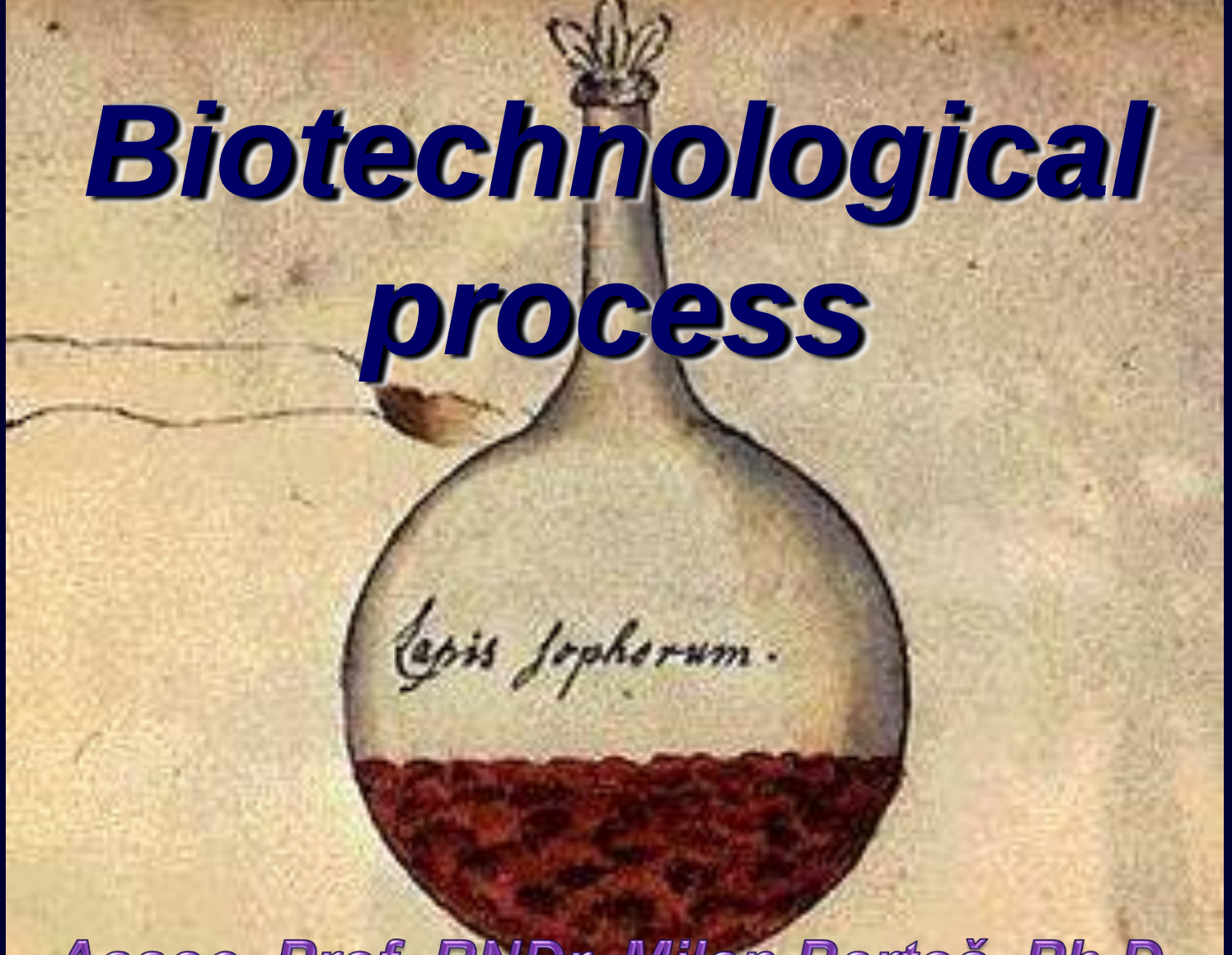
Biotechnological process



Assoc. Prof. RNDr. Milan Bartoš, Ph.D.

Medecina tertii ordinis

Biotechnology of Drugs 2024

Content

1) **Basic definition**
2) **Materials for the fermentation process**
3) **Sterilisation**
4) **Bioreactors and fermenters**
5) **Cultivation of microorganisms**
6) **Downstream processing**
7) **Separation and isolation methods**

Biotechnological process

The main products are

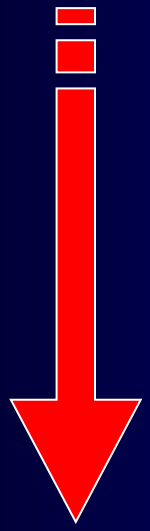
- ***Biomass***

- ***Extracellular product, i.e. metabolite***

metabolite - primary
- secondary

Phases of biotechnological process

SUBSTRATE

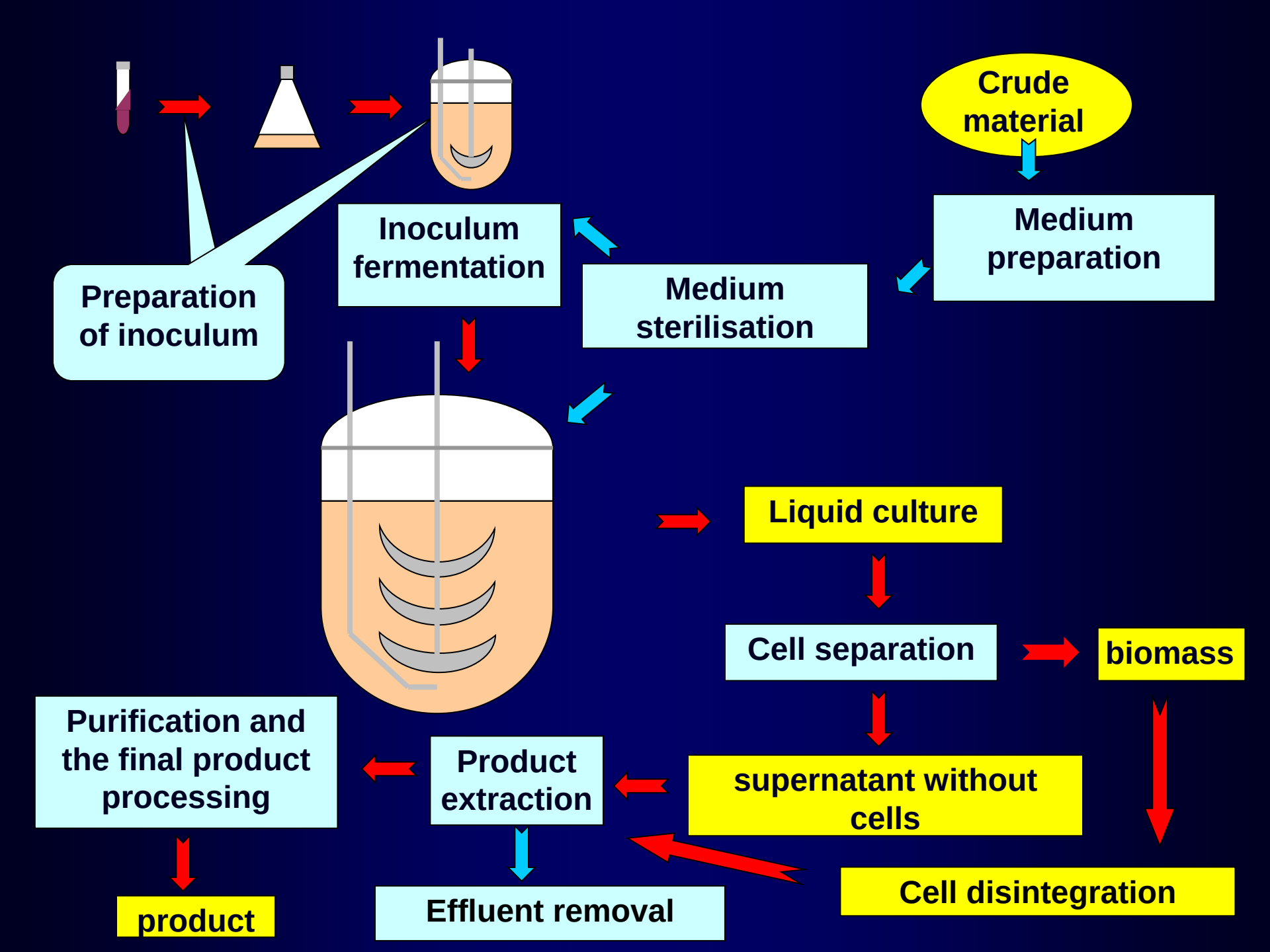


PRODUCT

Phase 1 (“upstream processing“)

Phase 2 - bioprocess

Phase 3 (“downstream processing“)


Crude material
Medium preparation
Medium sterilisation
Inoculum fermentation
Preparation of inoculum
Liquid culture
Cell separation
biomass
supernatant without cells
Product extraction
Purification and the final product processing
product
Effluent removal
Cell disintegration

Materials for the fermentation process

1. **Water**
2. **Air**
3. **Sources of carbon**
   - **Saccharides**
   - **Complex substrates**
   - **Plant oils and animal fats**
   - **Petrochemical sources**
   - **Synthetic alcohols**
   - **Organic acids**

Water in the fermentation process

Normal drinkable, treated water

(deionised water)

- **Preparation of growth media**
- **Washing of biomass**

Technical water

- **Cooling of the growth media**
- **Regulation of the cultivation temperature**
- **Washing of the equipment**

Macro elements in the fermentation process

Air

- Aerobic processes, mixing, aeration

Sources of nitrogen

- Ammonia, ammonium salts
- Amino acids, urea
- Corn-steep liquor, plant flours
- peptone, yeast extract

Phosphate sources

- Inorganic phosphorus (K_3PO_4, Na_3PO_4, $(NH_4)_3PO_4$)
- Natural sources (corn-steep liquor, peanuts flour, soya flour, waste from meat and fish processing, bones

Microelements in the fermentation process

Sources of other important elements

- **Biogenic elements (K, S, Ca, Mg, Na)**
- **Trace elements (Fe, Zn, Mn, Cu, Co…)**
- **Inorganic salts, mostly sulphates and chlorides**
- **industry (Corn-steep liquor, soya and peanuts flours, beet molasses, whey …)**

Growth factors, precursors, protective compounds

- **Vitamins (B = food yeasts), amino acids (as pure chemicals or natural sources)**
- **Precursors (adding of phenylacetic acid or phenyl acetamide) improve the yield of penicillin G**
- **Buffers (maintenance of pH, e.g. $CaCO_3$)**
- **Antibiotics (if they don't interfere with production and purification processes)**

Antifoam agents

- **Foaming is a typical accompanying feature of most industrial substrates used for fermentation in which high concentration of compounds is present**
- **Foam structure is influenced by several factors (pH, temperature, viscosity, …)**
- **Each substrate influences foaming**

- **Antifoam agents (natural plant and animal or synthetic) are frequently used as sources of carbon**
- **They usually work at low concentrations and for a long time, must not be toxic to the organism**

- **Natural oils and fats, higher alcohols, derivatives of sorbitol, polyether's and various silicones are used**

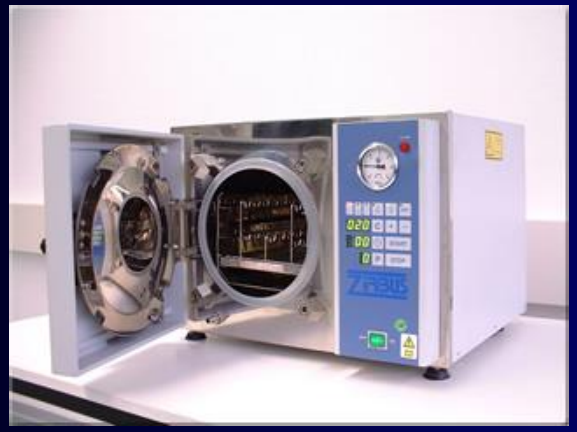
Sterilisation

The aim of sterilisation is to remove all microorganisms

Methods of sterilisation

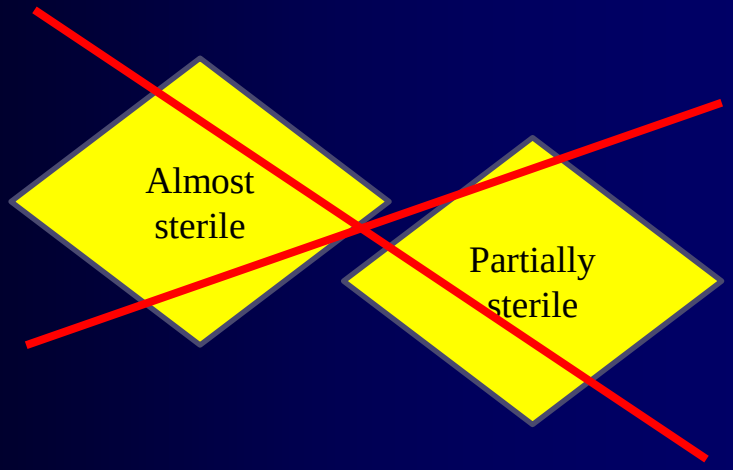
- **Heat**
- **Filtration**
- **Chemically (β-propiolactone, ethylenoxide, propylenoxide and glutaraldehyde)**
- **Radiation (RTG, β-waves, UV light and ultrasound)**

Sterilizers



The concept of sterilisation

Sterilisation is an absolute concept = there are no degrees of sterility



Partially?

