Classical biotechnological processes in pharmacy

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

Biotechnology of Drugs 2024

Content

1) Industrial microbiology
2) Enzyme engineering
3) Immobilized enzymes
4) Plant biotechnology
5) Animal cell culture
6) Cell crossing, hybridoma

Industrial microbiology

Modern industrial microbiology has its origins in penicillin production technology developed in the 1940s

- **Bacteria**
- **Actinomycete**
- **Algae**
- **Yeasts**
- **Fungi**

"O Lord, I fall on my knees and beg You not to make my syntheses worse than those of bacteria!„

Ode to the Organic Chemist

Key issues in microbial production

1) Selection and modification of the characteristics of the production organism
2) Selection and provision of suitable starting material
3) Optimization of the conditions for growth of the microorganism and product formation
4) Design of suitable production facilities

1) Selection of the production organism

Objectives of breeding

1) Increase yields of the desired substance
2) Suppression of the formation of accompanying (contaminating) substances (especially those with side effects)
3) Changing metabolism so that an expensive inducer is not required
4) Forcing the organism to produce even under repressive or inhibitory conditions

Methods of breeding microorganisms

Induction and selection

Mutagens – chemical and physical

Hybridisation

Degenerate production strain x viable strain with low production

Most commonly spheroplast/protoplast fusion

Recombination of DNA

The most modern method of breeding

Disadvantages of microbial production

genetic variability and trait instability

- **Mutations can result in a less productive variant in the population that outgrows the original culture**
- **Periodic testing of inoculum properties is necessary**
- **It is advisable to maintain the culture under selection pressure - antibiotics, auxotrophy**

2) Starting raw materials

Primary

Directly from starting materials - corn (starches), carbohydrates (sucrose, glucose, xylose), cellulose, vegetable oils, milk, petroleum

Secondary

By-products or waste from the processing of primary raw materials - molasses, corn liquor, whey, potato water, lignocellulosic waste, animal waste, ...

Products of other microbial manufactures

Used to produce ethanol, methanol, glycerol or for biotransformation

Lignocellulosic waste

Preliminary adjustment

Division into lignin, cellulose and hemicelluloses

- **chopping or grinding into smaller particles**
- **removal of lignin and hemicelluloses in an acidic or basic environment**

Hydrolysis of cellulose to simple sugars (saccharification)

- **using sulphuric acid or**
- **enzymatically - amylase, cellulase, galactase, xylanase and others**

Fermentation of glucose

Production of ethanol or biomass (fodder yeast)

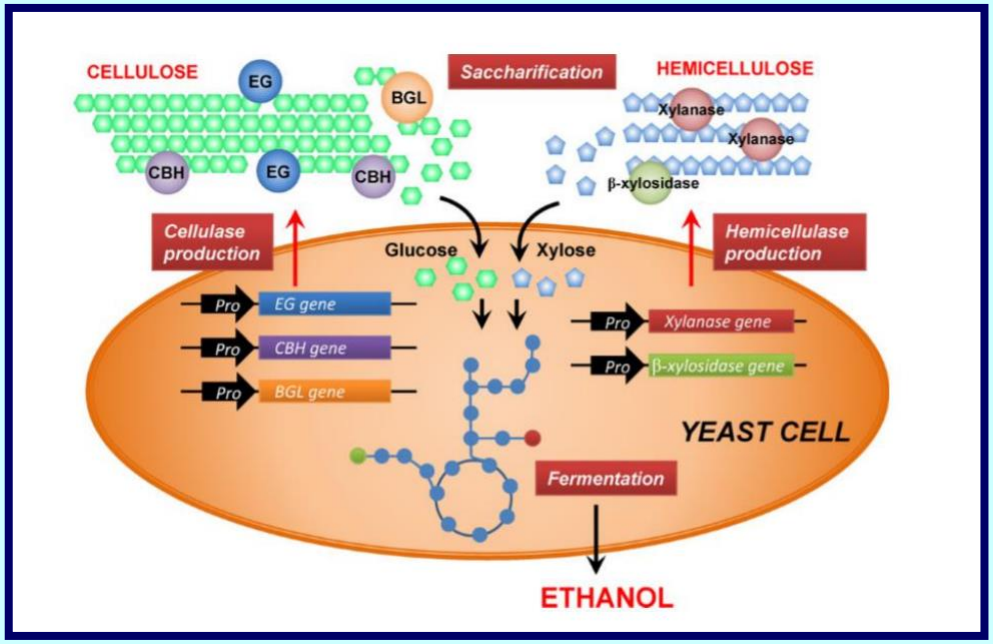
Simultaneous saccharification and fermentation (SSF)

