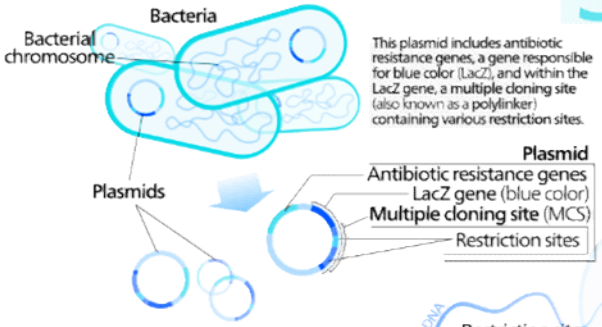
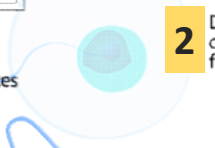
PREPARATION OF RECOMBINANT PROTEINS

Bi7430c Molecular Biotechnology Practicals

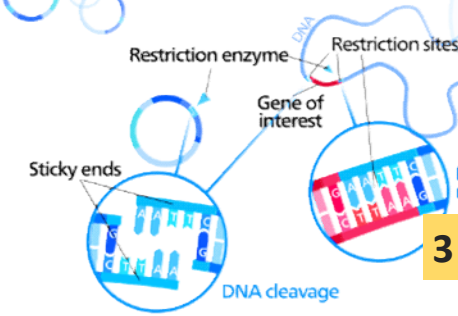
gene cloning

1 Small, circular DNA molecules called **plasmids** are removed from bacterial cells. These plasmids serve as **vectors**—molecules which will carry genes of interest.



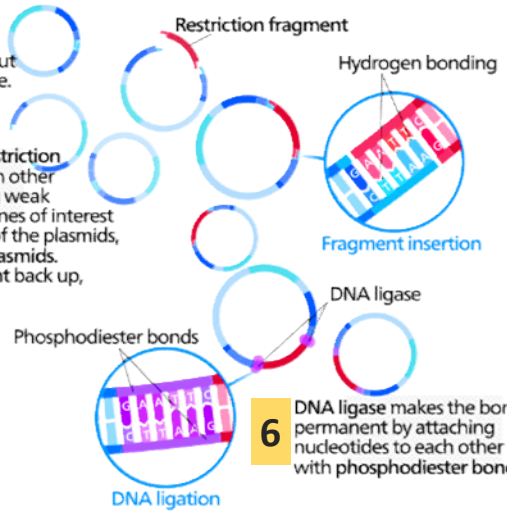


2 DNA containing the gene of interest is also taken from its cell.



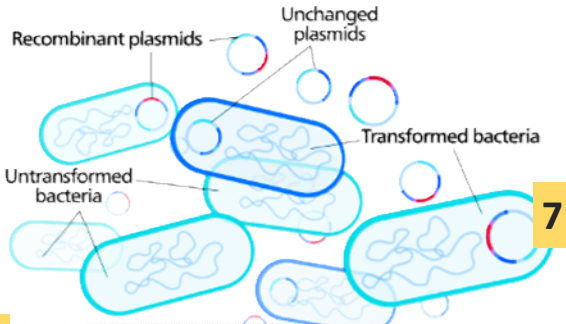
3 A restriction enzyme (also called a restriction endonuclease) recognizes its specific **restriction site**—a short sequence about 4–8 base pairs long.

4 It breaks apart the DNA, leaving overhangs called **sticky ends**. The restriction enzyme cuts open the circular plasmids. The same enzyme cuts out the gene of interest from its DNA molecule.



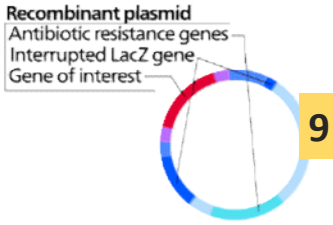
5 The sticky ends of the **restriction fragments** attach to each other via base pairing, forming weak hydrogen bonds. The genes of interest get included into some of the plasmids, forming **recombinant plasmids**. Other plasmids close right back up, remaining unchanged.

6 **DNA ligase** makes the bond permanent by attaching nucleotides to each other with **phosphodiester bonds**.

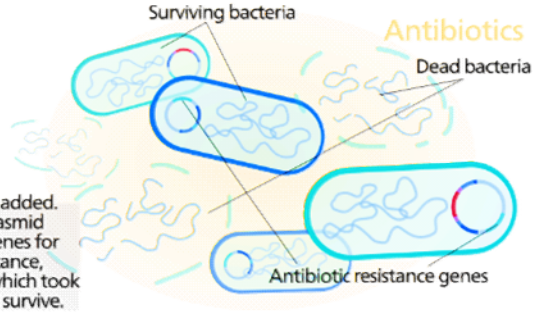


7 The plasmids are mixed with the bacteria. Some of them take up the plasmids in a process called **transformation**.

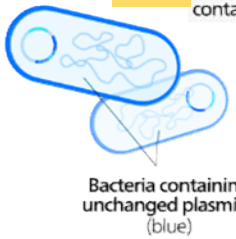
8 Plasmids with an uninterrupted LacZ gene turn their bacteria blue. In the recombinant plasmids, the inserted gene interrupts the Lac Z gene, and the bacteria remain their original color. Bacteria which did not take up any plasmids also remain uncolored.

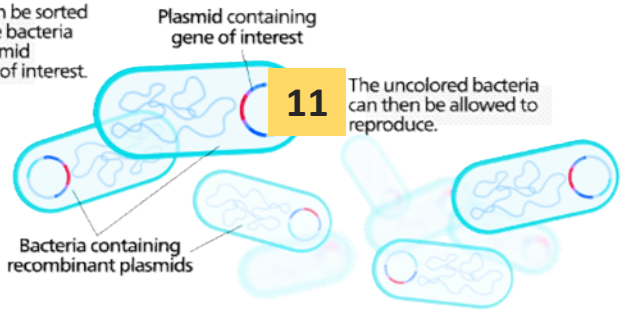


9 Antibiotics are added. Because the plasmid contains the genes for antibiotic resistance, only bacteria which took up the plasmid survive.



