Hold Vessel**
- 3,000L

**Viral Filtering**

**Anion Exchange Chromatography (QFF - Fast Flow)**

**Column**

**Eluate Hold Tank**
- 5,000L

**Ultra Filtration Diafiltration**

**Bulk Fill**

**Purification**

**Upstream/Downstream Manufacturing Overview**

**24 days**

**31 days**

**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

NBC²
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
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
Spinner Flasks

Placed in a CO₂ incubator to provide a controlled environment for CHO cell scale-up

- Temperature: 37°C
- CO₂: 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)
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. CO₂ 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

\[ u = \frac{(\ln \text{OD}_2 - \ln \text{OD}_1)}{T_2 - T_1} \]

Or

\[ u = \frac{(\ln X_2 - \ln X_1)}{T_2 - T_1} \text{ (where } X=\text{live cell count)} \]

Doubling Time

\[ T_d = \frac{\ln 2}{u} \]
Live Cell Count: Spread Plate Method

```
1.0 1.0 1.0 1.0 1.0

1.0 ml

9.0 ml

??? CFU/ML

1 ml

"TMTC" 311 colonies

27 colonies

3 colonies

```

Image shows petri dishes with bacterial colonies.
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
Spectrophotometers 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.
A uv-visible spectrophotometer 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 $\text{H}_2\text{O}$, $\text{CO}_2$, and energy (ATP and heat).

- **Analyte analysis** - Glucose concentration measurements using an analyte 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 anaerobic 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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Microbial Cell Culture</th>
<th>Mammalian Cell Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth Rate (u)</td>
<td>Doubling Time = minutes to hours</td>
<td>Doubling Time = days</td>
</tr>
<tr>
<td>Temperature (T)</td>
<td>Great diversity: -0 to +100 degrees (plus/minus 1) C often by cooling</td>
<td>Most: 37 to 42 degrees C Control to plus or minus .1 degree C</td>
</tr>
<tr>
<td>pH</td>
<td>Great diversity: pH 2-10 (<em>Pichia</em> = pH 5.8) Control by adding acid or base</td>
<td>Narrow range: pH 6.8 to 7.2 (<em>CHO</em> = pH 7.2) Control by sparging CO²</td>
</tr>
<tr>
<td>Dissolved Oxygen (DO)</td>
<td>Air or Oxygen sparged Robust cell walls allow rigorous sparging</td>
<td>Air sparged Extremely shear sensitive use sintered sparger</td>
</tr>
<tr>
<td>Agitation Rate</td>
<td>Agitation rates can be &gt;800rpm Use Rushton impeller</td>
<td>Agitation rates &lt;150rpm Use maine impeller</td>
</tr>
<tr>
<td>Foam</td>
<td>Foam probe, anti-foam agent required</td>
<td>No foam probe or anti-foam required</td>
</tr>
</tbody>
</table>
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

- sterilised alkali reservoir
- pump
- stirred tank reactor
- pH probe
- pH meter
- controller
- recorder

DO Process-Control Loop

- filter
- air\textsubscript{O\textsubscript{2}} pump
- oxygen meter
- controller
- recorder
- oxygen probe
- sparger