- setting compromise conditions for saccharification and fermentation
- most often carried out in several tempered vessels in succession,
- within the first vessel (or in the first few vessels) the conditions are set closer to the optimum for saccharification
- in the last vessel (or in the last few vessels) the conditions are closer to the optimum for fermentation

A prerequisite for SSF is the availability of cells (yeast) tolerant to higher temperatures. Temperatures up to 40-60°C + enzymes that guarantee the breakdown of long chains of cellulose and hemicellulose

CBP – Consolidated bioprocessing

A method where saccharification and fermentation are carried out by a single species of organism - yeast, which is capable of both producing the necessary enzymes (cellulase, xylanase) and metabolizing sugars into ethanol.



Recombinant yeast cells developed for CBP. The CBP cells have the capability to produce sufficient cellulases (EG, CBH and BGL) and hemicellulases (xylanase and β-xylosidase) for hydrolysis of biomass and efficient ethanol production from both glucose and xylose.

HASUNUMA, Tomohisa a KONDO, Akihiko. Consolidated bioprocessing and simultaneous saccharification and fermentation of lignocellulose to ethanol with thermotolerant yeast strains. Process Biochemistry. 2012, 47

3) Production process

See separate lecture –

Biotechnological process

4) Design of production facilities

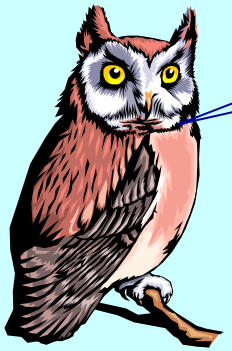
See separate lecture –

Biotechnological process

Enzyme engineering

Acquisition of enzymes and their modifications suitable for practical applications, optimization of their use in various fields of human activity and efforts to construct artificial enzymes

Is it chemical technology or biotechnology?



How are enzymes obtained?