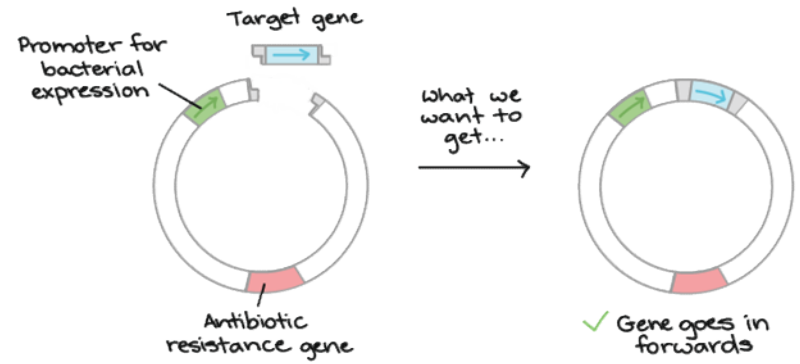
10 The bacteria can then be sorted by color, isolating the bacteria which took up a plasmid containing the gene of interest.



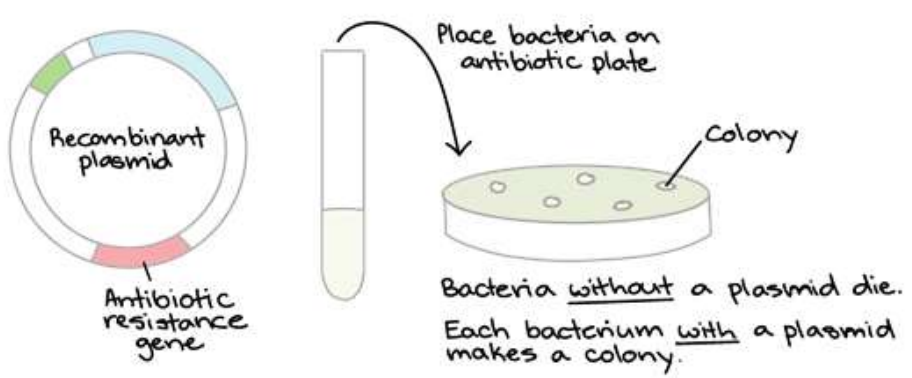


11 The uncolored bacteria can then be allowed to reproduce.

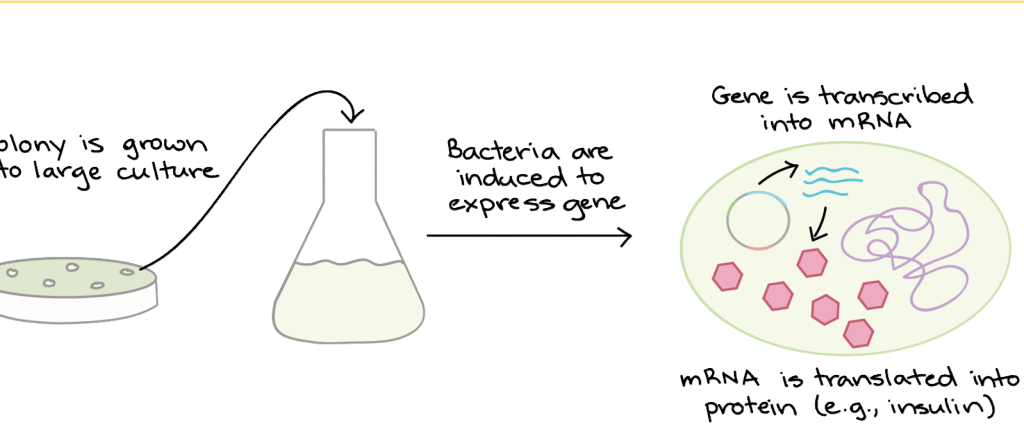
LIGATION OF TARGET GENE INTO PLASMID



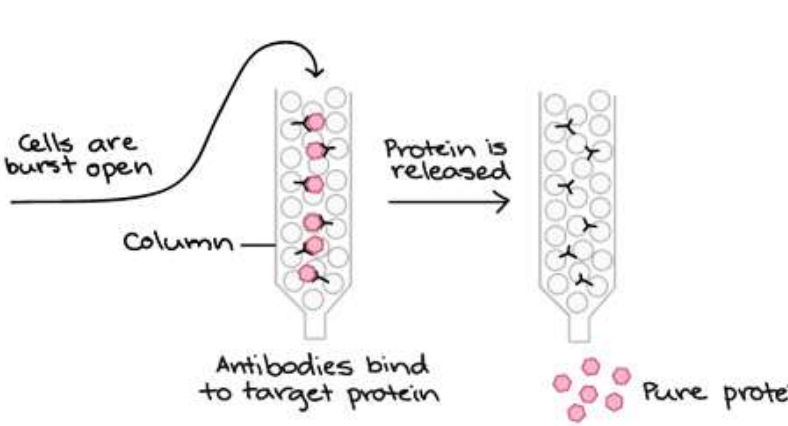
TRANSFORMATION AND SELECTION



CULTIVATION AND EXPRESSION



PURIFICATION

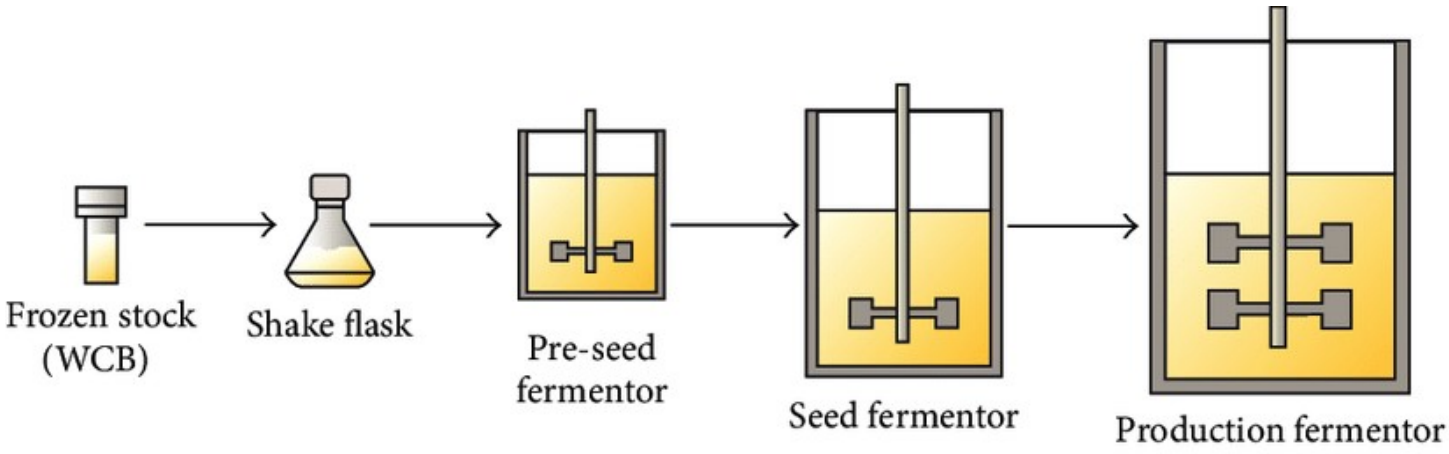


SCALE-UP

- shake flasks – up to 1L (uncontrolled conditions)
- laboratory fermentor – up to 10L (sterilized whole with medium, tubes, filters...)
- pilot scale – hundreds of L