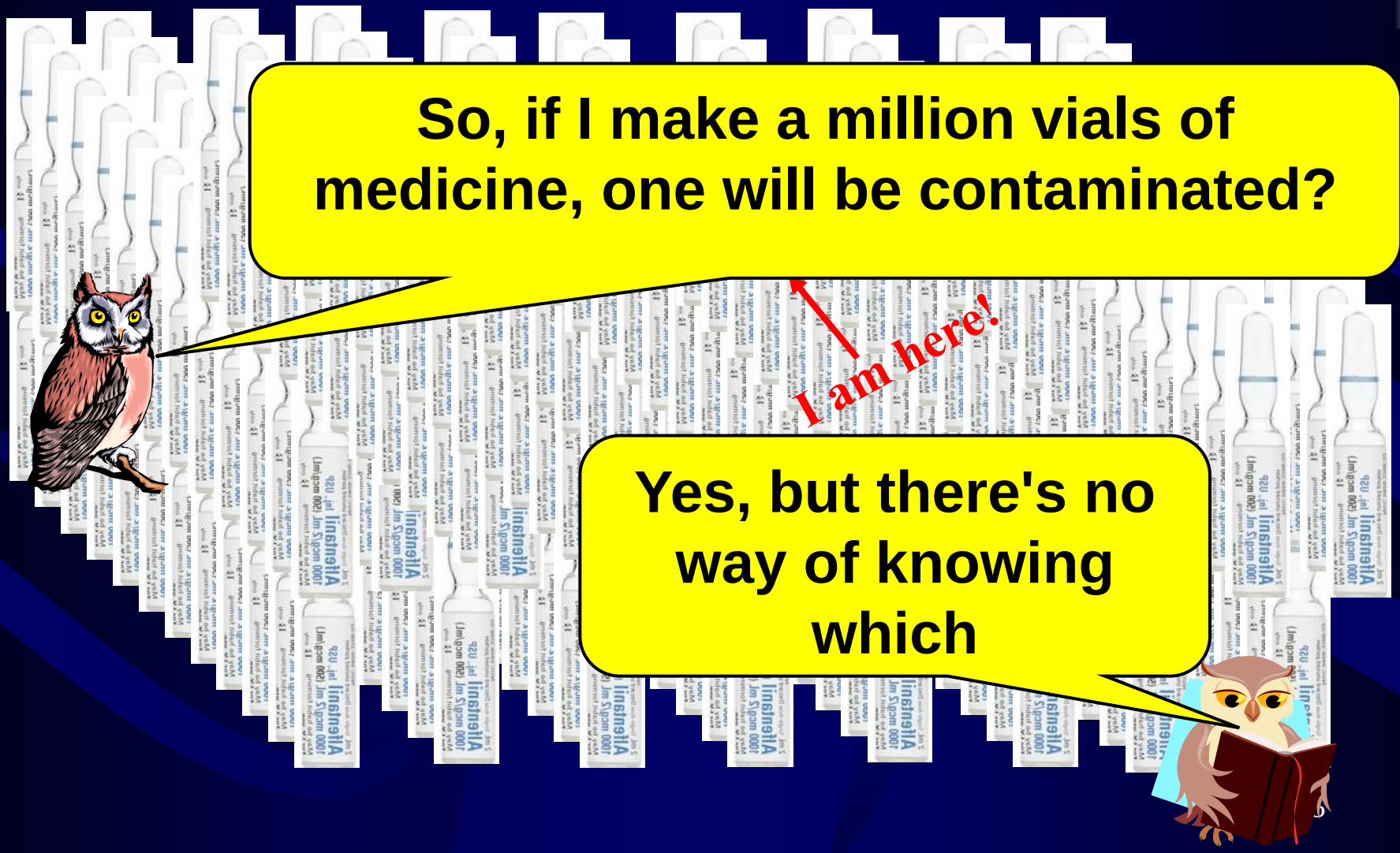
!!! We cannot guarantee sterility, only express the probability of sterility !!!

Probability of sterility

It is expressed as 10^{-6}, i.e. 1 surviving microorganism per million

A sterility probability of 10^{-6} means that there is a 1 in a million chance that the item is still contaminated

Probability of sterility



Sterilisation of growth media and tools - I

Destruction of microorganisms by heating

- **Temperature denaturation of one or more enzymes, which have essential functions in the organism**
- **The speed of resulting inactivation is influenced by:**
  - **Environment (amount of water, growth medium pH, concentration of solutes, etc.)**
  - **Physiological state of cells**

Spores are highly heat resistant

Sterilisation of growth media and tools - II

Batch sterilisation by heating

- **The growth medium is heated directly in a reaction vessel (bioreactor)**
- **After the exposure to a high temperature, the content of the reaction vessel is cooled**
- **Heating is direct by hot steam or by heat exchanger**

- **Efficiency of sterilisation depends on the temperature and time of sterilisation**

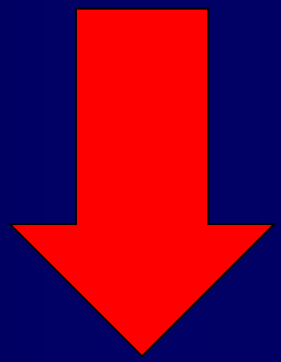
121 °C…… 15 min
126 °C…… 10 min
134 °C…….. 3 min

Given temperatures correlate with the pressure of saturated vapour

Sterilisation of the growth media and tools - III

Continuous sterilisation by heating

- **Cost effective method, less steam and cooling water is necessary**
- **Shorter sterilisation time = 5-8 min**
- **Higher temperature = 135°C**
- **Heat-labile compounds in medium are less degraded**
- **More correct and automatic regulation of the process**



Sterilisation of the growth media and tools - III

Continuous sterilisation by heating

1) **Direct steam is transported to the liquid medium through pipes, then the material travels into the expansion tank, where it is rapidly cooled**
2) **Heating and cooling of the plate heat exchanger, time is shortened to approximately 20 s – 5 min, sterilisation temperature 135 °C is sustained for about 2 – 3 min**

Continuous sterilisation is suitable for complex media, which don't contain solid phase but may contain heat-labile growth factors

Sterilisation of the growth media and tools - IV

Medium sterilisation by filtration - I

- **Only in media, which contain heat-labile compounds and sterilisation by heating is therefore not possible**
- **Only soluble compounds may be present**
- **Filters have pores 0.2 μm in diameter**

Sterilisation of the growth media and tools - V

Medium sterilisation by filtration - II

- **It is necessary to sterilise the equipment before the medium sterilisation**
- **Sterilisation is performed by heating, vaporing, chemically or by UV lighting**
- **The equipment is usually distributed sterile directly from producers**

Sterilisation of the growth media and tools - VI

Bioreactor sterilisation

- **Bioreactor must be sterilised when empty if continuous sterilisation of media or filtration are used**
- **Hot steam (121 °C)**
- **Hot air (150 – 180 °C)**
- **Chemically**

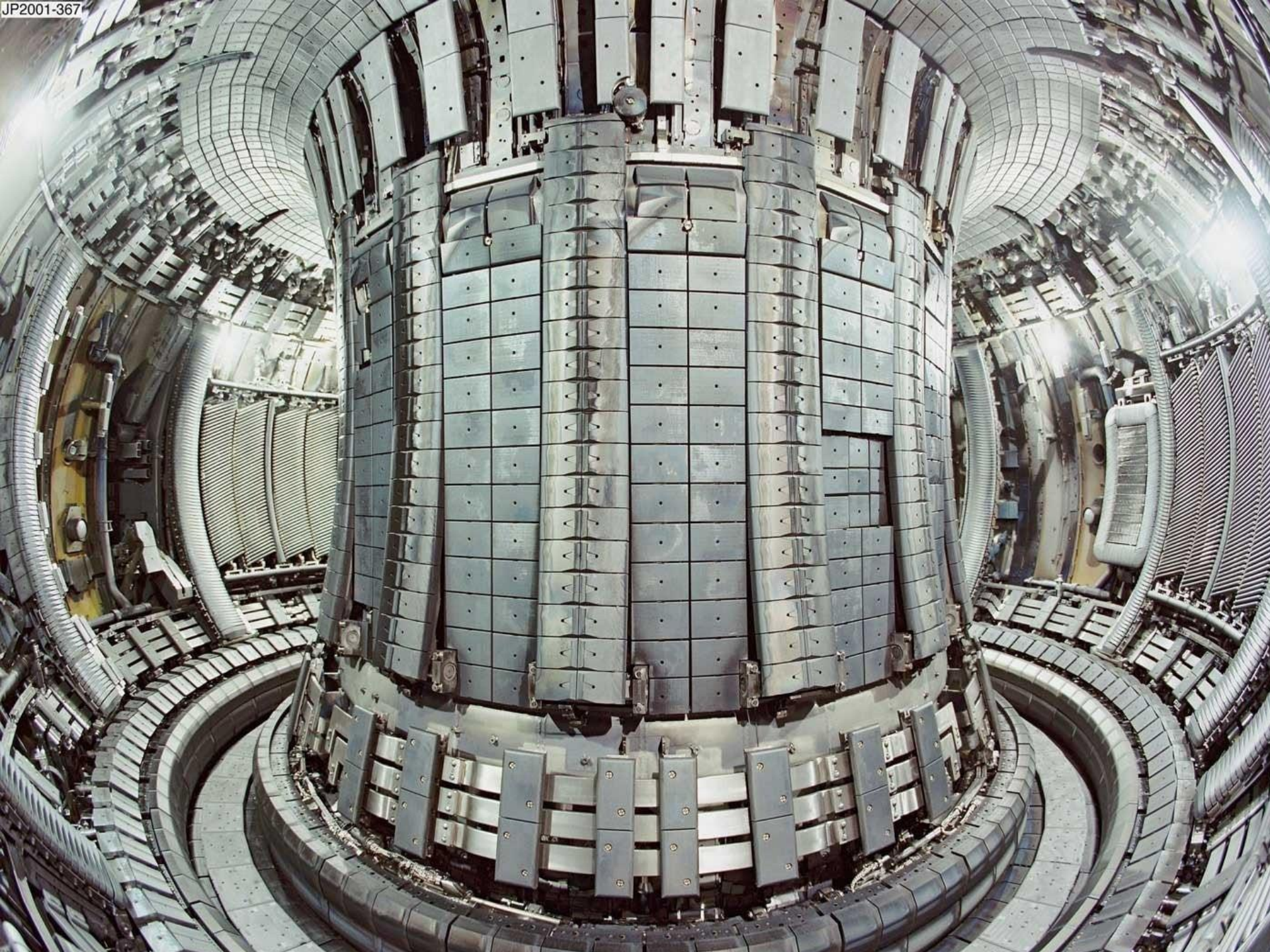
Air sterilisation

- **heating,**
- **UV lighting**
- **Electromagnetic waves**
- **Filtration – mostly in industry due to financial reasons**

Rough pre-filtration of air – porous materials, e.g. Powder coal and lignite coke, glass fibres

Filtration

- **On membranes (nitrocellulose)**
- **In-depth = air flows through a thick layer (several dozens of cm) of filter (glass fibres, nitrate cellulose, teflon, nylon or polyacryl)**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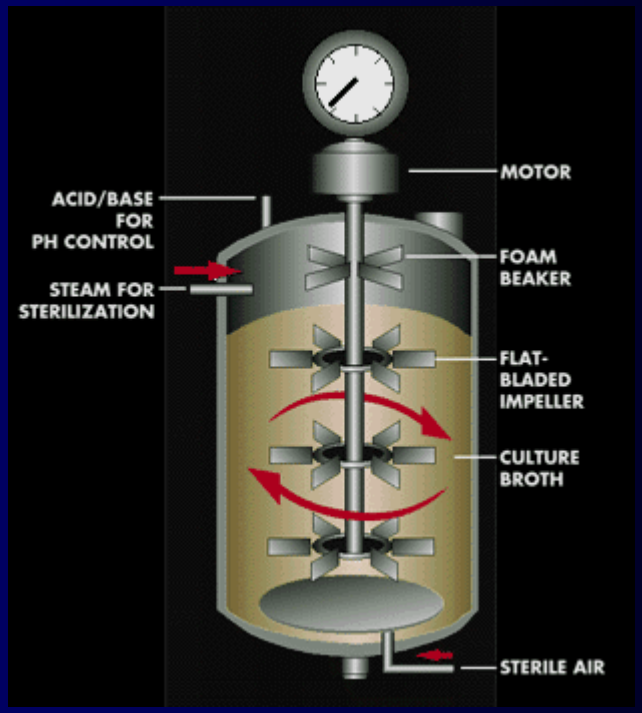
Bioreactors and fermentors

