Microbes as factories for protein production

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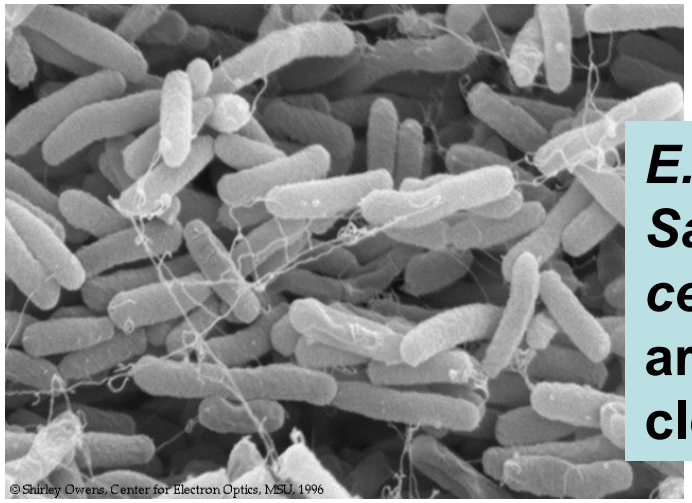
Microbes as protein factories

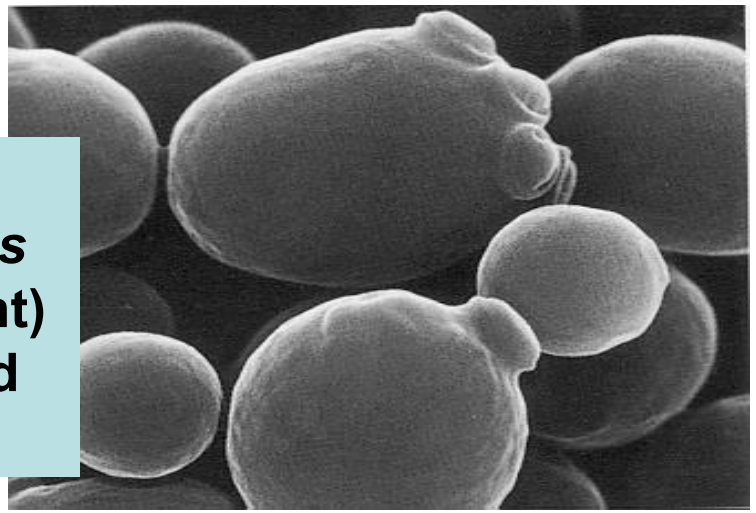
Learning outcomes. Be able to…

- **Explain why microbes are useful for protein production**
- **Discuss use of wild-type vs. mutants vs. recombinants**
- **Describe the advantages and disadvantages of different methods used for microbial cell lysis**
- **Describe the principles and methods involved in three stages of protein purification:**
  - **Precipitation**
  - **Ion exchange chromatography**
  - **Affinity chromatography**

Microbes as a source of proteins

- **Proteins:** amino acid polymers, enzymes / structural
- Humans need protein in diet; but also proteins used for:
  - **domestic** eg. proteases in washing powder
  - **industrial** eg. amylases to convert starch to sugars
  - **medical** eg. hormones like insulin
- Bacteria and fungi are excellent **sources** of proteins and useful as **cloning hosts** for expressing foreign proteins