- industrial bioreactor – thousands of L (sterilized with steam through pipes)

MANUFACTURE

- a cell line is established from a single clone and is used to make-up the master cell bank (MCB)
- MCB must be characterized and tested for contaminants (bacteria, fungi, mycoplasmas, viruses)
- cells from MCB are expanded to form the working cell bank (WCB), which is characterized for cell viability prior to use in the manufacturing process

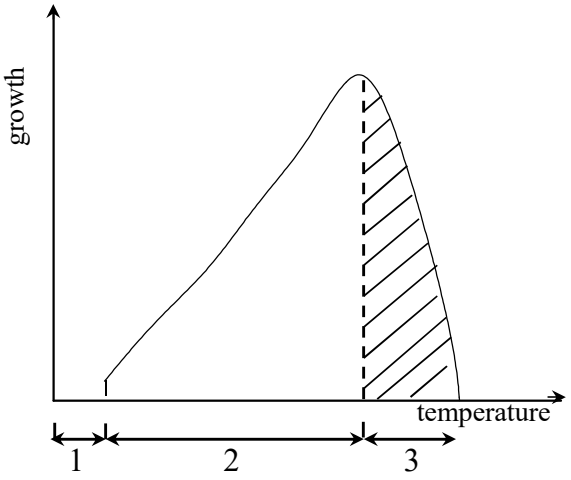


CONDITIONS TO CONTROL

- factors influencing the growth of microorganisms and production of metabolites
    - temperature
    - concentration of oxygen
    - pH
    - pressure
    - extracellular concentration of substances and water
    - agitation
    - production medium