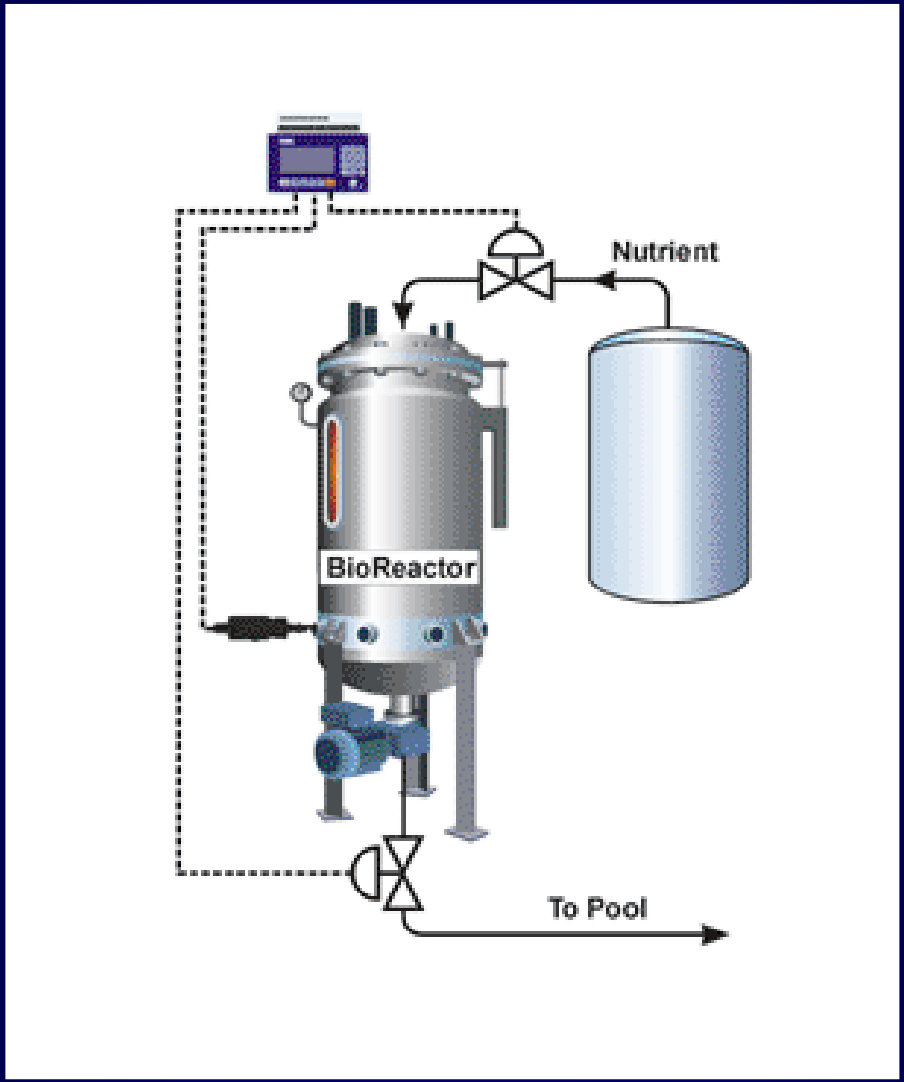
Bioreactor (fermentor) = heart of any production line in the biotechnological process

Basic parts of the bioreactor

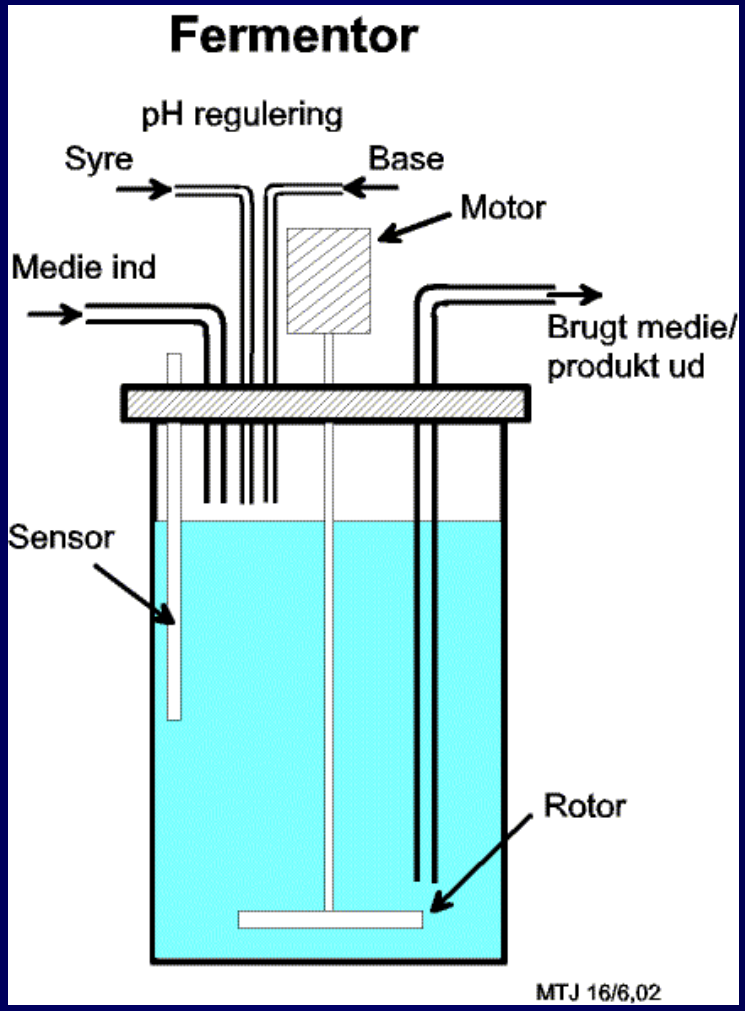
- Inlet and outlet of the growth media
- Inoculum supply
- Motor-driven impeller
- Air supply valve
- Sample collector
- Heating system
- Thermometer, manometer, pH, O_2 and CO_2 levels controlling devices, etc.

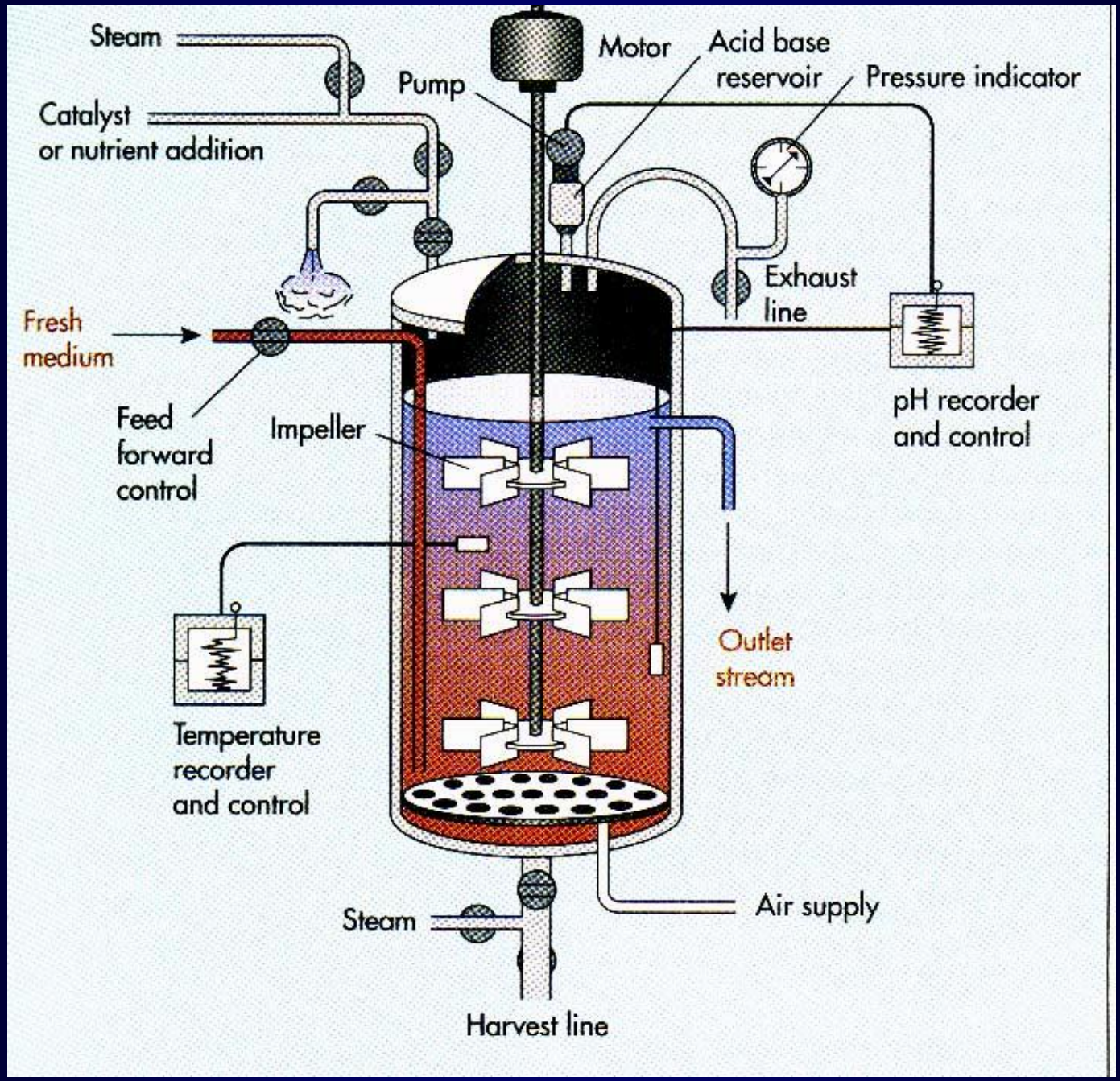


Material flow through the bioreactor/fermentor



Bioreactor/fermentor scheme




Steam
Catalyst
or nutrient addition
Pump
Motor
Acid base
reservoir
Pressure indicator
Exhaust
line
Fresh
medium
Feed
forward
control
Impeller
pH recorder
and control
Outlet
stream
Temperature
recorder
and control
Steam
Air supply
Harvest line

Types of bioreactors

For cultivation of freely growing cells,
immobilised cells,
enzymes

According to the process type: batch, semi-continuous (fed) and continuous

According to the size: laboratory, pilot plant and operational

According to the shape: cylindrical, with spherical bottom, circulatory, tower-like

According to the manner of stirring: fitted with mechanical, pneumatic or hydrodynamic stirring

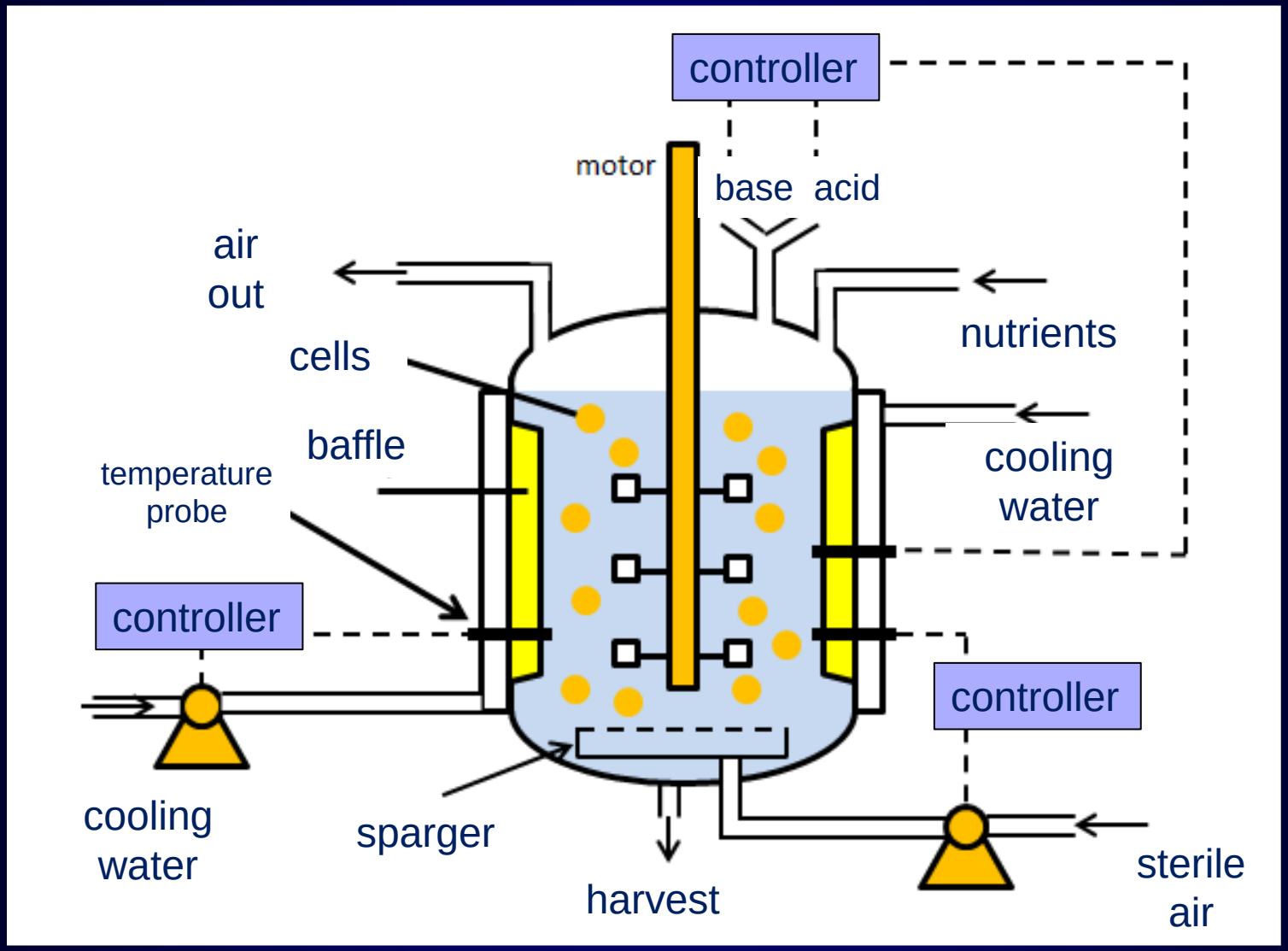
Non-sterile, sterile (special)

Liquid medium, solid medium etc.