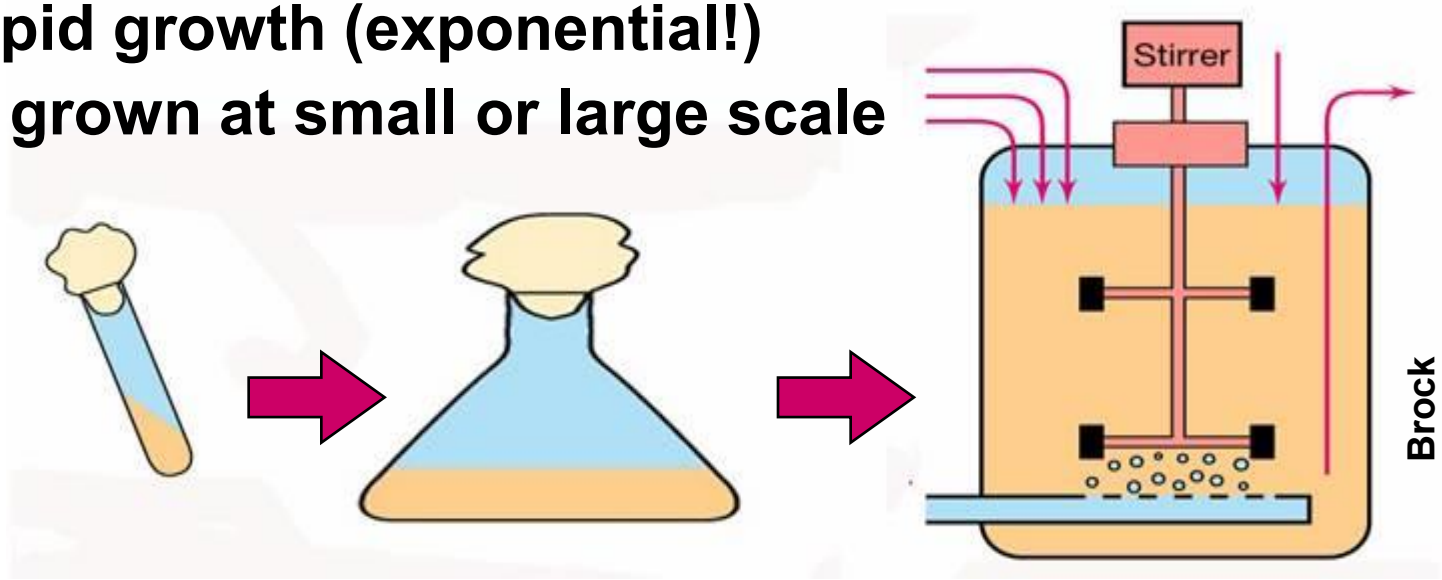


E.coli (left) and *Saccharomyces cerevisiae* (right) are widely-used cloning hosts

Microbes as protein factories

- Microbes have advantages over macroorganisms as sources and hosts for protein production:
- <u>Easy to grow</u>
  - simple nutrient requirements
  - very rapid growth (exponential!)
  - can be grown at small or large scale

Microbial protein factory:



Animal protein factory:

Microbes as protein factories

- **More advantages of microbes :**

  Easy to genetically manipulate

  - **prokaryotes are haploid, no introns***
  - **many host/vector systems available**
  - **easy to get DNA in and out of cells**

Electroporation machine

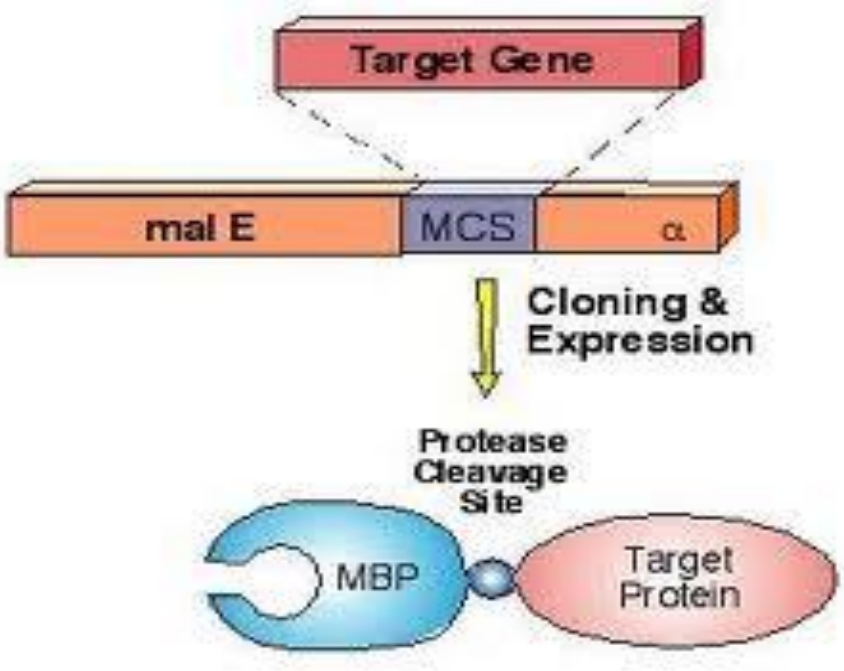
Electroporation cuvettes

Microbes as protein factories

- **More advantages of microbes:**

  Many heterologous expression systems available

  - **high protein expression level**
  - **easy control of gene expression**
  - **affinity tags and other tags to aid purification**



eg. ligating your gene of interest to the *E.coli* maltose binding protein gene (malE) can enhance the solubility of the target protein.

More than a 'tag' – a gene fusion.

Wild-type vs. mutant vs. recombinant microbes

- **Wild-type: naturally-occuring strain**
- **Mutant: strain subject to mutagenesis and selection**
- **Recombinant: strain with foreign DNA introduced**

- **Choice depends on:**
  - **Amount of protein produced: most wild-type microbes don't make enough target protein for commercial use**
  - **Time and money: is it worth investing in mutagenesis or genetic engineering to increase yields?**
  - **Application: recombinants or potential pathogens face difficulties with public perception and legal regulations**

Wild-type microbes as protein factories

- What does "wild-type" mean?

1. Exactly the same as found in Nature ?
2. Not intentionally genetically modified ☆
3. Not subjected to genetic engeering

- All microbial populations will change genetically over time (even if we don't want them to)

- Microbes evolve to suit the conditions
  - Vaccine strains: 'tamed' by growth in lab
    eg. *Mycobacterium bovis* BCG
  - Industrial strains: subjected to intentional selection, may also include mutation / genetic engineering

Wild-type microbes as protein factories

- **Microbial cells for industrial synthesis of single proteins are almost always recombinants and/or mutants.**

- **But there are several examples of wild-type microbes as sources of protein-containing biomass :**