TEMPERATURE

- psychrophiles
- mezophiles – *E. coli* (5 to 40°C)
- thermophiles

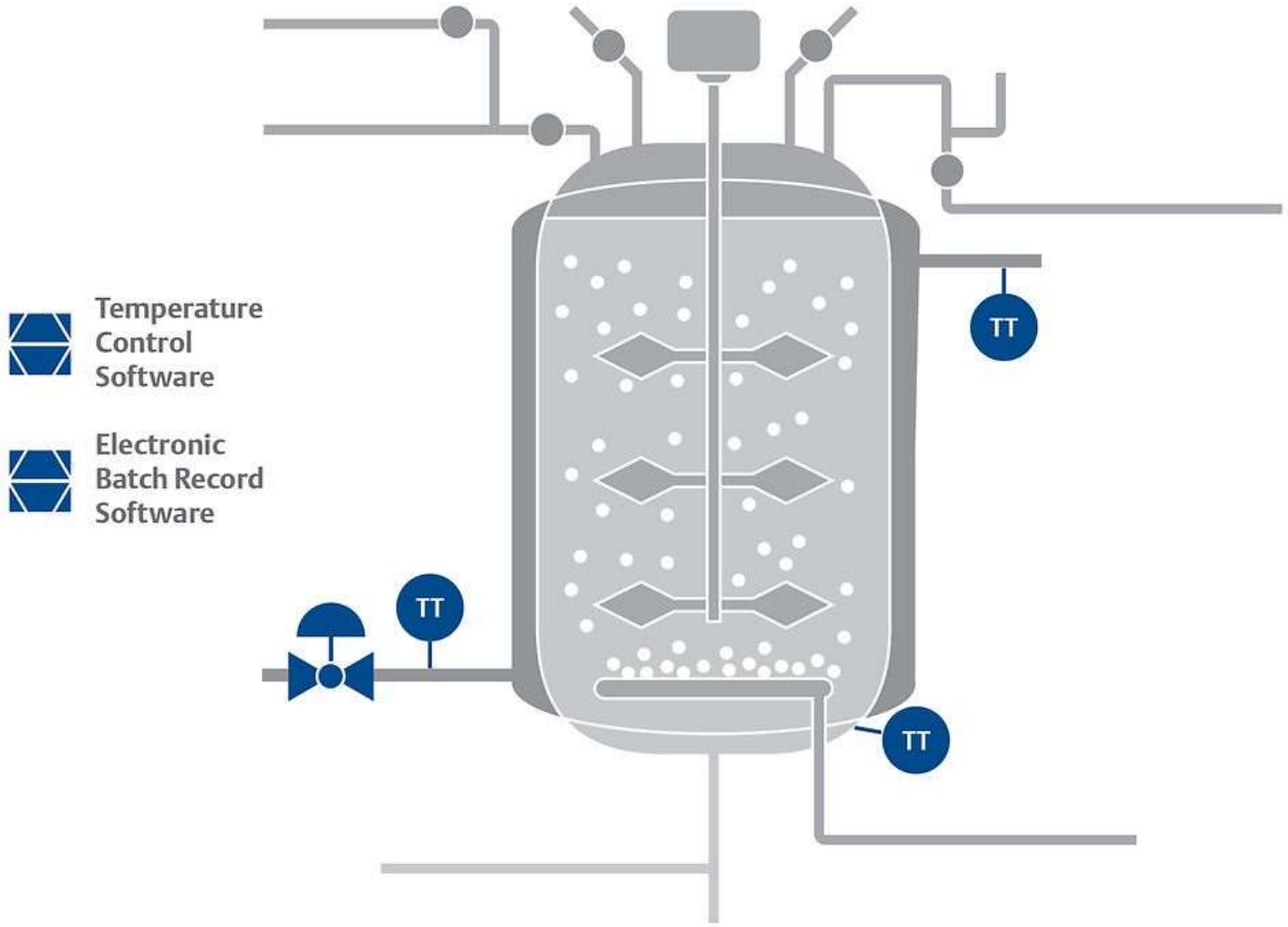


- ↑t = ↑growth and synthesis of metabolites
- above 45°C proteins losing 3D structure
- above 60°C half of proteins denatured

1. solidification of lipids in membranes
2. growth is proportional to temperature
3. denaturation of proteins

TEMPERATURE

- t measurement
  - thermometer
- t maintenance
  - thermal jacket
  - water in/out
- across all operating processes, temperature variations of as little as 1°C can limit production



OXYGEN

- aerobic – supply of oxygen necessary
  - agitation and aeration
  - shape of cultivation flask
  - increased pressure
  - additives

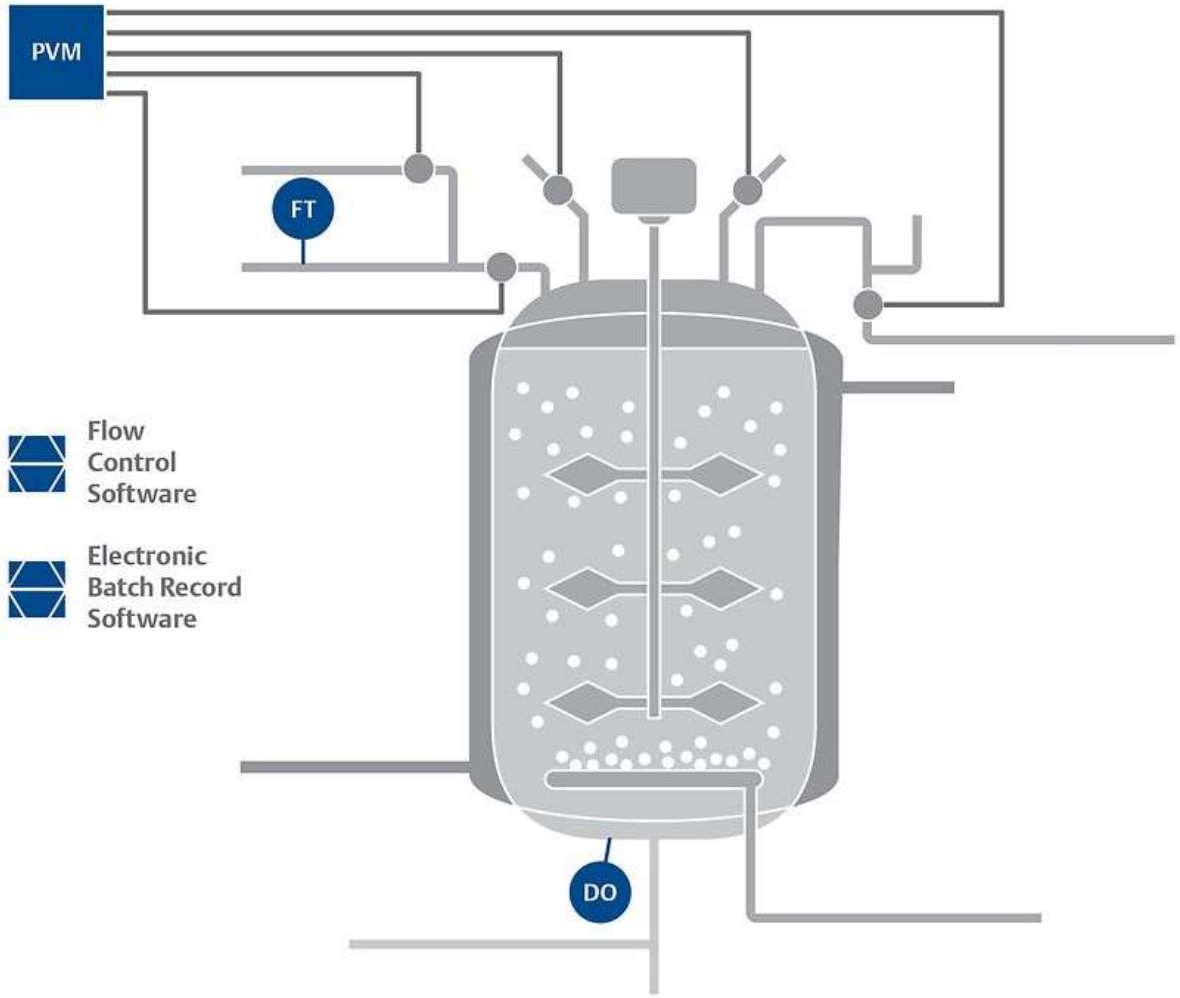
- microaerophile
  - hard to maintain standard conditions