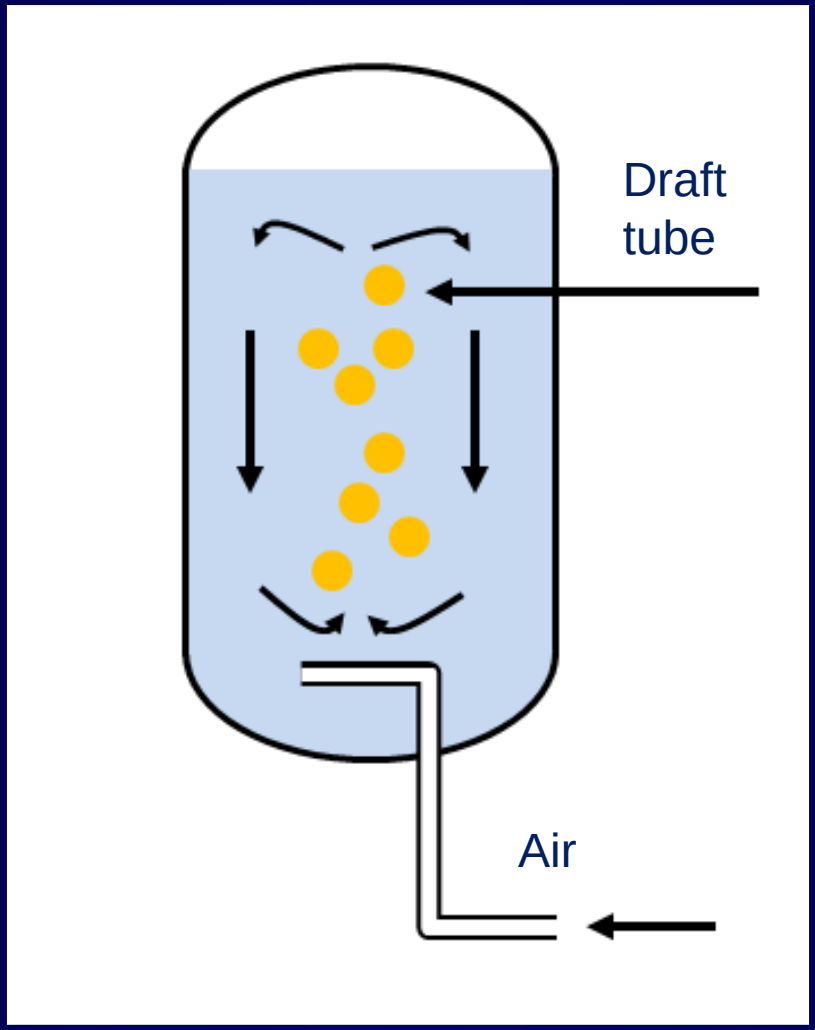
Division of bioreactors according to cultivation method

- **Stirred-tank**
- **Airlift bioreactor**
- **Fixed-bed bioreactor**
- **Membrane bioreactors (e.g. hollow fibre perfusion bioreactor)**