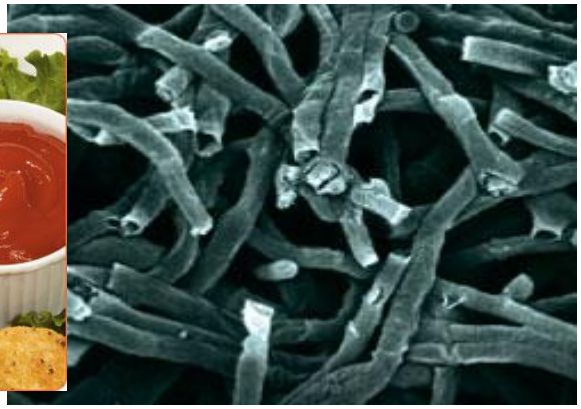
Saccharomyces cerevisiae

brewers/bakers yeast

***Fusarium sp.* "Quorn"**

fungus, but not mushroom

Hyphae similar in size to animal muscle fibres



Mutant microbes as protein factories

- Mutation and selection can greatly improve protein yield, and the methods are simple, cheap, widely applicable

Example - β-Amylase from *Bacillus cereus*

- *B.cereus* BQ10 grows on starch as carbon source via extracellular β-amylase, but secretion level is low
- Improved mutants can be easily identified by iodine-screening on starch agar plates (→ larger clearing zone)

Detection of amylase activity on starch agar: Starch+iodine→black

Clear zones around colonies indicate starch hydrolysis

Mutant microbes: β-Amylase in *Bacillus cereus*

Nanmori et al (1987)
Appl.Environ.Microbiol

BQ10

wild type
- 1 x activity,
- glucose-repressible

UV irradiation

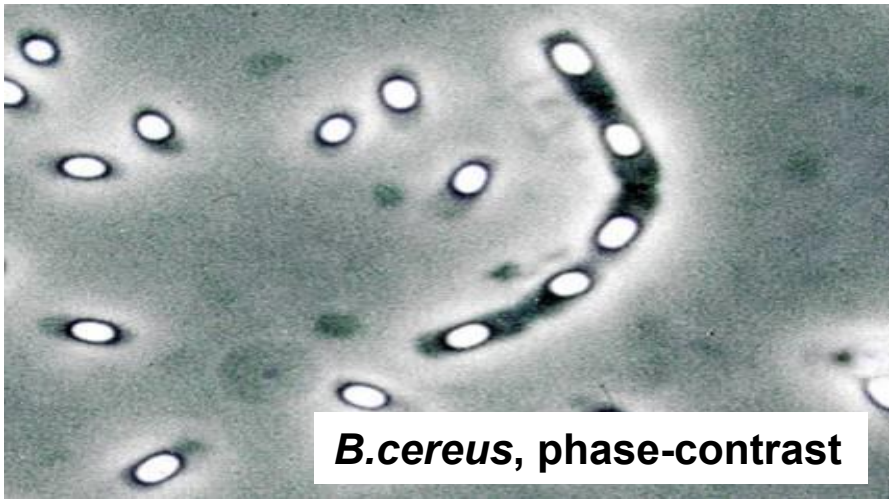
BQ10-S1

1st generation mutant:
- 10 x more activity,
- glucose-repressible

Spontaneous mutation

BQ10-SpoII

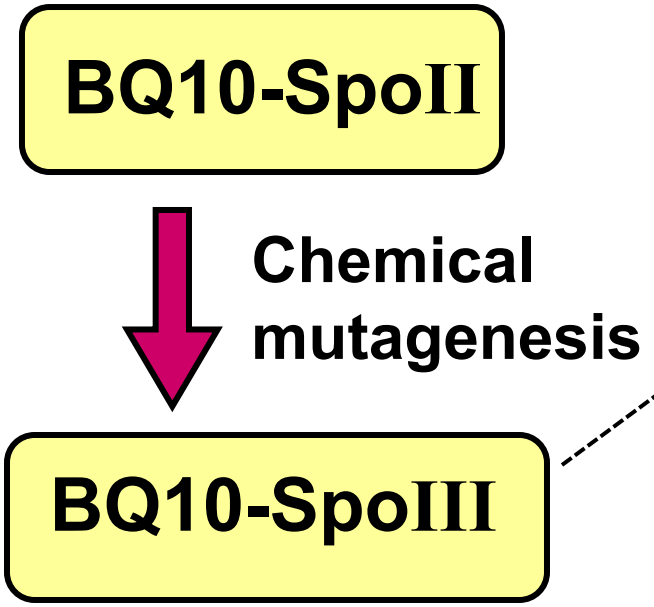
2nd generation mutant:
- 4 x more activity
- glucose-repressible



B.cereus, phase-contrast

Mutant microbes: β-Amylase in *Bacillus cereus*

… continued



3rd generation mutant:

- 5 x more activity
- constitutive expression

- Total increase in β-amylase activity from wild-type → S1 → SpoII → SpoIII strain = 10 x 4 x 5 = 200-fold !
- Expression in SpoIII no longer inhibited by glucose
- What about *B.cereus* pathogenicity? Perhaps clone improved amylase into *B.subtilis* ? ("GRAS*" microbe)