- anaerobic
  - facultative (*S. cerevisiae* +O_2 = growth and production of acids, -O_2 = ethanol)
  - obligatory – complete elimination of O_2 during cultivation is necessary; N_2 and CO_2 atmosphere

OXYGEN

- fostering the optimal growth environment within a bioreactor requires adding the right amounts of reactant gasses to a vessel to support a cell culture

- the amounts of gasses (oxygen) vary depending on the size and growth stage of a culture



pH

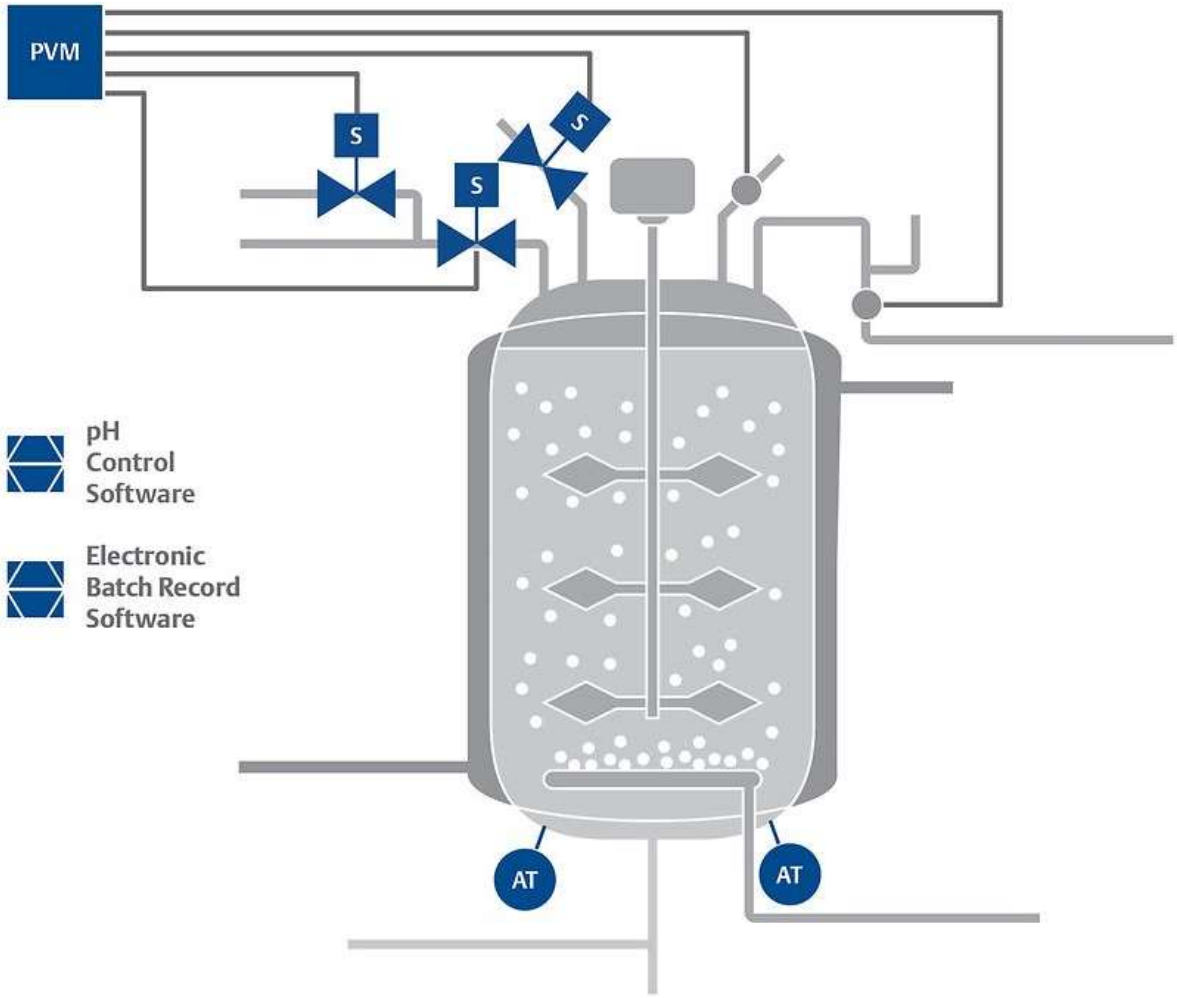
- H^+ interact with proteins and lipids in the cell membrane
- if the difference between extracellular and intracellular pH increases, energetic demand on cells grow to maintain the intracellular pH

- filamentous fungi – can withstand even lower pH
- yeasts – pH usually 4 to 7
- bacteria – pH usually 4 to 9
- mammalian cells – very sensitive to pH changes

- some microorganisms have different pH optimum for growth and production of metabolites (*A. niger* growth 5-7, citric acid production 2-3, gluconic acid 5-6)

pH

- pH measurement
  - electrode
- pH maintenance
  - acid (phosphoric acid)
  - base (ammonium)
- metabolic changes in organisms and improperly calibrated sensors can result in ineffective pH control and negatively impact an operation