Sources

- plant netting
- animal tissues
- microorganisms

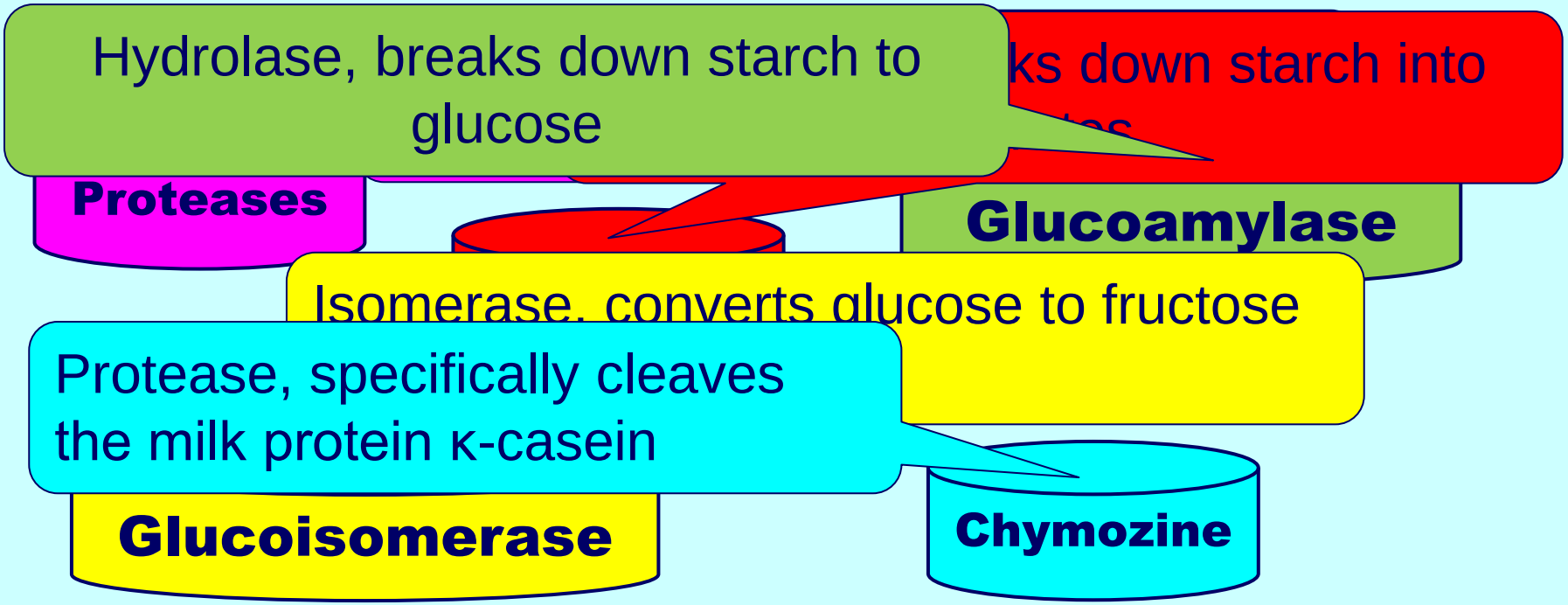
Isolation

- HPLC
- Electromigration methods
- Affinity chromatography

Most enzymes of microbial origin are extracellular

- They are found in the culture medium
- Their isolation is easier

The most commonly produced enzymes



- **First large-scale production – 1890**
- **Hydrolysis of starch to glucose by mould extract**
- **Wider introduction into industry after World War II**

The most common industrial producers

Bacteria	Fungi	Yeasts
Bacillus subtilis var.	*Aspergillus oryzae*	*Saccharomyces*
B. licheniformis var.	*A. niger*	
B. coagulans	*Rhizopus oryzae*	
Micrococcus lysodeikticus	*R. stolonifer*	
Streptomyces olivaceus	*Mucor hiemalis*	
S. olivochromogenes	*Tricoderma reesei*	
S. rubiginosus	*Mucor miehei*	
	M. pusillus	
	Claviceps purpurea	
	Penicillium chrysogenum	

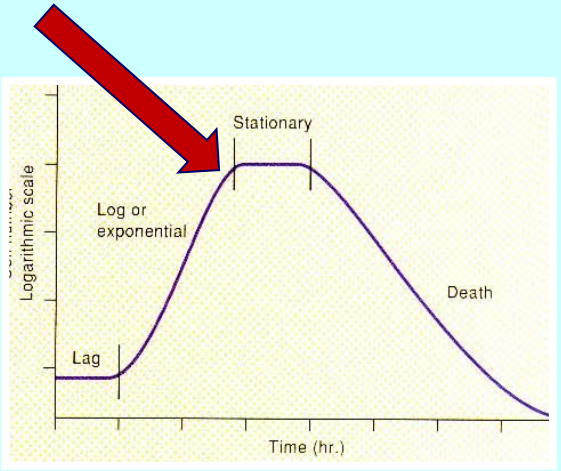
Production of bacterial α-amylase

- It is an intracellular enzyme
- *Bacillus subtilis* or *B. amyloliquefaciens* or *B. licheniformis*
- Submerged cultivation
- Inoculum = pure culture with 250 times the production of the original strain
- Expression induced by lactose = low/high glucose concentration in the medium

Recap lactose operon regulation from MolBiol lectures

The α-amylase fermentation process

- The volume of the fermenter is 4 500 to 135 000 litres
- Fermentation takes 4-6 days
- pH of the medium = 7.0
- The buffer is calcium carbonate
- Medium temperature 30-40°C
- Production of α-amylase starts when the density of the culture is 10^9-10^{10} cells/ml
- Peak production at the end of the log phase of growth before cell sporulation begins