*Generally Recognised As Safe

Recombinant microbes as protein factories

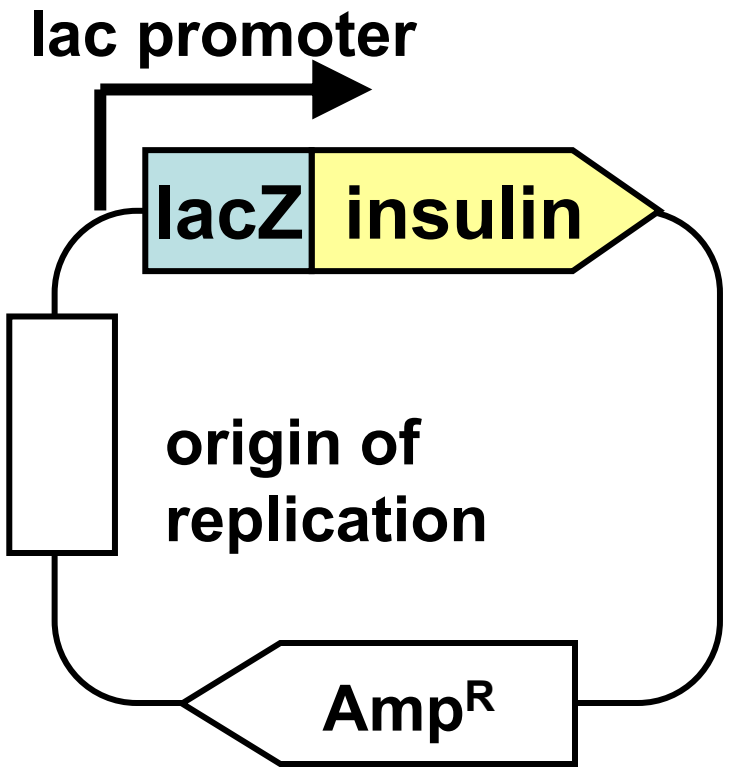
- Recombinants are especially useful for:
  - low-abundance proteins
  - proteins from difficult-to-manipulate hosts

eg. production of human insulin in *E.coli*

- synthesize insulin gene, join to plasmid pBR322 (RE / ligase)

- Transcriptional fusion to lac promoter for gene expression

- Translational fusion to LacZ protein: stability, solubility

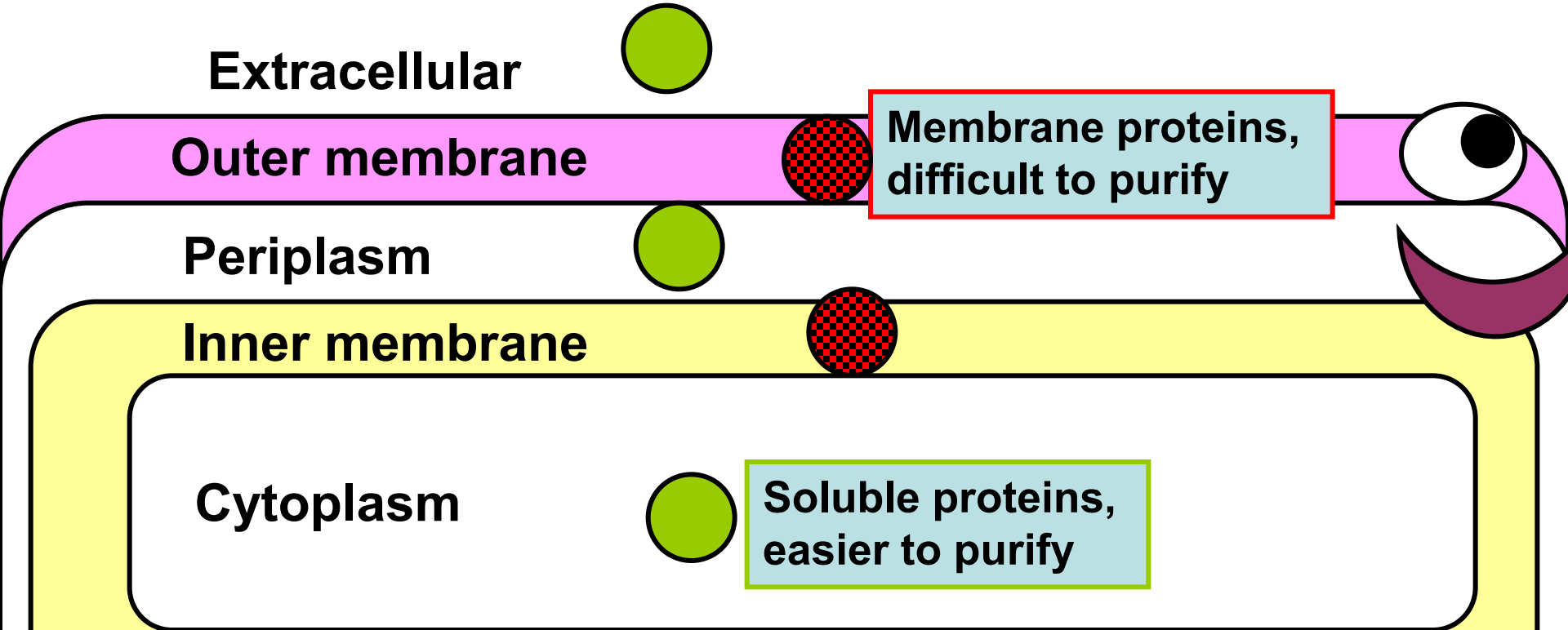


More examples of using recombinants in prev. 3042 lecture; also see later 3032 lecture.