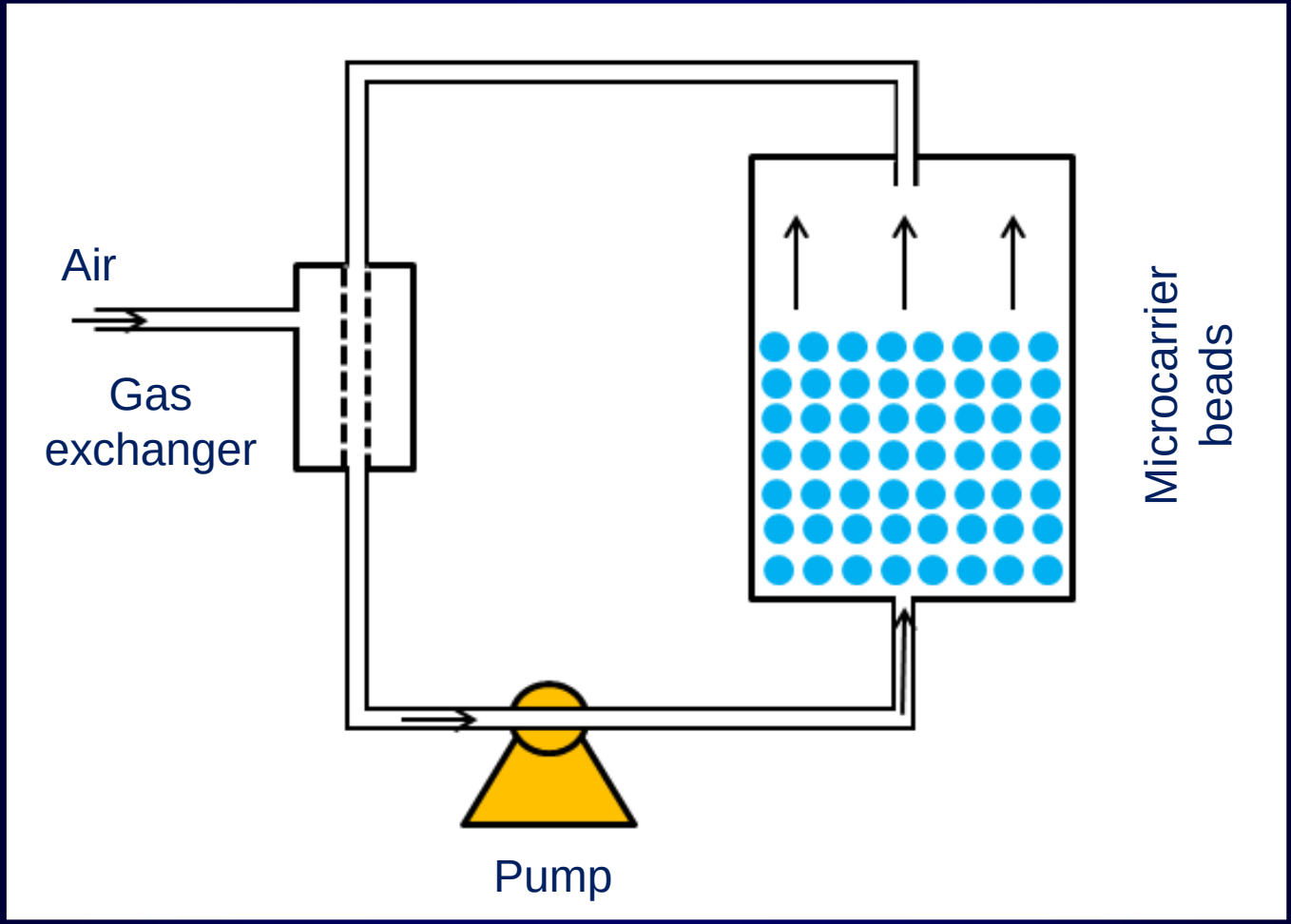
Stirred-tank



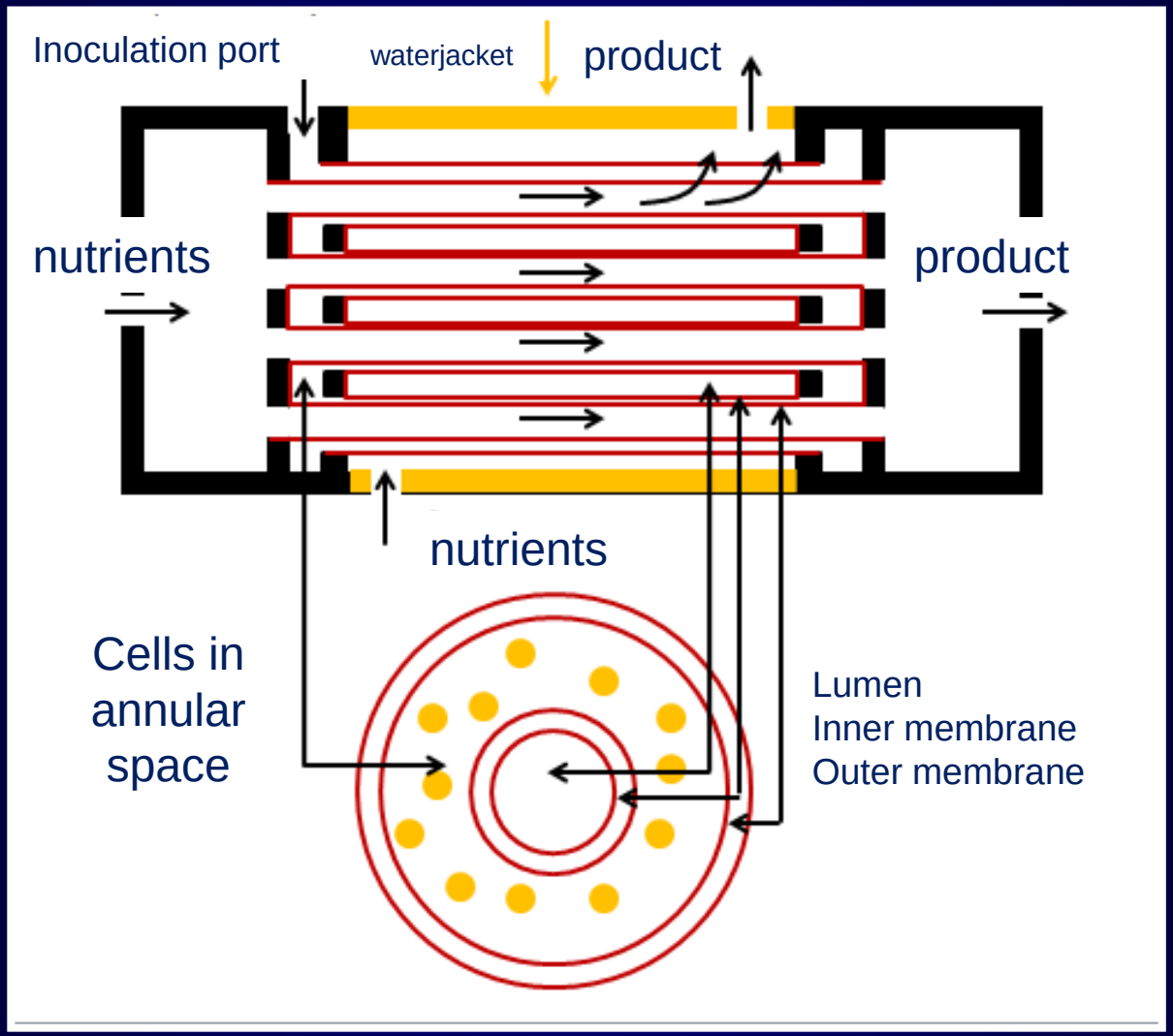
Airlift bioreactor



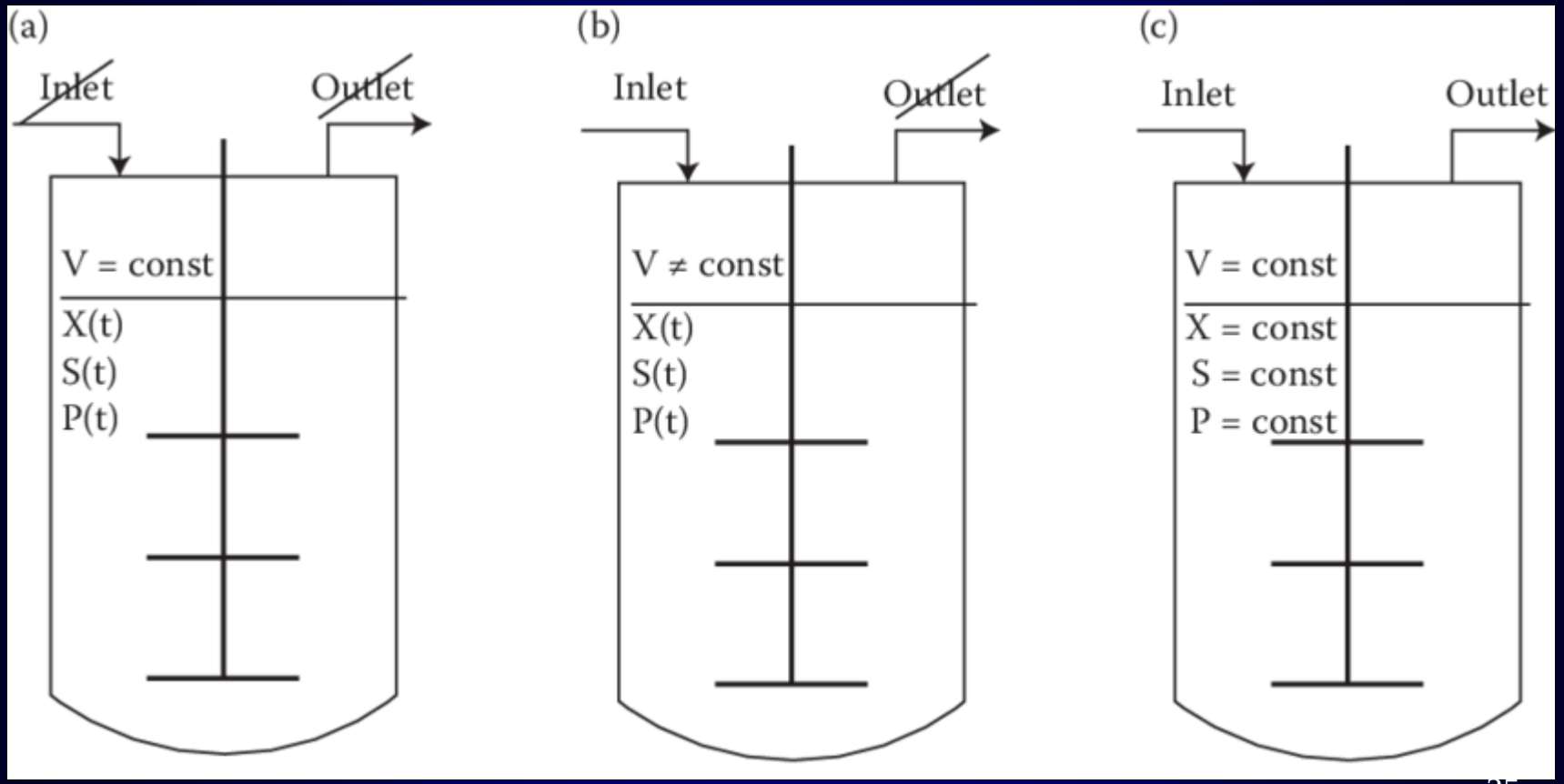
Fixed-bed bioreactor



Membrane bioreactor

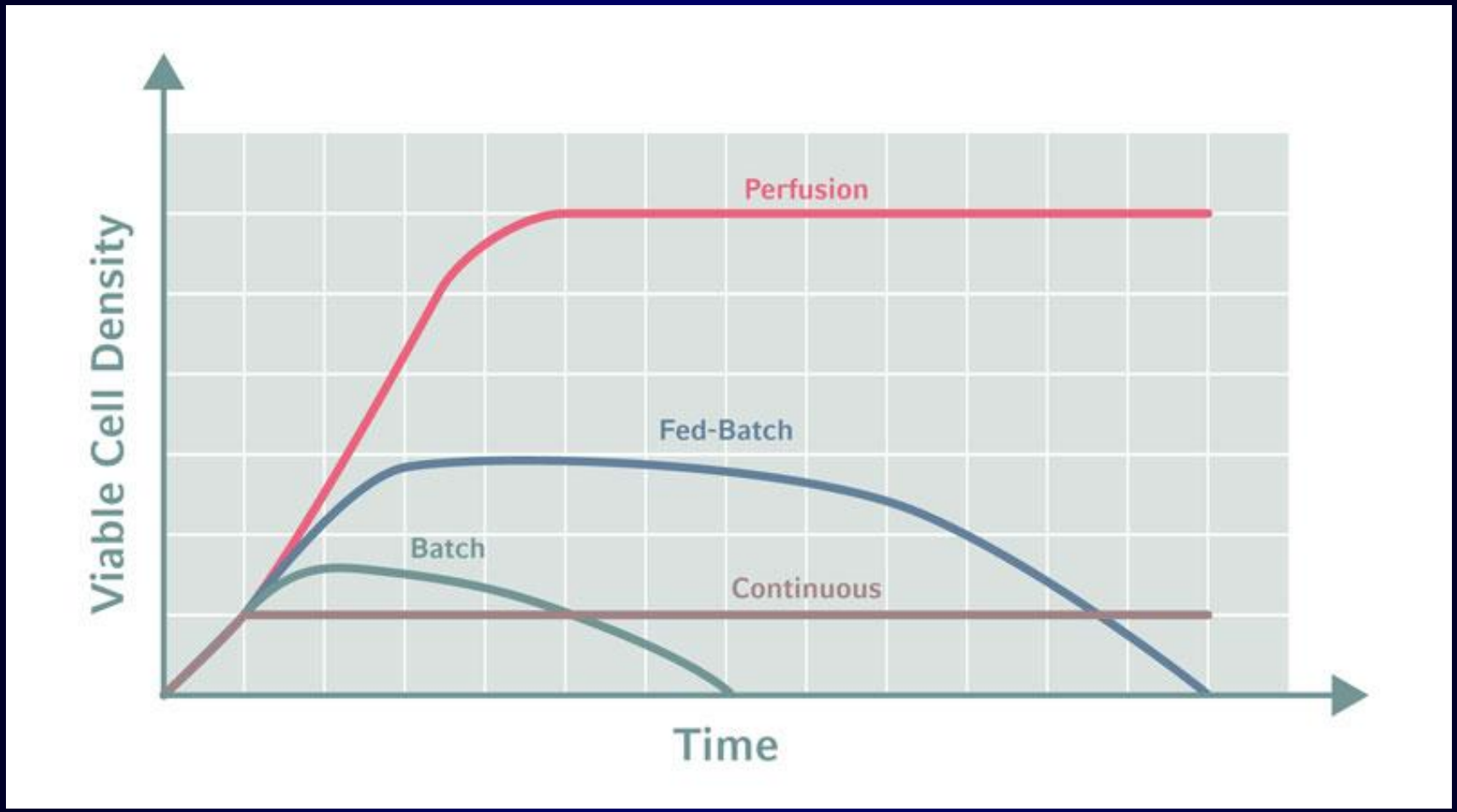


Classification of bioreactors according to cell growth kinetics and product formation



Batch **Fed-batch** **Continues**

What do the growth curves look like then



Examples of bioreactors

Cultivation of microorganisms

- **Continuous- log phase is prolonged → steady state**

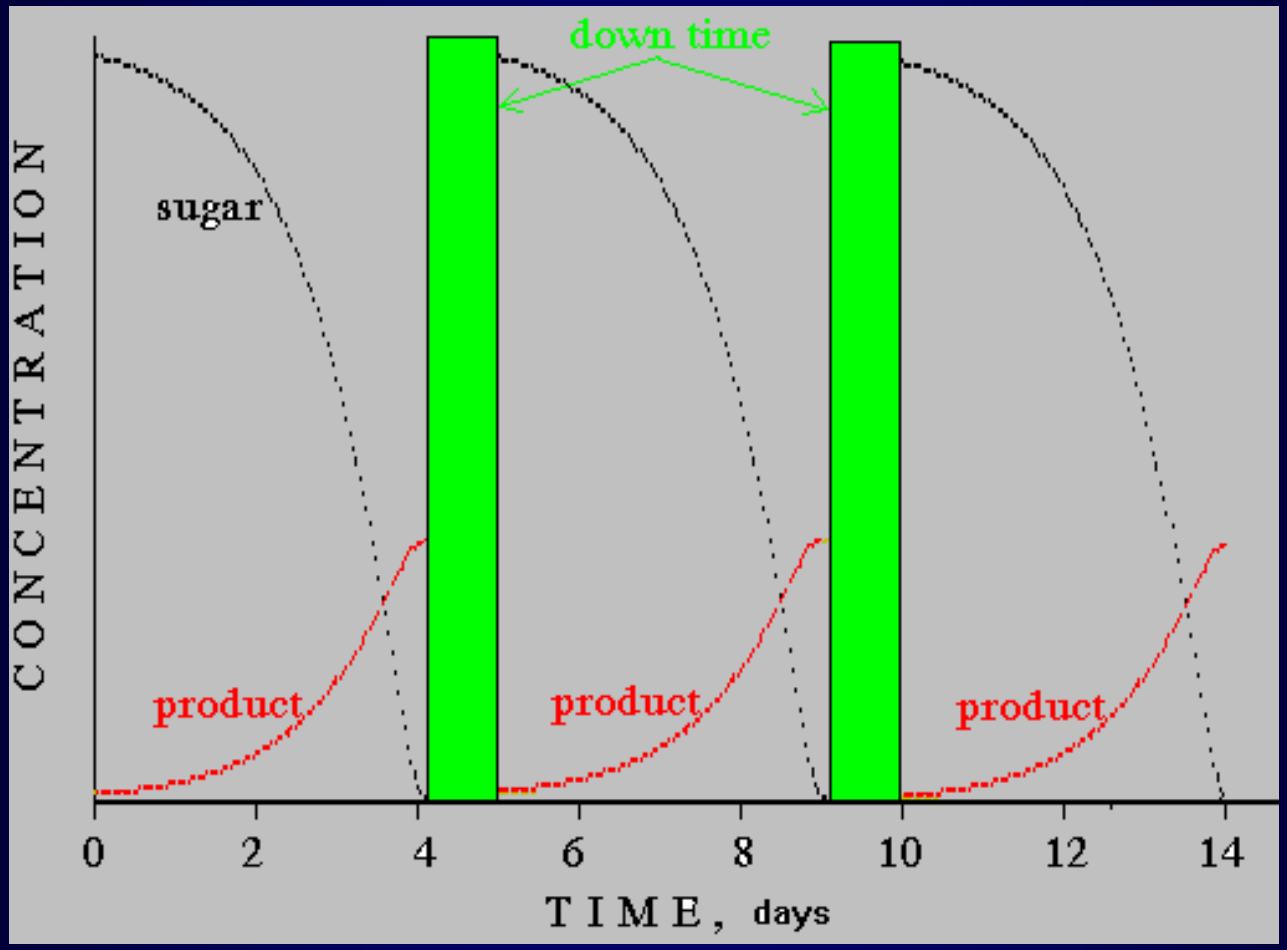
  (large volume of cultivation media, sewage purification)

  - **chemostat**
  - **turbidostat**

- **Dis-continuous (one-shot, "batch") → after nutrients exhausting the growth is slowed**

- **Semi-continuous ("fed batch") → periodical adding of nutrients, gradual growth slowing (production of yeast)**

Cultivation with continuous exchange



Types of cultivation

- **Submersion**

- **Surface cultivation on liquid medium – especially for filamentous microorganisms, e.g. *Aspergilus niger* – producing citric acid**

Requirements for materials used in the construction of fermenters

- **Corrosion resistant – no metals must be released into the media**
- **Non-toxic to the cell population**
- **Can be sterilised by highly pressurized steam**
- **Resistant to deformation – stirrers, inlet valves**
- **Transparent materials (glass)**

Inoculum preparation

- **Inactivated cell cultures are transfered, which is in into the growth medium, where the cells start to proliferate and reproduce**
- **From several thousands of cells → to several hundreds liters of culture**
- **Transfer into bioreactor is performed at the end of the log growth phase**
- **Inoculum is used in an amount of approximately 5% of the volume of the growth media**

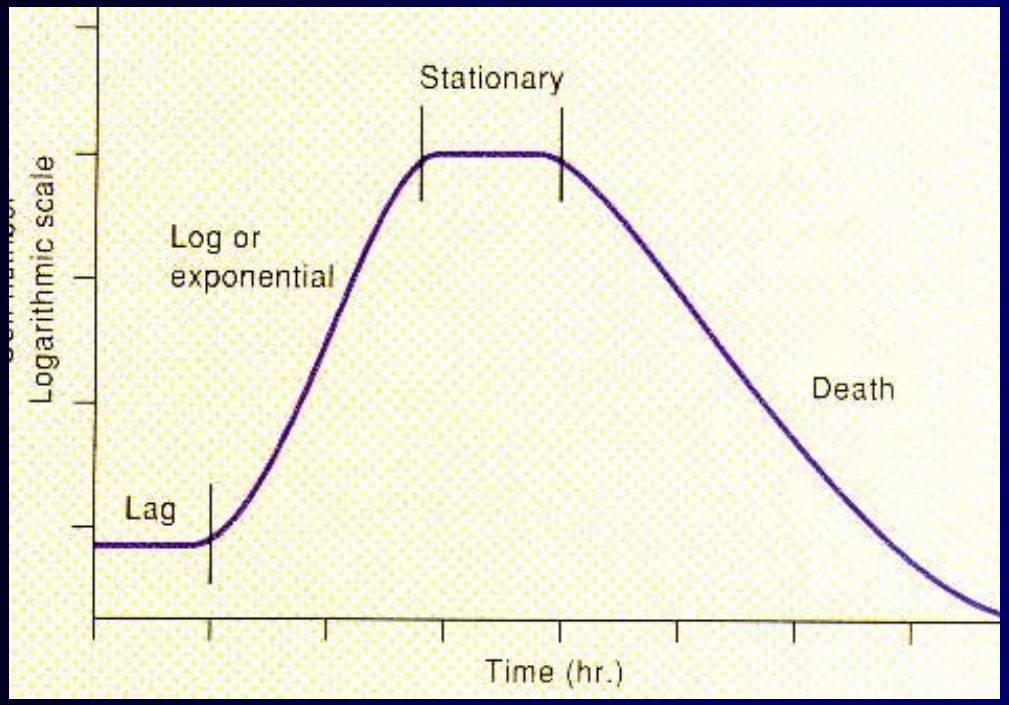
Process of inoculation

- **Transfer of inactivated cells into the fresh media**
- **Prevention of contamination of the media**
- **Monitoring of quantity and physiological stage of cells (growth curve)**