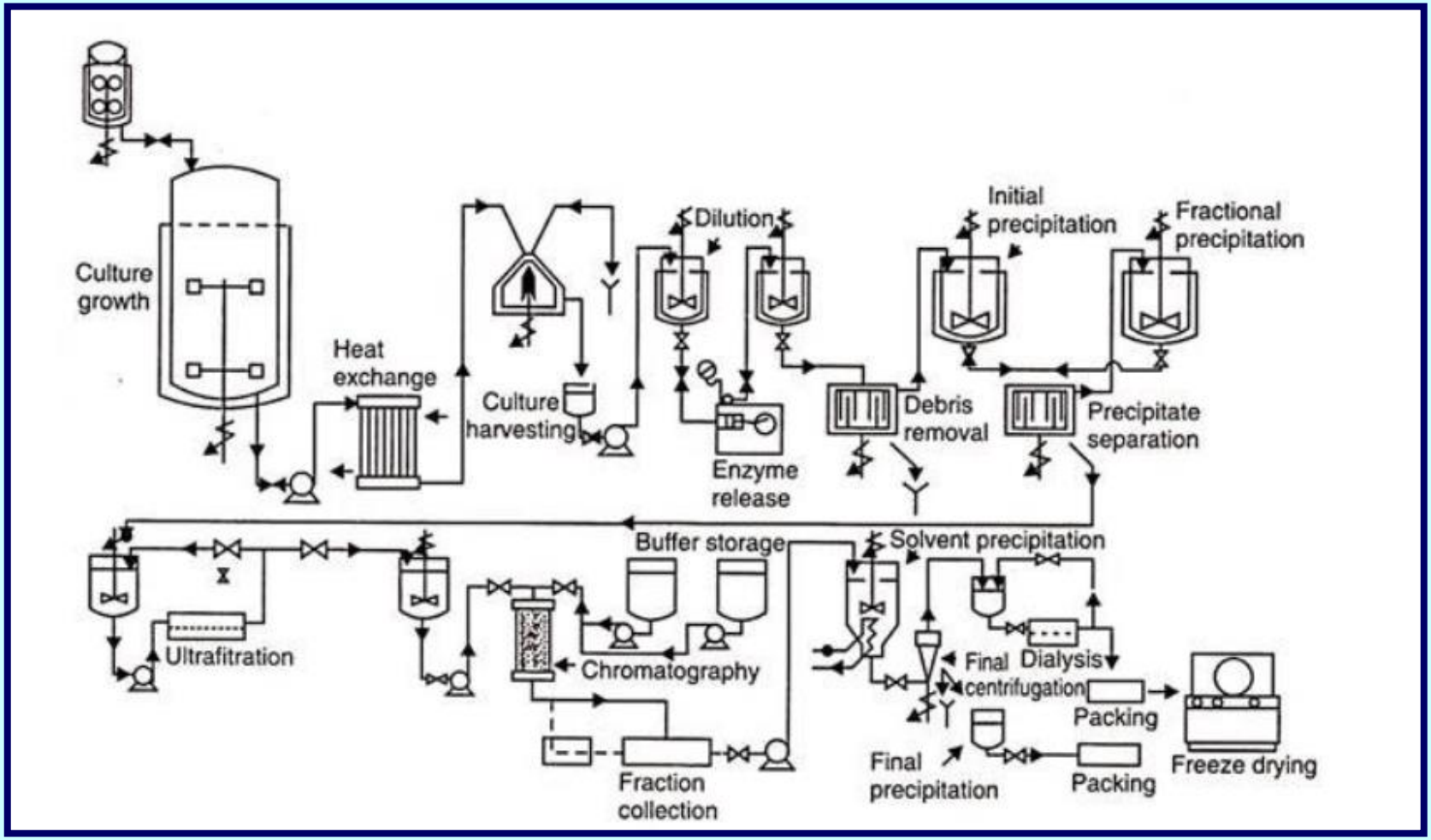


Harvesting and preparation of α-amylase

- Prevention of enzyme degradation = rapid cooling of the medium to 5°C
- Biomass collection by centrifugation
- Degradation of cells, flocculation of residues with calcium phosphate
- Precipitation of enzyme with acetone or ethanol or ammonium sulphate or sodium sulphate
- Fractional precipitation = purest product