Goeddel et al. PNAS, 1979

Protein purification

- Target protein's source and properties determine the type of purification methods that are appropriate:
  - Source: prokaryote/eukaryote, wild type/recombinant
  - Location: cytoplasm, periplasm, inner membrane, outer membrane, extracellular
  - Solubility: soluble/insoluble



Protein purification

- **Target protein's source and properties determine the type of purification methods that are appropriate:**
  - **Stability: pH, temperature, oxygen, ionic strength**
  - **Size: molecular weight, monomers/multimers**
  - **Charge: Positive/neutral/negative → isoelectric point?**

eg. Buffer for sMMO purification:

- **potassium phosphate: pH control**
- **glycerol: protection from O_2**
- **dithiothreitol: protection from O_2**
- **PMSF: protection from proteases**

Soluble methane monooxygenase (sMMO): an important enzyme for biocatalysis and bioremediation

Protein purification – first stage: Cell lysis

- Lysis methods may be physical, chemical or enzymatic
- Lysis method must be compatible with target protein
- Need to get good lysis but avoid damage to proteins
- First, separate cells by centrifugation or filtration
  → Keep cell pellet, discard growth medium (always?)

How to centrifuge
100,000 litres of culture ?

Cell lysis – physical methods

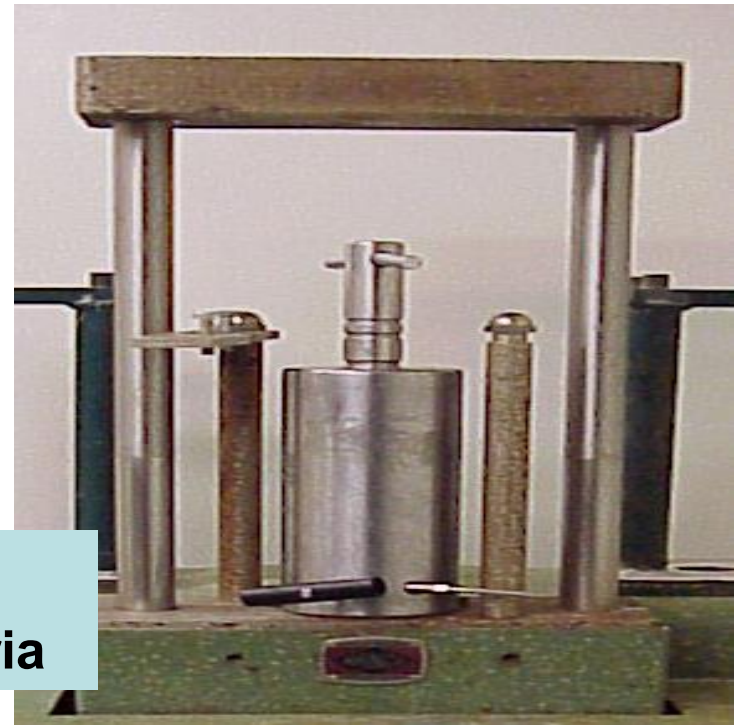
- **Sonication**: most commonly used method for *E.coli*
- **French Press**:
- **Beadbeater**:

} More rigorous than sonicator, can also lyse G+ve cells & yeast

- **Freeze/thaw**: used in combination with other methods

BEWARE!

- Heat generated by bead-beater or sonicator can denature proteins, or activate proteases



French Press:
Hydraulic shear forces lyse bacteria

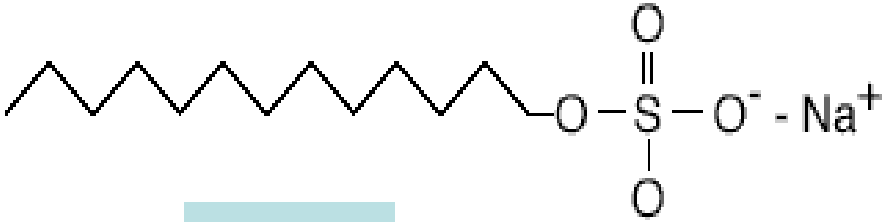
Cell lysis – chemical methods

- **Detergents (ionic) eg. SDS, CTAB. Good for DNA / RNA extraction from cells, not good for protein extraction**

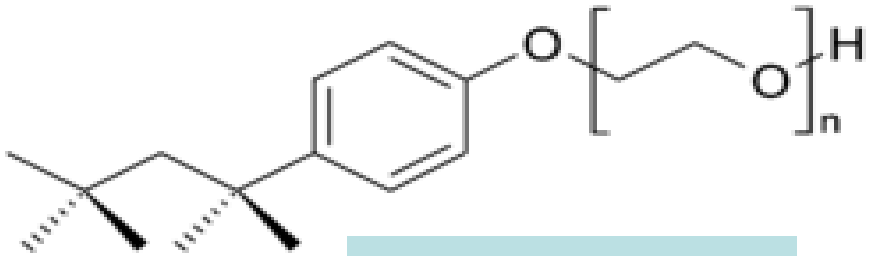
  → strong detergents DENATURE proteins !