Growth monitoring

- **Changes of pH**
- **Oxygen depletion**
- **Change of cell weight after centrifugation**

Growing curve



- **Lag-phase**
- **Phase of accelerated growth**
- **Log or exponential phase**
- **Phase of decelerated growth**
- **Stationary phase**
- **Death phase**

Log phase

- **The most suitable from the technological perspective**
- **Time-limited**

Measurement and regulation of the biotechnology process

Physical

- Temperature, steam, water and air pressure, power input, foam formation and its amount, gases and liquids flows

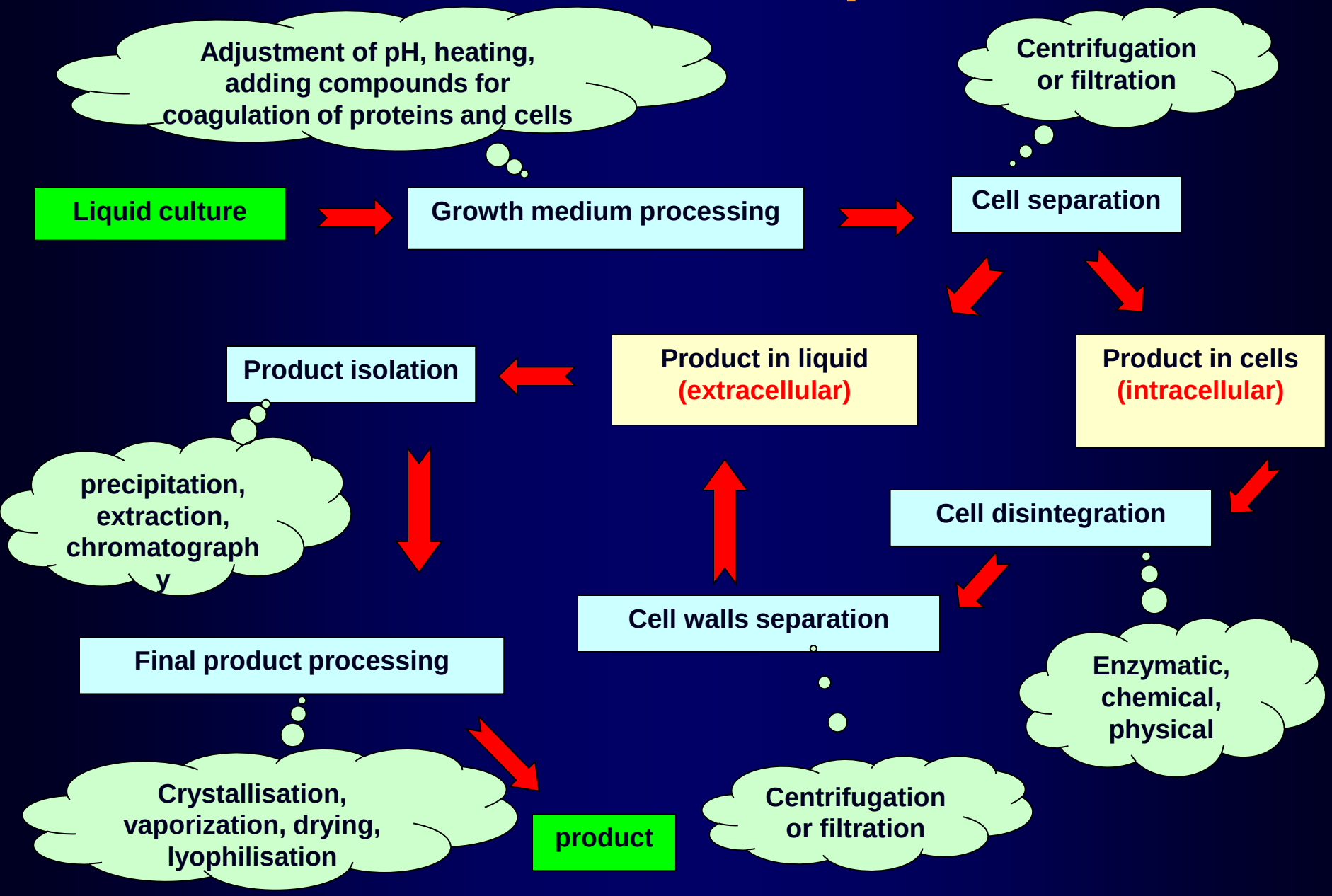
Physico-chemical

- pH, redox potential, amount of soluble oxygen, chemical agents (measurement of concentration of growth stimulators and inhibitors or products formation - C, N, P, S, Mg, K, Na, Fe, growth regulators, precursors, etc. Measurement of NH^{4+}, Mg^{2+}, Na^{+}, Ca^{2+} and PO_4^{3-} concentration by specific electrodes)

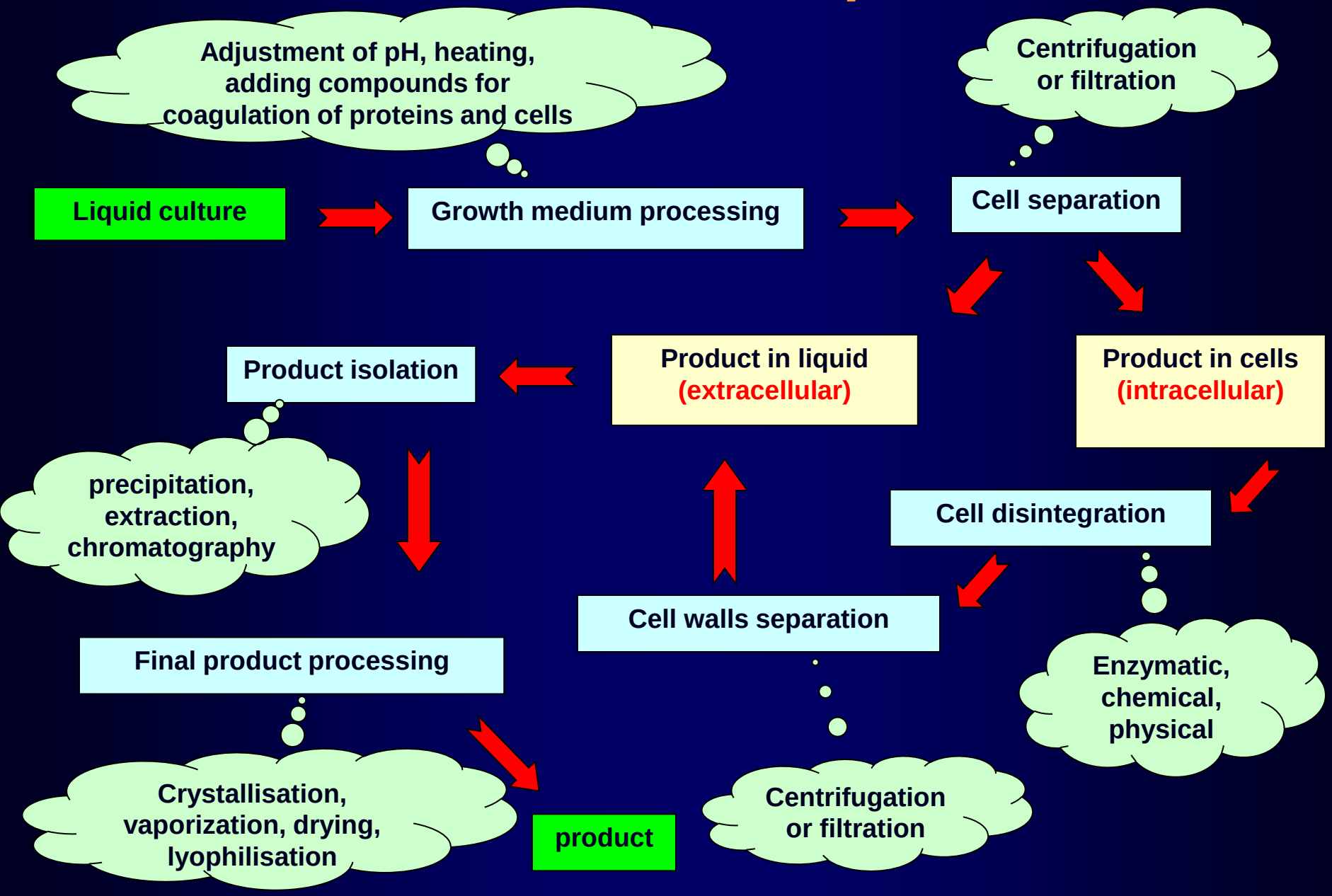
Downstream processing

Saccharomyces cerevisiae

Products isolation and purification

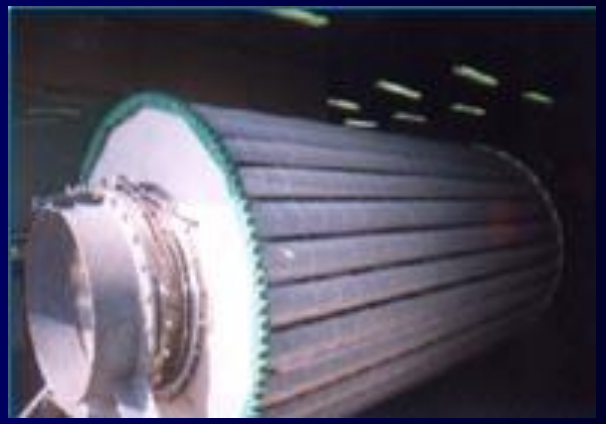


Products isolation and purification



Cell separation

- **Centrifugation (batch or continuous)**
- **Filtration (rotary vacuum drum filters)**

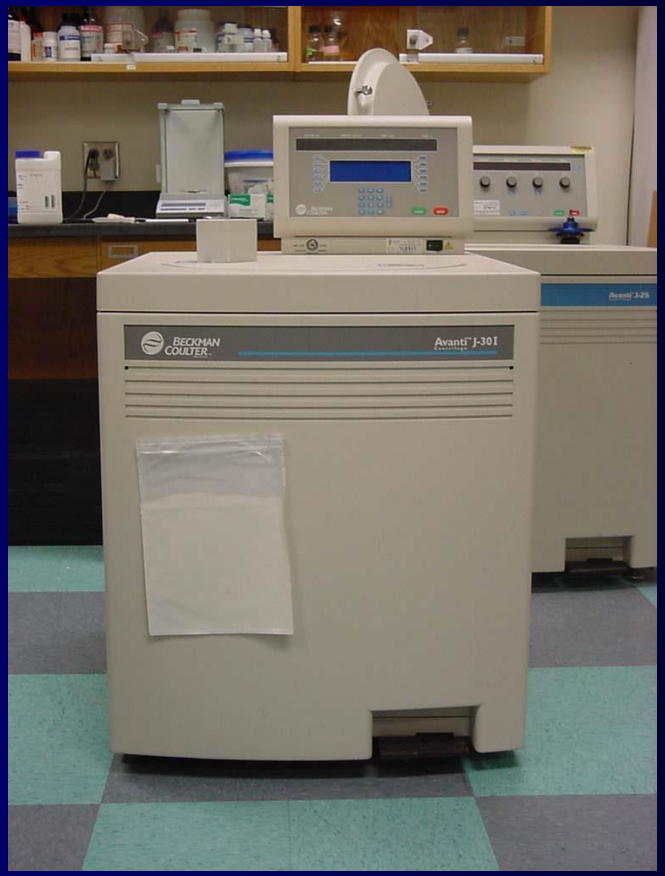


Cell disintegration

- **enzymatic: lysozyme**
- **chemical: alkali, detergents**
- **physical: osmotic shock, cell disintegration by abrasives, ultrasound**

Separation of cell walls

- centrifugation
- filtration



Isolation of product from liquid phase

- **Extraction**
  - System of two miscible solvents
  - Protein isolation = PEG and dextran or PEG and specific salts, such as K_3PO_4 or NH_4SO_4
- **Precipitation**
  - Protein salting by NH_4SO_4
  - Precipitation by organic solvents (ethanol, acetone, …)
- **Chromatography** (gel, ionex, bioaffinity, adsorption)
- **Electro migration** (electrophoresis, isoelectric focusing, isotachophoresis)

Final processing of the product

Evaporisation

- Vacuum vaporizer
- Mind heat-labile compounds!
- Desktop vaporizers are better for heat-labile enzymes

Drying

- Removing water and volatile compounds from the product
- Dryers – belt, tray, drum, spray
- Fluid-air heat exchanger (passage of warm air through the material) – frequent in pharmaceutical industry

Chromatography - I

- **Biologically active substances constitute a big group of compounds with special functions**
- **Changes of pH, ions forces, metal ions concentration, cofactors, etc. can strongly influence isolated biologically active molecules**
- **To avoid the loss of biological activity during the isolation process, it is necessary to use as mild separation methods as possible**

Chromatography - II

- **Development of effective isolation methods (gel, ionex, bio-affinity chromatography, etc.) enabled establishment of new branches of chemical industry**

- **No development of chemistry of proteins and nucleic acids – molecular biology, gene engineering, immunochemistry, etc. would be possible**

Purification strategy - I

- **Low concentration of biologically active compounds**
- **Mixture of many similar substances**

The first stage of isolation = adsorption

- **Bio-specific affinity chromatography**
- **Most proteins have negative charge at physiological levels of pH → sorption to annex**

Next stage of isolation

- **gel chromatography**
- **electrophoresis**

Purification strategy - II

Combination of several methods of separation is mostly successful for isolation of pure and biologically active substances

Don't repeat methods based on the same purification principle - when you prepare purification schedule

Gel chromatography

Separation of bio macromolecules based on different size of individual substances; the substances are separated on porous stationary phase (gel filtration)

Stationary phase – inert porous material saturated by liquid

- **agarose**
- **cross-linked dextran (Sephadex)**
- **polyacrylamide (BioGel P)**
- **cellulose (Cellufin)**
- **material based on Silica Gel or porous glass**

Principle of gel chromatography

Small molecules diffuse into pores of matrix during the flow of mixture of compounds through the porous stationary phase. It means that the movement of small molecules is slowed down. Large molecules are not captured by pores and move quicker. The bigger the molecule, the quicker the movement the through chromatography column is.