PRESSURE

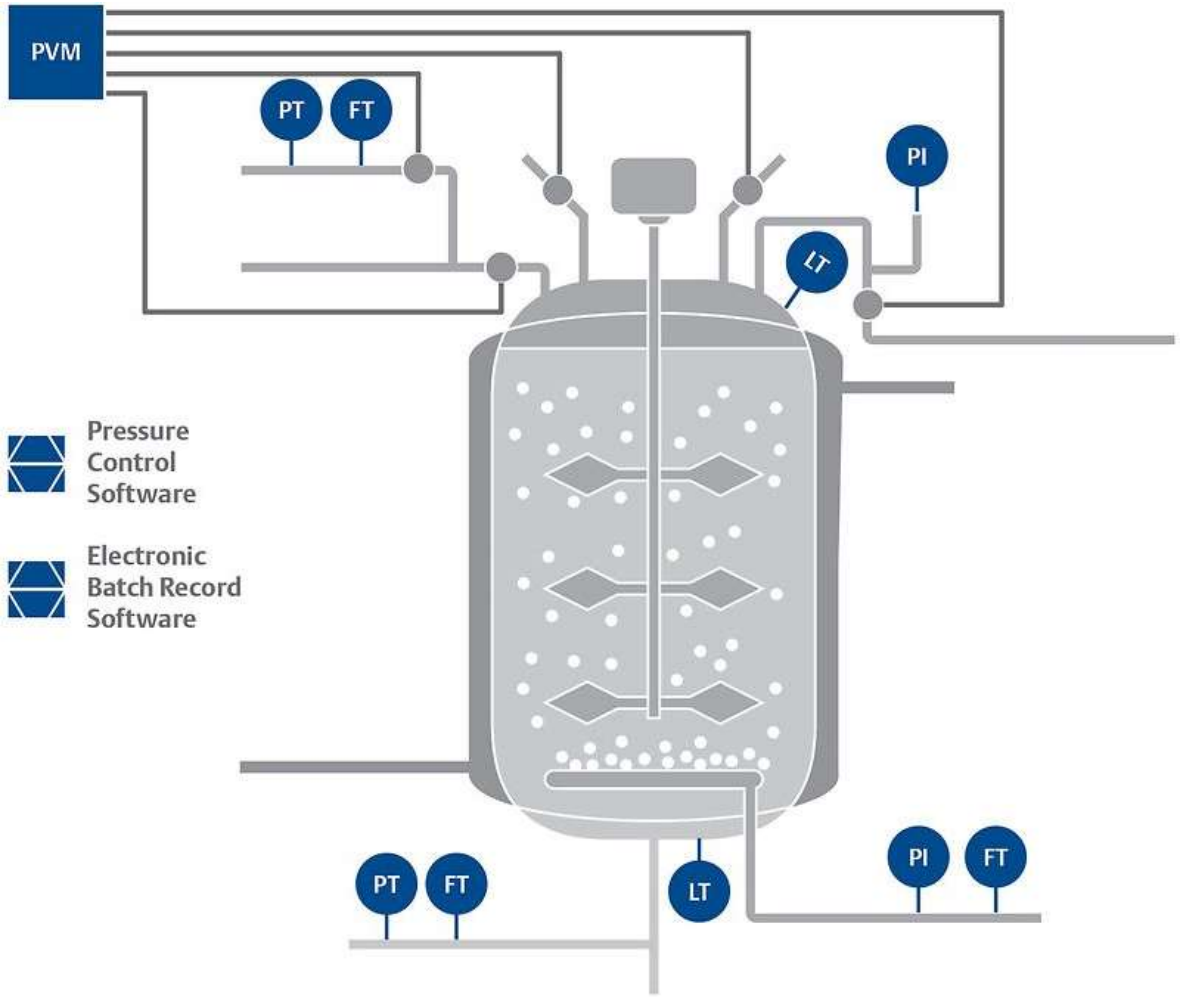
- obligatory basophiles
  - they can not withstand atmospheric pressure
  - marine microorganisms found in oceans are not suitable for laboratory/industrial use

- lots of microorganisms can form spores that are resistant to high pressures and temperatures – problems with sterilization

PRESSURE

- precise control of the pressure is necessary for ensuring adequate delivery of oxygen from a gas stream to cell culture

- even slight variations in local pressures can stunt cellular growth and limit product formation



EXTRACELLULAR CONCENTRATION OF SUBSTANCES

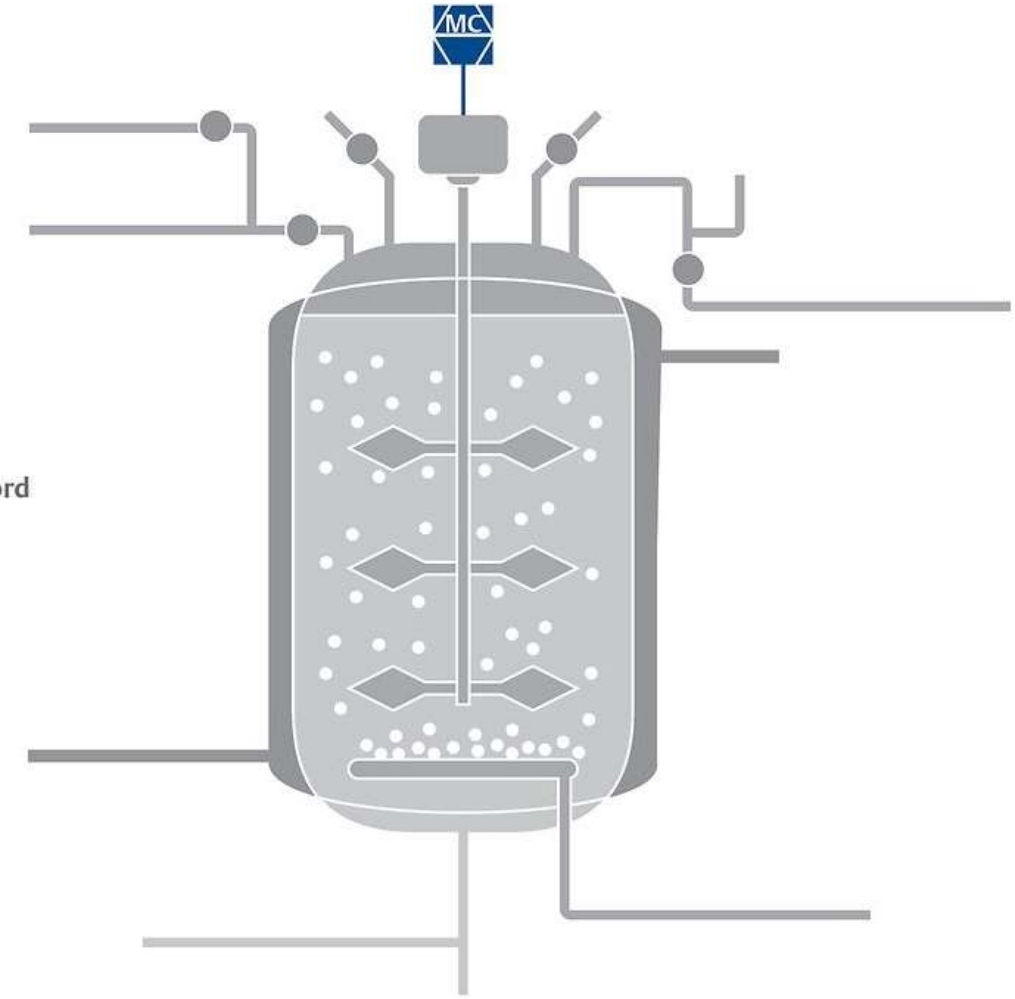
- halophiles – tollerant up to 25% of NaCl
- osmophiles – especially fungi (growth even on solid substrates like fermented soya, using only atmospheric moisture)