- **Detergents (non-ionic) eg. Triton X-100. Can be used for cell lysis during protein purification in some cases**

- **Organic Solvents : eg. chloroform, toluene. Limited usefulness. May precipitate or denature proteins.**



SDS



Triton X-100

Cell lysis – enzymatic methods

- Lysozyme – useful for weakening bacterial cell wall
  – used in combination with other methods

- Zymolase – attacks fungal cell wall, useful for yeast

- DNAse/RNase - not for lysis, but useful to remove nucleic acids from lysate

- Protease – Useful for lysis when extracting DNA/RNA, but not for protein purification!

- Enyzmatic methods are very gentle (good), but add a lot of unwanted protein to the lysate (not so good)

Protein purification – Precipitation

- **Selective precipitation is useful for removing impurities AND for concentrating target protein**
- **Principle: salts or solvents compete with protein for water molecules. If you take away enough water from the protein, it will precipitate.**
- **Precipitate protein: ammonium sulfate, ethanol, acetone**
- **Precipitate nucleic acids: streptomycin, protamine**

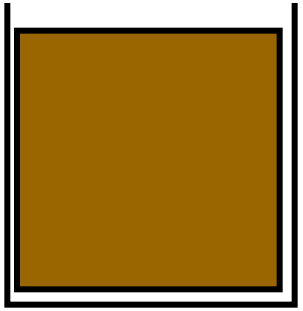
Acid precipitation of urine proteins

Many chemicals will precipitate protein, but for purification, this needs to be REVERSIBLE!

Protein purification – Precipitation

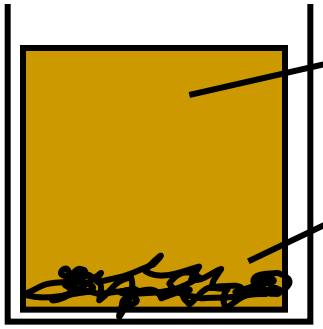
EXAMPLE

eg.