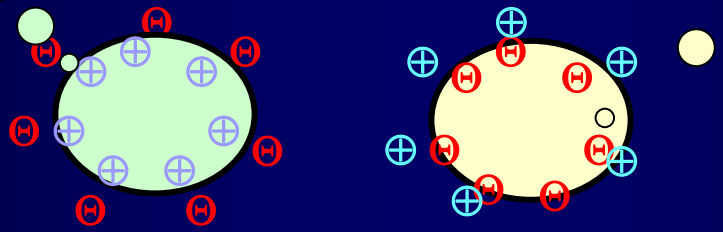
- **Repeated washing at mobile phase also washes out small molecules**
- **No interaction between the solute and matrix; no denaturation of separated substances**

Ion exchange chromatography

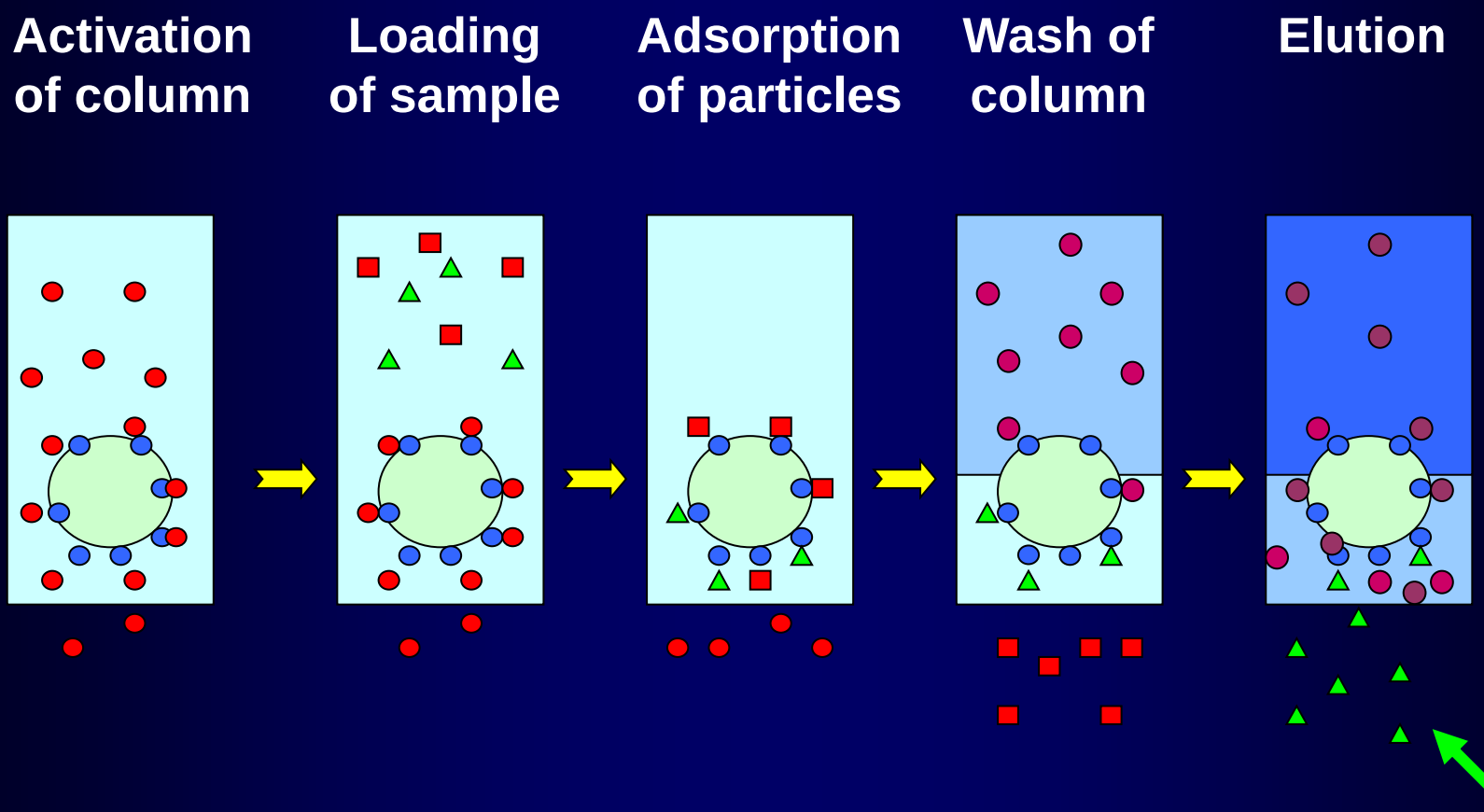
- **The method is based on a reversible exchange of ions between mobile liquid and stationary phases**
- **Stationary phase - ionexes (anion or cation exchangers)**

annex exchanges anions

Cation exchanger exchanges cations



Ion exchange chromatography process



Materials for ionexes

- **Modified cellulose**
- **Sephadex**
- **Ionexes derived from agarose (Trisacryl, Fractogel…)**
- **Ionexes based on silica gels and synthetic polymers**

Ion exchange chromatography is one of the most widespread methods, which were and still are used for the isolation of different biologically active substances (enzymes, NAs, AAs, antibiotics, vitamins, nucleosides and nucleotides, lipids, etc.)

Bio affinity chromatography

Based on an outstanding feature of biologically active substances to form tightly connected specific reversible complexes with other compounds called affinity ligands

enzyme – substrate, cofactor – effector, antibody – antigen, hormone – receptor, etc.

The principle of isolation method is interaction of isolated protein with ligand connected to solid surface

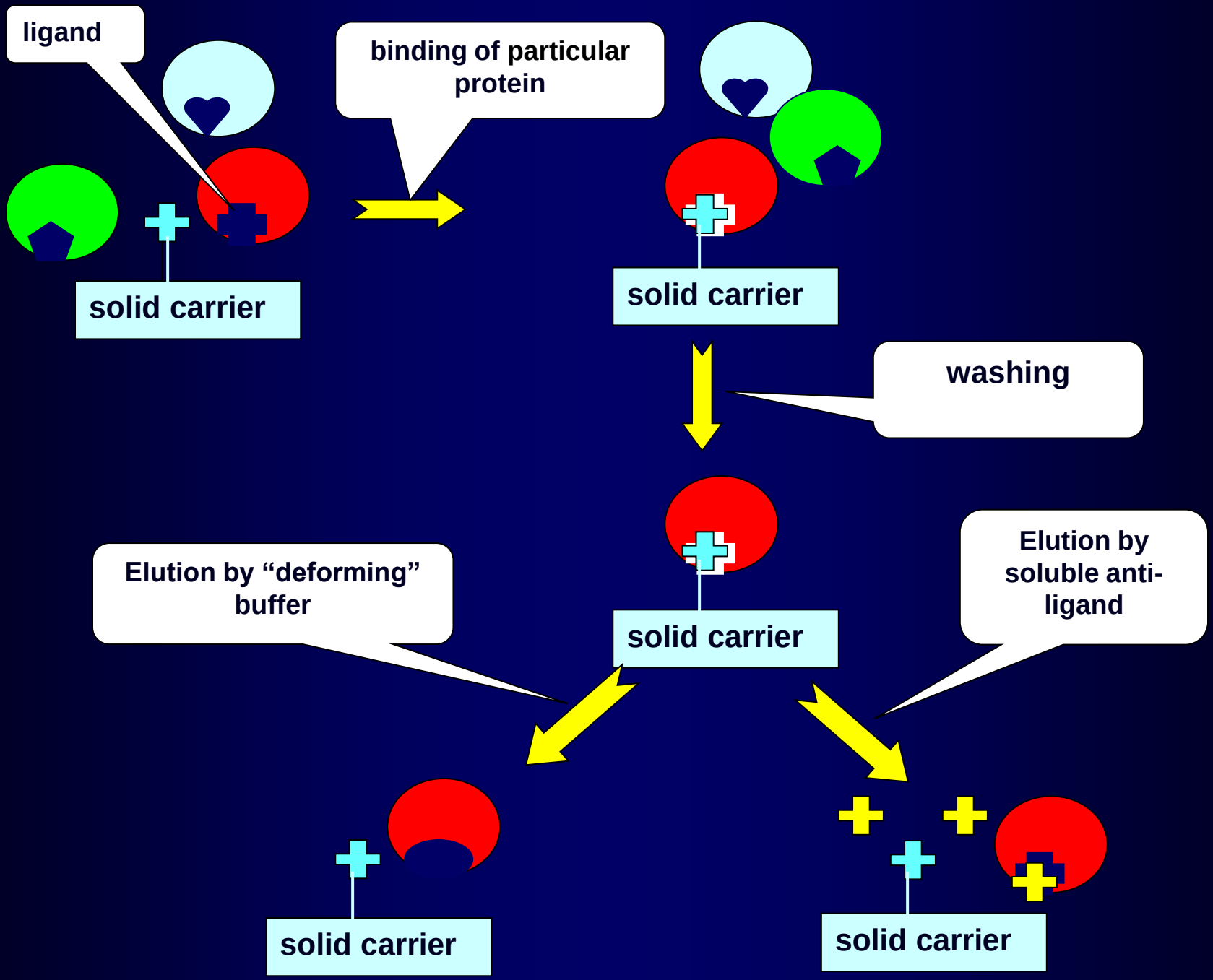
Ligand = compound which forms bio specific reversible complex with the isolated product

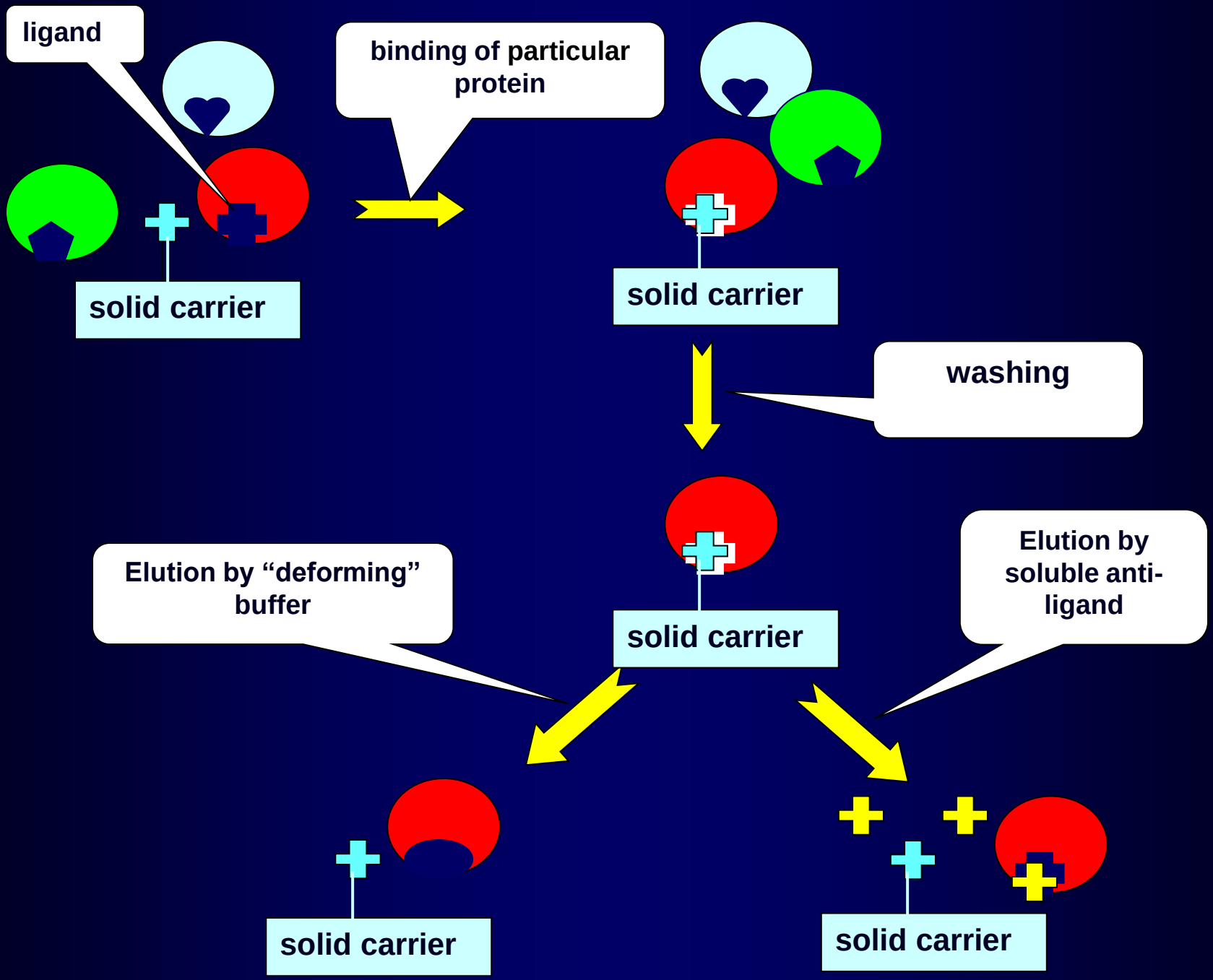
Ligands in affinity chromatography

Any compound, which is able to form bio-specific reversible complex with the given substance, can be used as a ligand