- very high concentration of substrates in extracellular environment
  - dehydration of cells
  - inhibition of important enzymes in cells

AGITATION

- agitation and rocking processes help transfer nutrients and oxygen to a cell culture within a bioreactor

- while these mixing processes are critical to achieving optimal productivity, imprecise and ineffective machinery can lead to excessive spinning or shaking – and destroy a cell culture

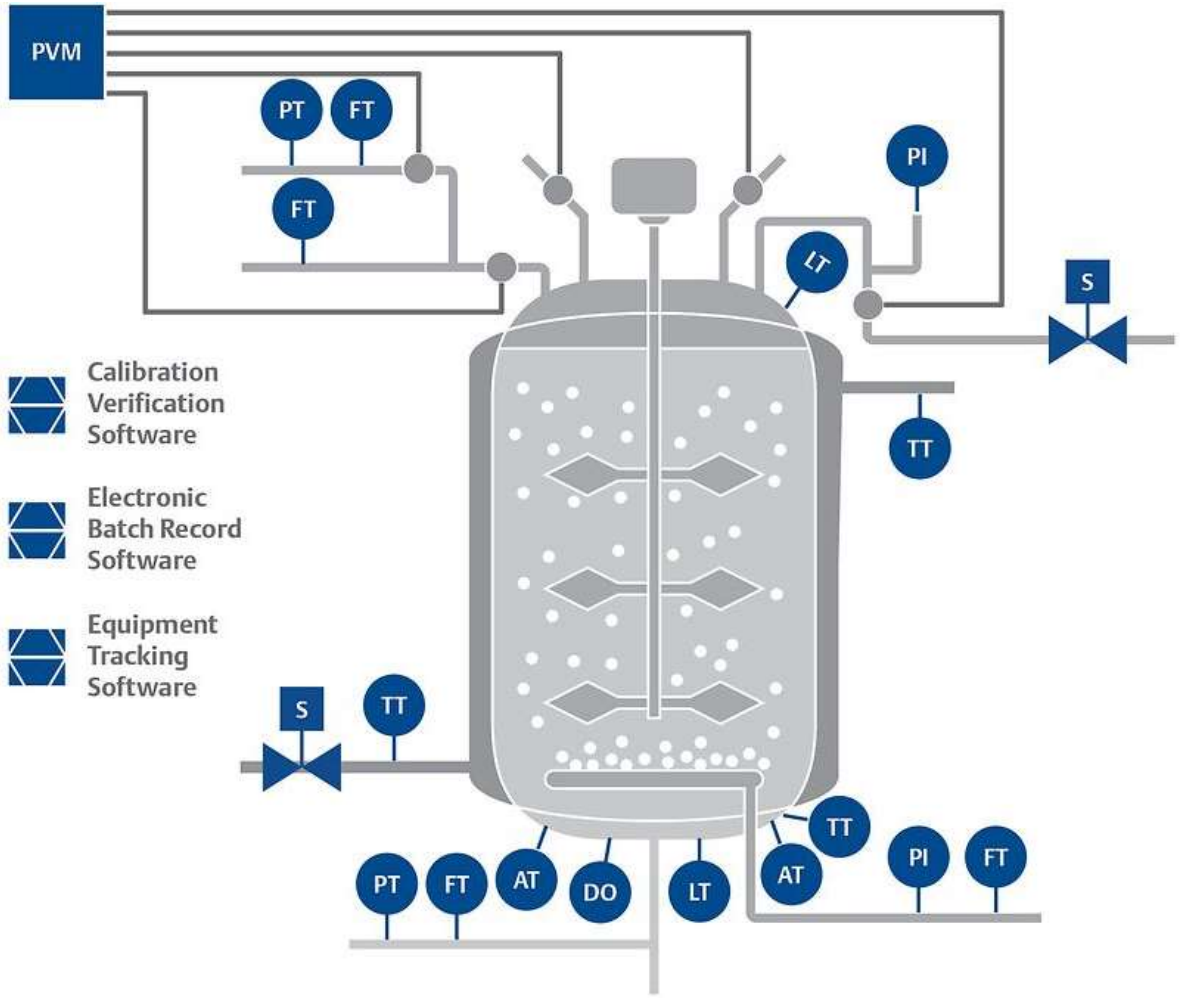


Electronic
Batch Record
Software

CALIBRATION!