intial cell lysate

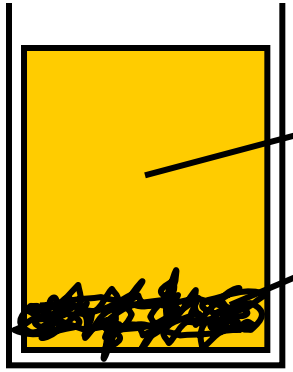
Add streptomycin to 10 mg/ml



supernatant → keep

DNA+RNA ppt. → discard

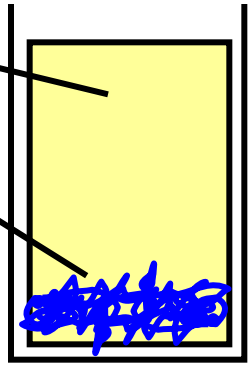
Add $(NH_4)_2(SO_4)$ to 30%



supernatant → keep

Non-target protein ppt. → discard

Add $(NH_4)_2(SO_4)$ to 60%



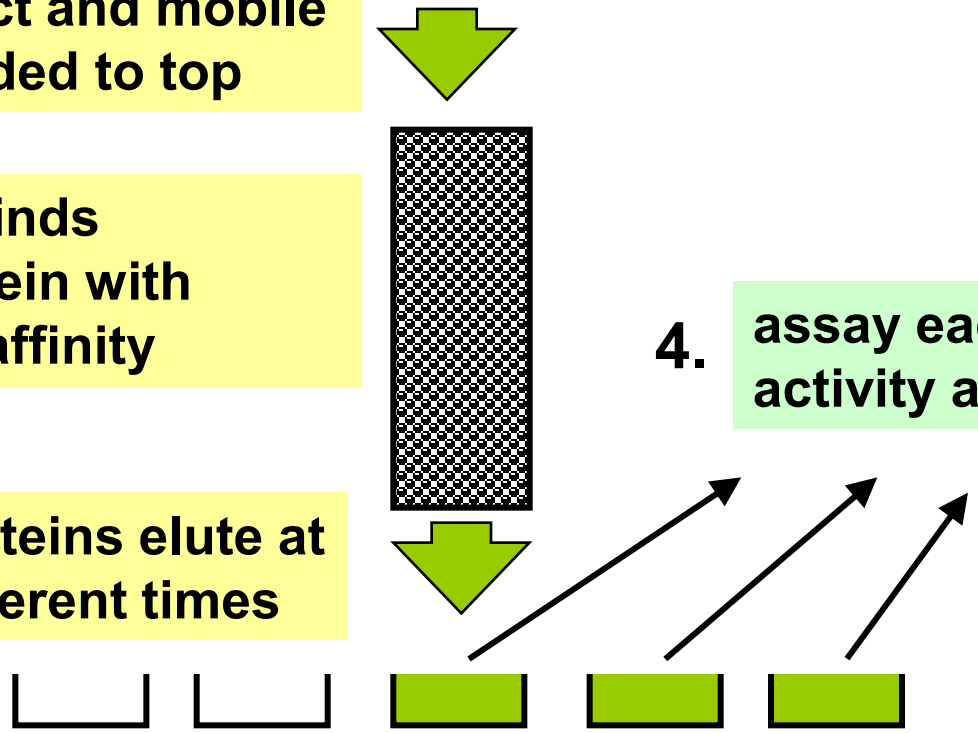
supernatant → discard

Target protein fraction in ppt. → keep

Protein purification – Chromatography

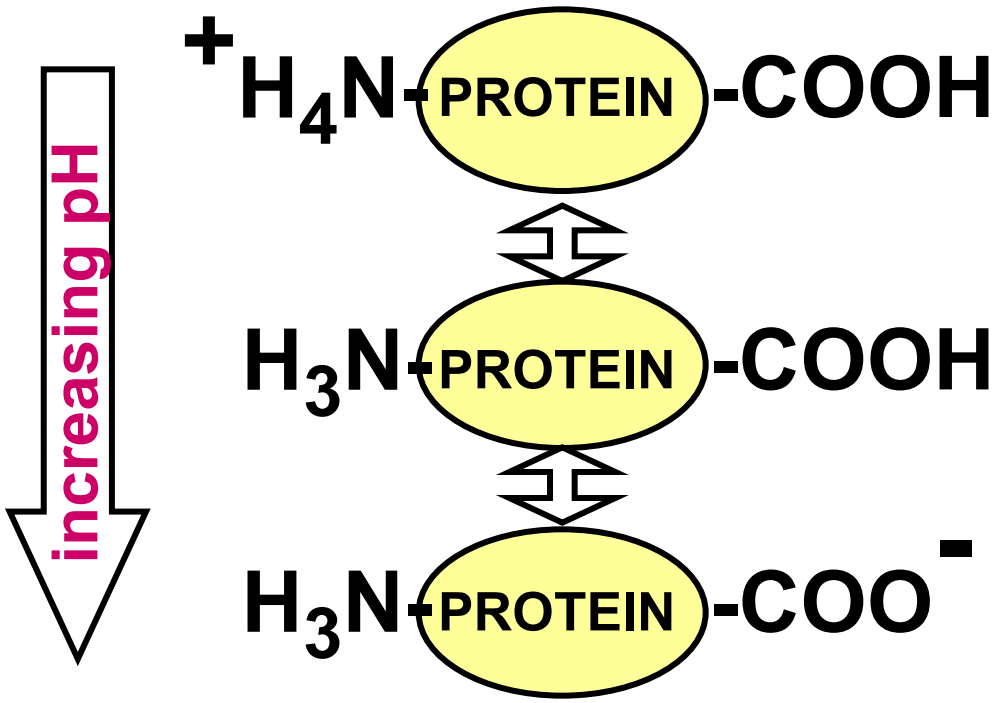
- Selective binding of proteins to a solid stationary phase, and elution with liquid mobile phase
- Depends on differences in charge, size, hydrophobicity or substrate/cofactor specificity between protein of interest and background proteins

1. cell extract and mobile phase added to top
2. column binds each protein with different affinity
3. proteins elute at different times
4. assay each fraction for activity and protein conc.



Protein purification – Ion Chromatography

- Separation based on CHARGE
- Stationary phase: anion- or cation-exchange resin
- Mobile phase: gradient of salt concentration
- pH and isoelectric point are critical: determine charge of protein, and which stationary phase is useful



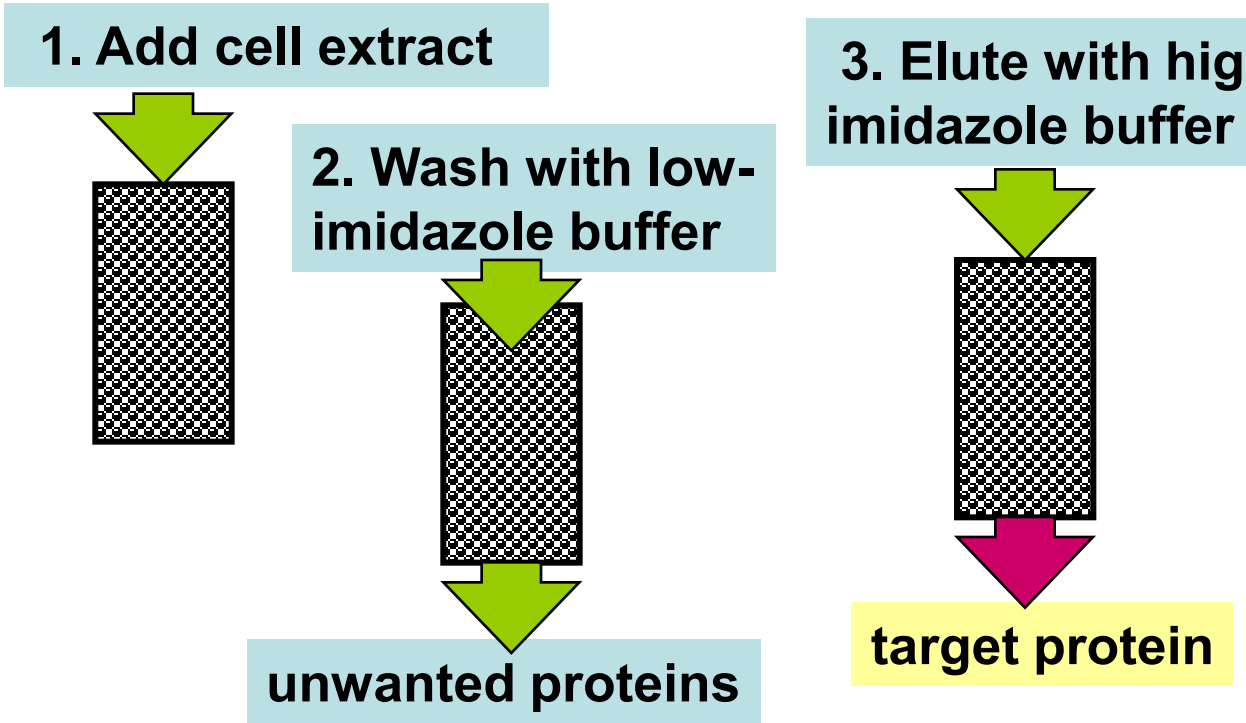
Below isoelectric point
→net positive charge
→use cation exchange