- Liquid form contains 2% of the enzyme
- Solid form contains 5% of the enzyme

Scheme of α-amylase production

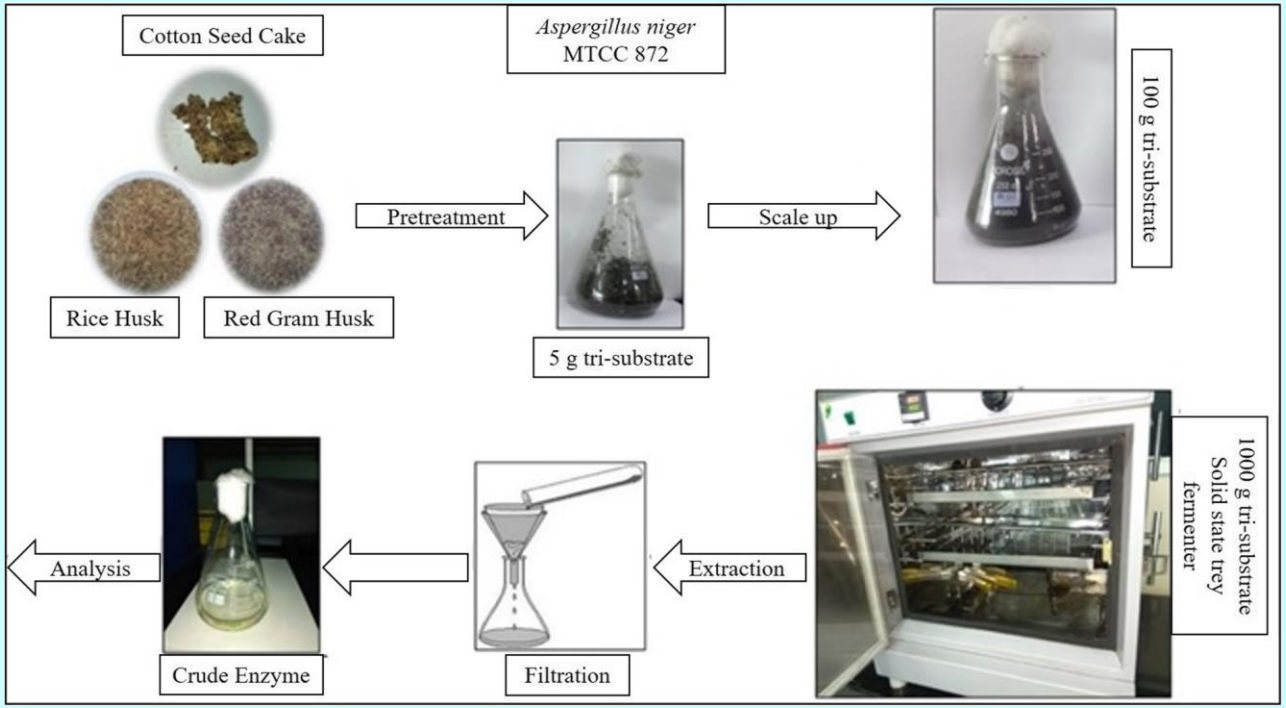


Industrial Production of Enzymes (With Applications) | Biotechnology (biotechnologynotes.com)

Production of α-amylase from fungi

- ***Aspergillus oryzae***
- ***Aspergillus niger***

- **Processes similar to bacterial α-amylase**



https://media.springernature.com/original/springer-static/image/art%3A10.1186%2Fs42269-019-0125-7/MediaObjects/42269_2019_125_Fig1_HTML.png

Other products of A. niger

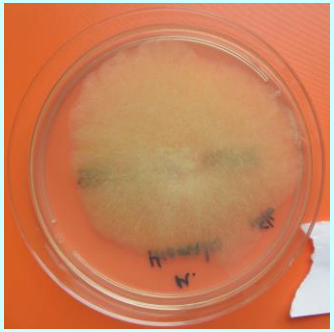
- *amylases*
- *hydrolases*
- *proteases*

- **Organic acids**
  - **Citric acid (E330)**
  - **Gluconic acid**

- **Products of fermentation**
  - **Sake**
  - **Soy sauce**

Other important fungi

- ***Rhizopus stolonifer***
  - ***fumaric, citric, lactic acid***
  - ***steroids***



- ***Mucor hiemalis***
  - ***fumaric, citric, lactic acid***
  - ***steroids***

- ***Claviceps purpurea***
  - **Alkaloids**
  - **ergotamine and ergometrine**

Stabilization of enzymes

The stability of the enzyme is often a limiting factor in its use

- By the addition of low or high molecular weight substances
- Chemical modification
- By forming cross-links in the molecule
- Denaturing and refolding the molecule
- Binding to a polymeric carrier
- Protein engineering

Free x immobilized enzymes

Disadvantages of free enzymes include

- **Instability**
- **Disposability of use**
- **Transfer of enzymes into the reaction mixture and need for removal**

Immobilized enzymes = biocatalysts

Immobilized biocatalysts

Biocatalysts = biological material that can transform a reactant into a product without changing the reactant itself

Immobilization = a process in which an enzyme or cell, or part of it, is converted into a form of heterogeneous catalyst