- for life sciences manufacturers, calibration issues can result in deviations and quarantined batches and requires increased maintenance

- lack of traceability can result in potential compliance issues



MEDIUM

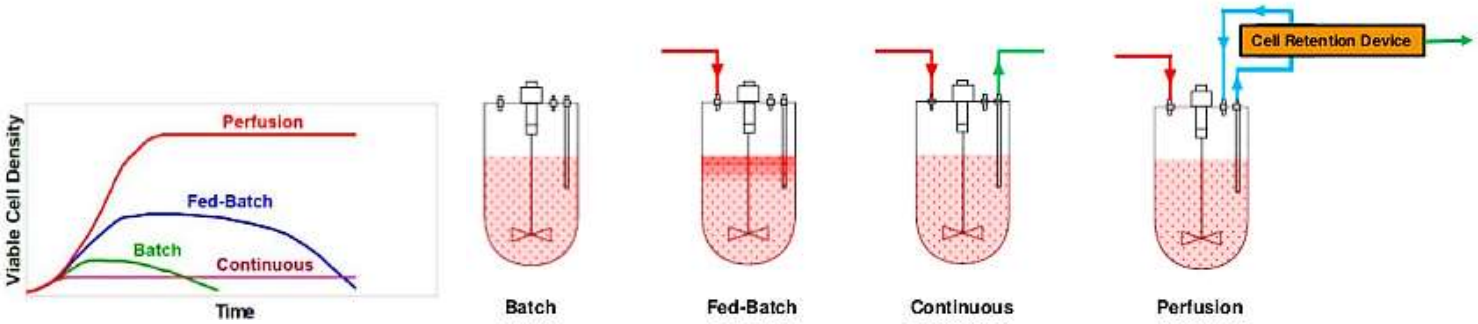
- nutrition sources
  - sources of proteins, vitamins, mineral and carbohydrates
  - peptone (hydrolysed protein), tryptone (digested casein), yeast extract
- energy sources
  - sources of carbohydrates
  - glucose, glycerole
- essential minerals
  - sources of micro and macro minerals
  - present in peptone and meat extract
- buffering agents
  - maintain optimum pH
  - specific amino acids, phosphates, citrates and zwitterions
- selective agents
  - allow the growth of only specific bacteria
  - antibiotics, tellurites, azides, bile salts
- solidifying agents
  - agar plates, gelatin
- specific substances such as growth factors, enzymes may be incorporated into the medium for specific bacteria

- commercial or DIY
  - LB (Miller/Lennox/low salt)
  - minimal salt/mineral (M9)
  - Terrific Broth TB
  - SOB
  - SOC

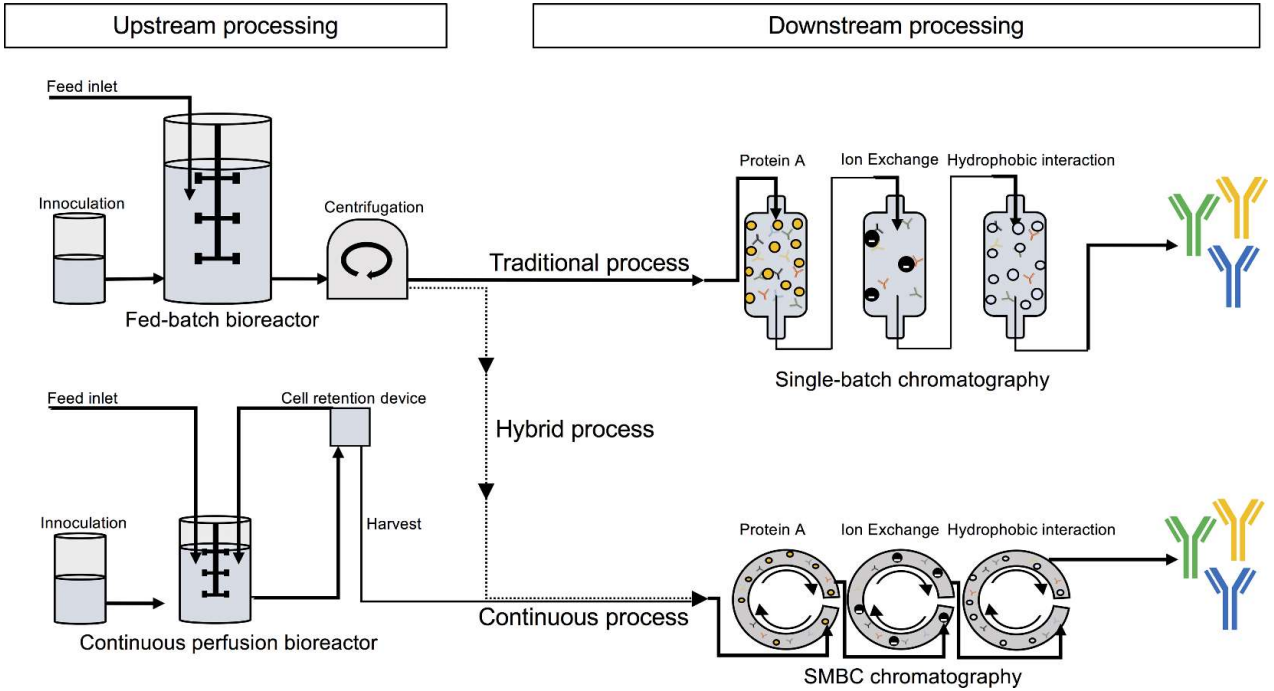
- commercial
  - EnPresso™ B Growth System (5x more protein compared to traditional culture media)

OPERATION MODES

Characteristics	Batch	Fed-batch	Continuous
Cultivation system	Closed-type	Semi-closed type	Open type
Addition of fresh nutrition	No	Yes	Yes
Volume of culture	Constant	Increases	Constant
Removal of wastes	No	No	Yes
Chance of contamination	Minimum	Intermediate	Maximum
Growth phase	Lag, log, stationary, decline	Lag, log, stationary, decline	Lag and log
Log phase	Shorter	Longer	Longest, continuous
Density of bacteria	Changes with time	Changes with time	Remains same
Product yield	Low	Medium	High



PROCESSING


Upstream processing
Downstream processing
Feed inlet
Innoculation
Centrifugation
Fed-batch bioreactor
Traditional process
Protein A
Ion Exchange
Hydrophobic interaction
Single-batch chromatography
Feed inlet
Cell retention device
Hybrid process
Innoculation
Harvest
Continuous perfusion bioreactor
Continuous process
Protein A
Ion Exchange
Hydrophobic interaction
SMBC chromatography