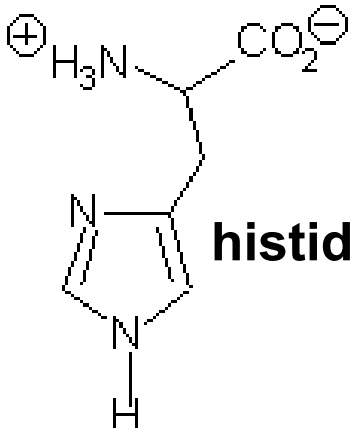
At isoelectric point
→ charges balance

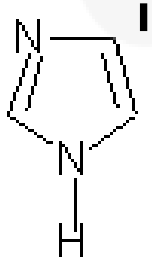
Above isoelectric point
→net negative charge
→use anion exchange

Protein purification – Affinity Chromatography

- Separation based on SPECIFIC biological interactions
- Stationary phase: gel beads linked to substrate/cofactor
- Mobile phase: increasing gradient of agent that competes for binding of substrate/cofactor or binding of enzyme
- eg. nickel-agarose specifically binds to histidine residues in protein, or to imidazole in mobile phase

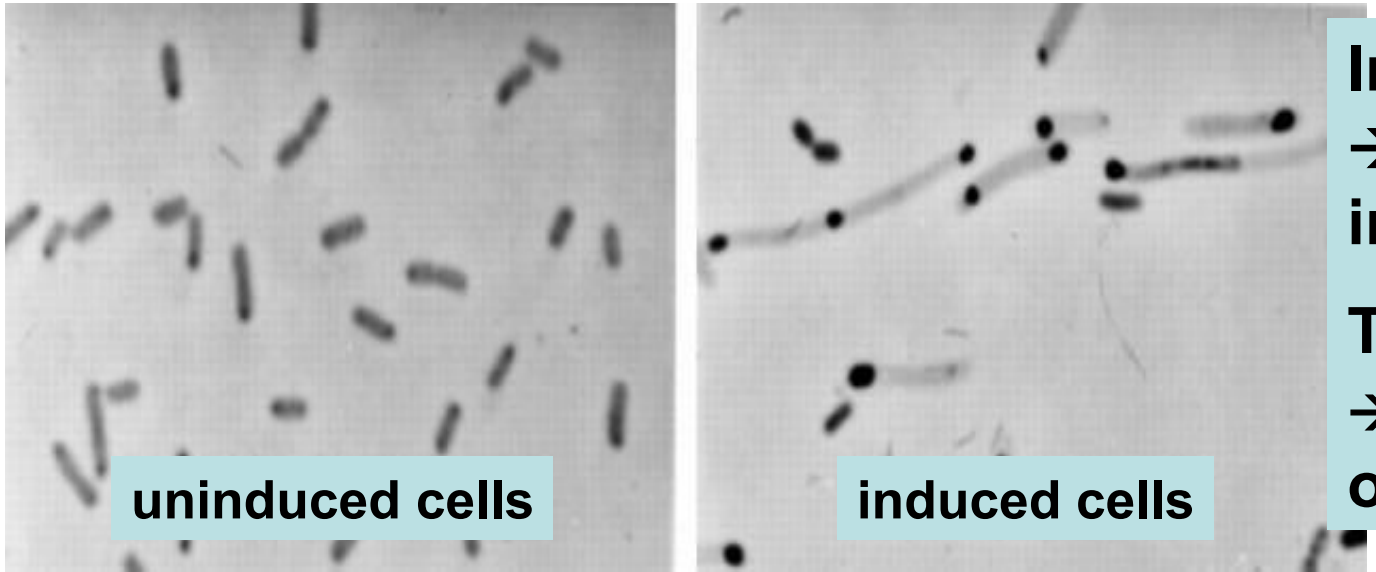






Challenges for protein purification

- **Membrane proteins – toxic and insoluble !**
- **many important proteins are located in cell membrane eg. transporters for nutrients (in) and toxins (out)**
- **overexpression of membrane proteins in recombinants is difficult due to high host toxicity**
- **purification of membrane proteins also difficult → insolubility, irreversible binding to columns**



Insolubility → Formation of inclusion bodies

Toxicity → Filamentation of cells

Picture credits

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