- **Ligand must contain a function group by which it binds to the solid carrier**
- **It must have sufficient affinity to the isolated substance**

- **Immobilised pyridine or adenine nucleotides**
- **Dyes with antraquione structure**
- **Immobilised haemoglobin or casein for proteolytic enzymes**


ligand
binding of particular protein
solid carrier
solid carrier
washing
Elution by “deforming” buffer
solid carrier
Elution by soluble anti-ligand
solid carrier
solid carrier


ligand
binding of particular protein
solid carrier
solid carrier
washing
Elution by “deforming” buffer
solid carrier
Elution by soluble anti-ligand
solid carrier
solid carrier

High performance bio affinity chromatography (HPLAC)

- **New method used only in laboratories so far**
- **Fully automated system working on high pressure**
- **Better resolution than in classical methods**

Electro migration methods

Zonal electrophoresis – separation based on the differences in total charge, volume and shape

- **Mostly in gels – agar or polyacrylamide**
- **Gradient gels (PGE) – *electric-field*-induced *movement* of *macromolecules* in a medium with gradient**
- **SDS-electrophoresis (SDS-PAGE)**

- **Isoelectric focusing (IF) – separation based on the isoelectric point**

The use of electro migration methods

- **Biochemistry, molecular biology, genetic engineering, bio analytical chemistry**
- **Medicine (diagnostics, immunochemistry, …)**
- **Accurate and effective analysis of**
  - **Simple organic compounds (amino acids, steroids, peptides, alkaloids, vitamins, dyes, antibiotics,…)**
  - **Complex macromolecular complexes (membrane receptors, enzymes, immunoglobulins, protein hormones, plasmatic proteins, allergens, etc.)**

Immobilised bio catalysers

Bio catalysers = biological material, which is able to transform any reactant into a product without changes of the bio catalyser itself

Immobilisation = process in which enzyme or cell (or its part) is transformed into the form of heterogenic catalyser

- **Enzymes**
- **Living cells**
- **Dead cells**

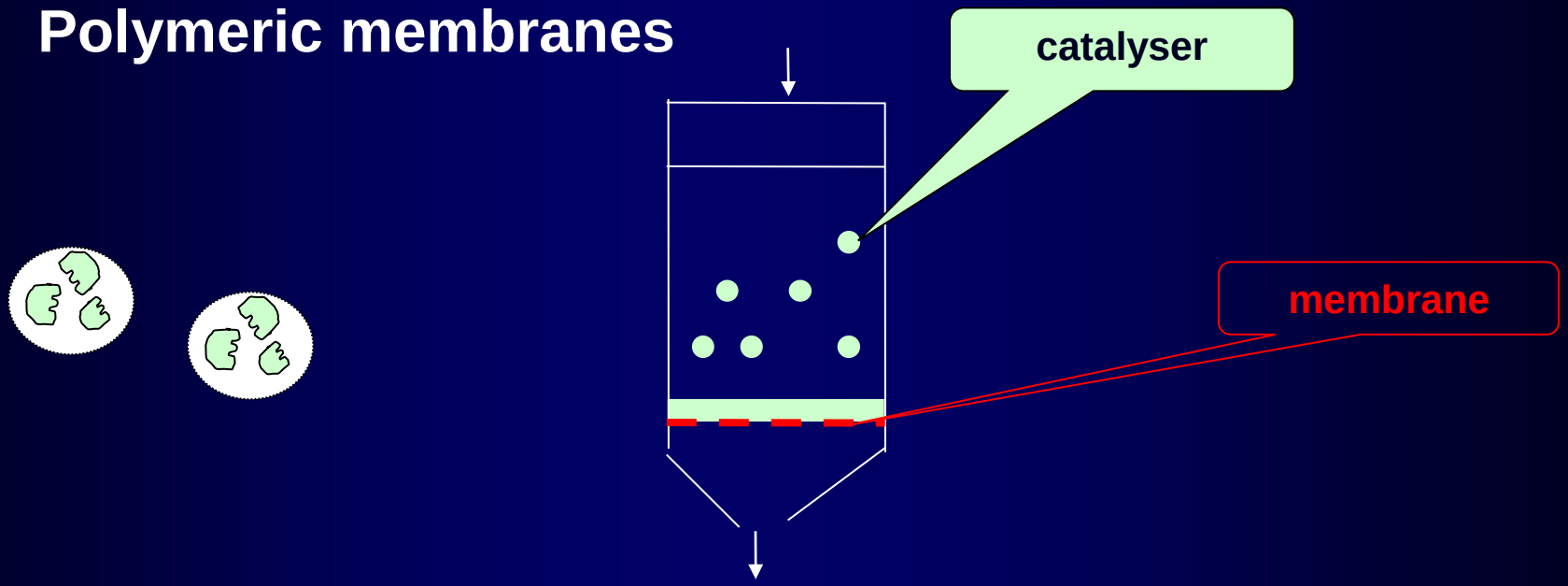
Advantages of immobilisation

- **Better economy of the catalysis**
- **Continuous process**
- **Better control of reactions**
- **Possibility to use non-compatible enzymes at the same time**
- **Longer enzyme activity**
- **Quicker separation of product and substrate**

Methods of immobilisation - I

1. Building into polymers

- **Polymerisation into gel matrix**
- **Dispersion in biopolymers**
- **Polymeric membranes**



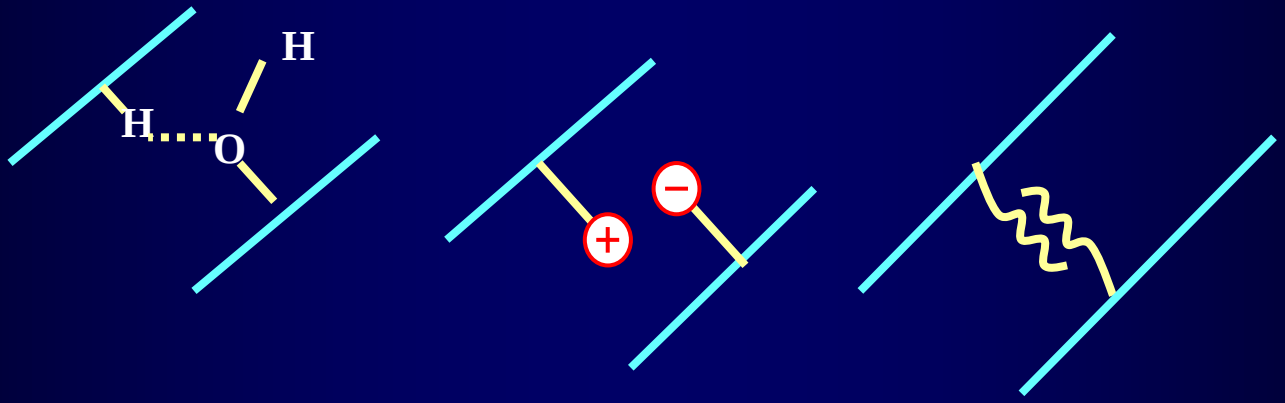
Microencapsulation – closing of a bio catalyser by a membrane into micro-capsules → forming of emulsion

Methods of immobilisation - II

2. Binding to solid carrier

adsorption

- Non-covalent binding by hydrogen bonds to inert carrier
- By electrostatic interactions to ion exchangers
- Non-specific interaction of hydrophobic groups, pseudoaffinity interaction, …

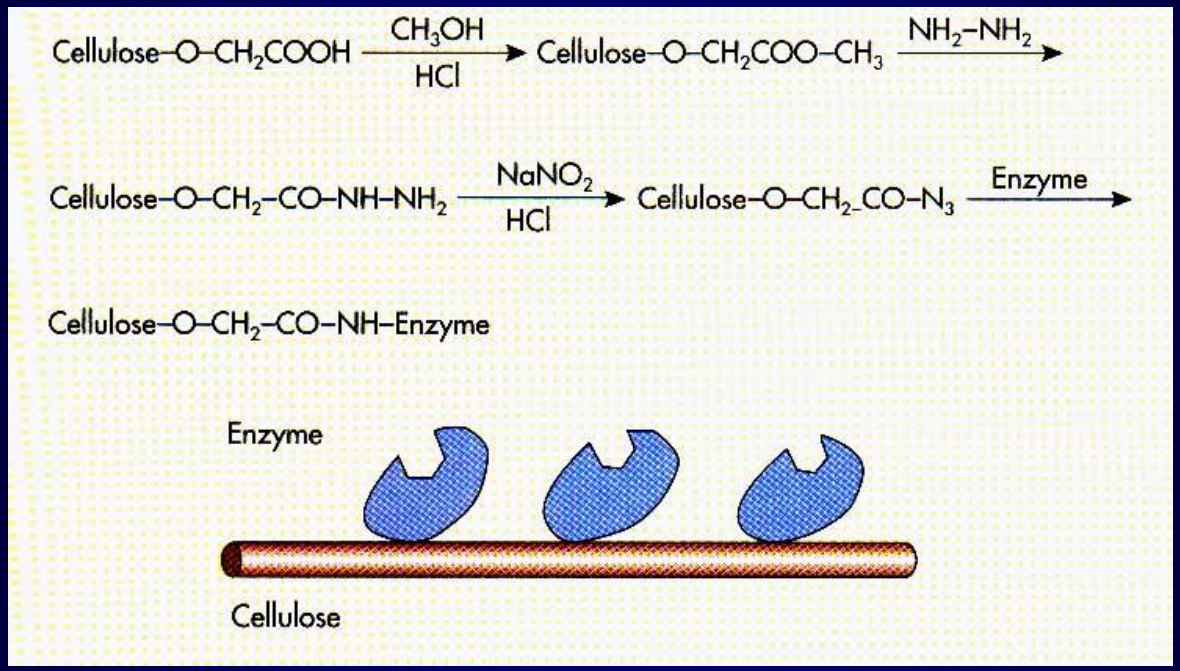


Methods of immobilisation - III

2. Binding to solid carrier

Covalent bond

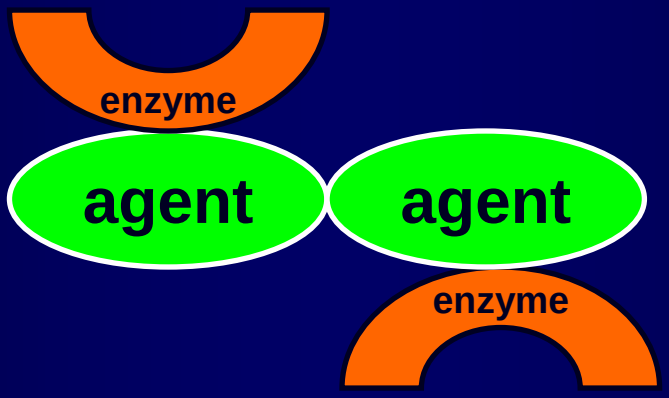
- **Modified natural polymers (cellulose, dextran, agarose, …), or also synthetic polymers (poly-acrylates, …)**



Methods of immobilisation - IV

3. Forming of aggregates without any carrier

- **Cross-linking of enzyme molecules by bi-functional agents or their binding to molecules of other inert proteins (inter-molecular bound)**



Summary

1) Basic definition
2) Materials for the fermentation process
3) Sterilisation
4) Bioreactors and fermenters
5) Cultivation of microorganisms
6) Downstream processing
7) Separation and isolation methods