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

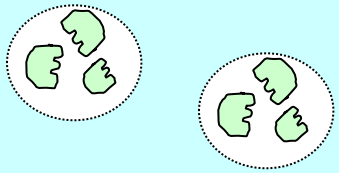
Benefits of immobilisation

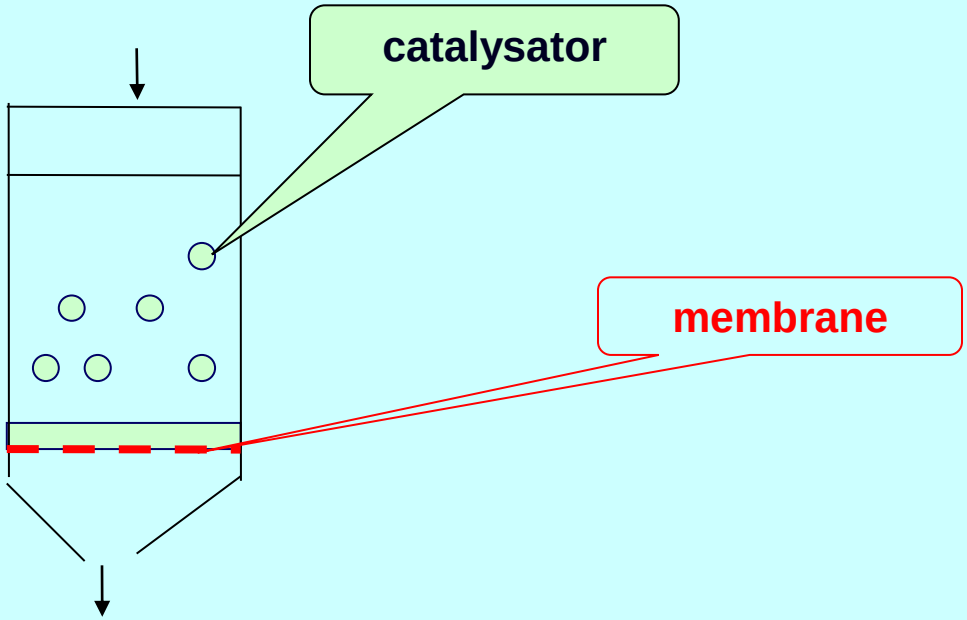
- **Higher process economy**
- **Continuous operations**
- **Better control of reactions**
- **Possibility to use incompatible enzymes simultaneously**
- **Longer enzyme activity time**
- **Faster separation of product and substrate**

Methods of immobilization - I

1. Embedding in polymers

- polymerisation into the gel matrix
- dispersion in a biopolymer environment
- polymer membranes





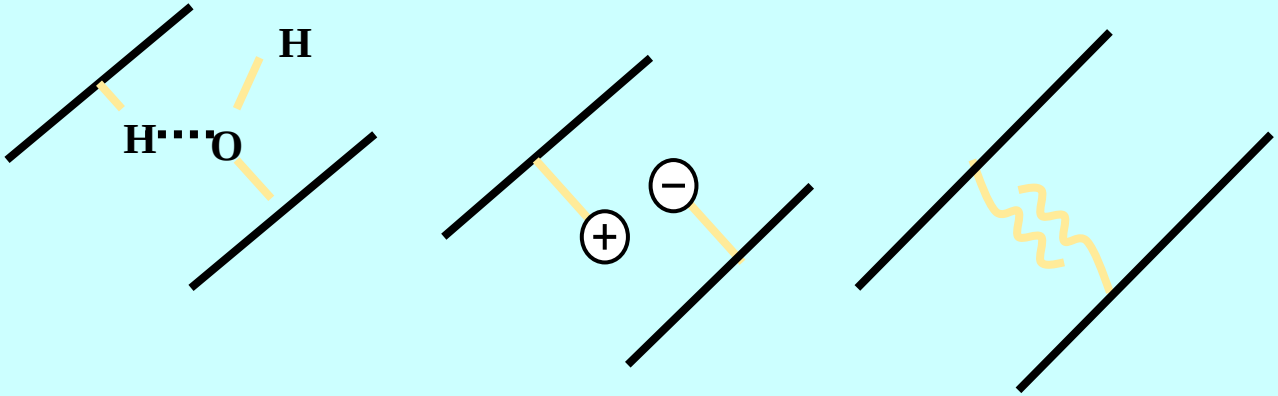
Microencapsulation - encapsulation of a biocatalyst by a membrane into microcapsules → emulsion formation

Methods of immobilization - II

2. Fixed carrier binding

adsorption

- non-covalent bonding via H-bonds to an inert support
- by electrostatic interactions on ion exchangers
- non-specific interactions of hydrophobic groups, pseudo affinity interactions...

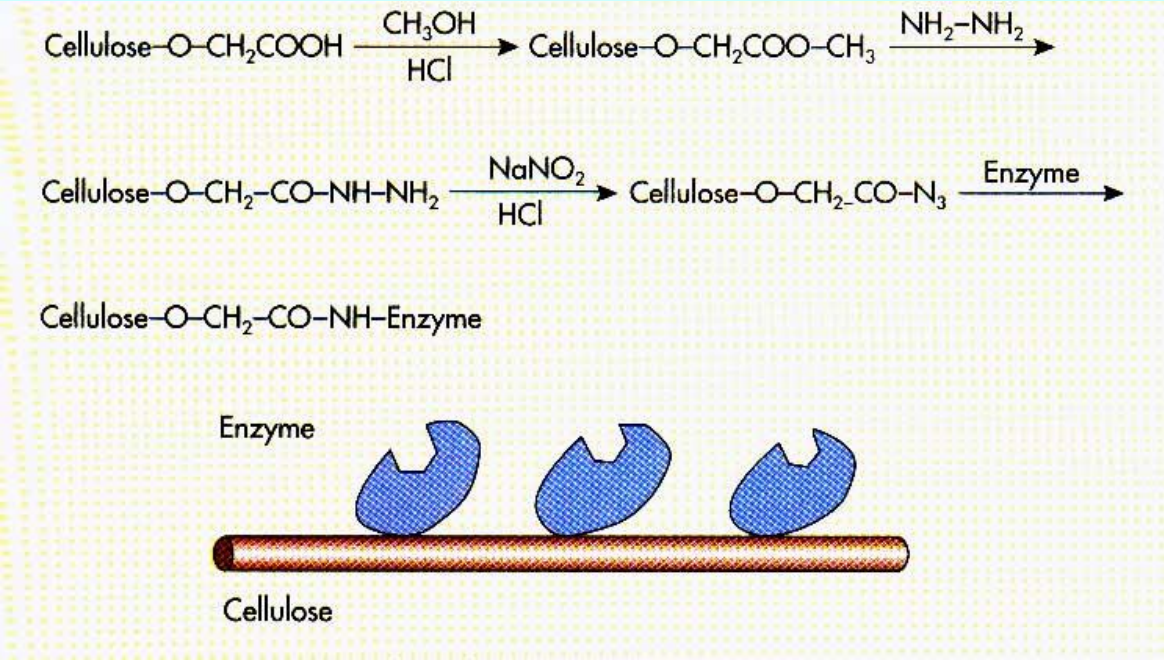


Methods of immobilization - III

2. Fixed carrier binding

Covalent bond

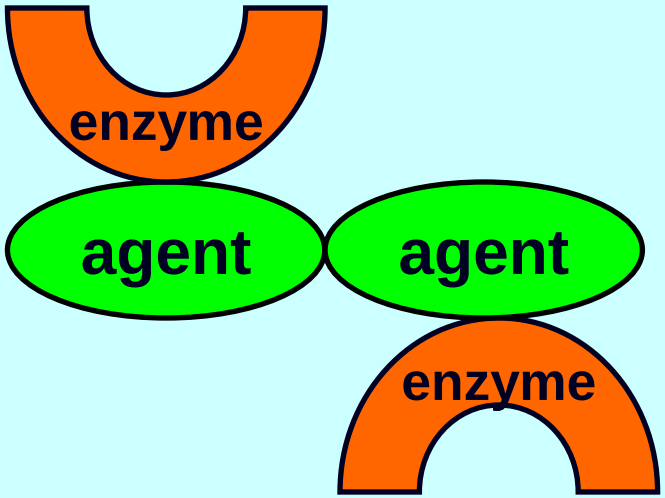
- **modified natural polymers (cellulose, dextran, agarose...), possibly also synthetic polymers (polyacrylates...)**



Methods of immobilization - IV

3. Creating aggregates without using a carrier

- **cross-linking of enzyme molecules by bifunctional reagents or by their binding to other inert protein molecules (intermolecular cross-linking)**



Multienzyme systems

Higher generation of enzyme catalysts

- Immobilized multi-enzyme systems
- Membrane-bound enzymes
- Immobilized organelles
- Immobilized whole cells

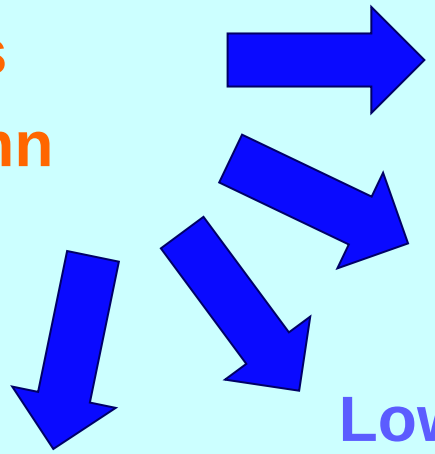
- Especially the cells of microbes
- Immobilised plant cells

  - After the growth phase (autolysed)
  - In growth phase (live)

Why immobilized cells?

- **Elimination of laborious and costly enzyme isolations and purifications**
- **Stabilization of enzymes in the natural environment of the cell**
- **Ability to utilize entire metabolic pathways**

- **Chemical reactors**
- **Continuous column processes**



Reducing the size and cost of production equipment

Better process control

Lower production costs

Higher product uniformity

Conclusion

The use of immobilized cells allows optimal utilization of enzyme systems and thus increased metabolite production using the same amount of cells

Where are immobilized cells used?

- Production of ethanol, beer
- Organic acids and amino acids
- α-amylase
- Bacitracin
- Disposal of toxic substances in environmental cleaning

Bacitracin is produced by *Bacillus subtilis* bacteria immobilized in PVA (poly(vinyl alcohol)) cryogels, although cultivation in liquid media is more common.Bacitracin is secreted into the medium

Do immobilized cells have any disadvantages?

- **Restriction of substrate access = not using the full capacity of the catalyst**
- **Optimum speed only on the surface**
- **Barrier is also a biomembrane = not suitable for macromolecules**
- **Undesirable reaction by admixture of other enzymes**
- **Various compromises are the solution**

- **Intensive cell growth = destruction of the system**
- **A medium that would keep cells alive while suppressing growth and division? DOES NOT EXIST**

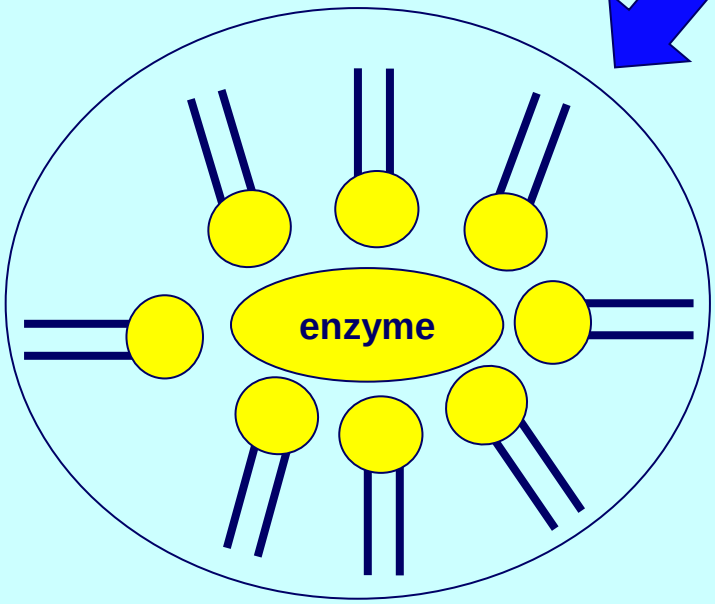
Enzyme micelles

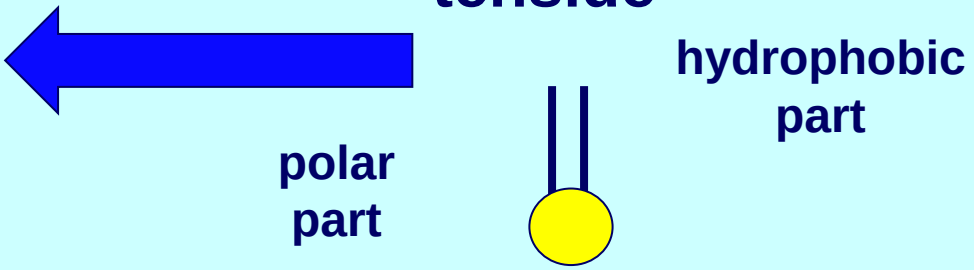
Aqueous environment

- Standard micelles
- Condensation reactions
- Hydrolases
- Oxidoreductases

Non-aqueous environment

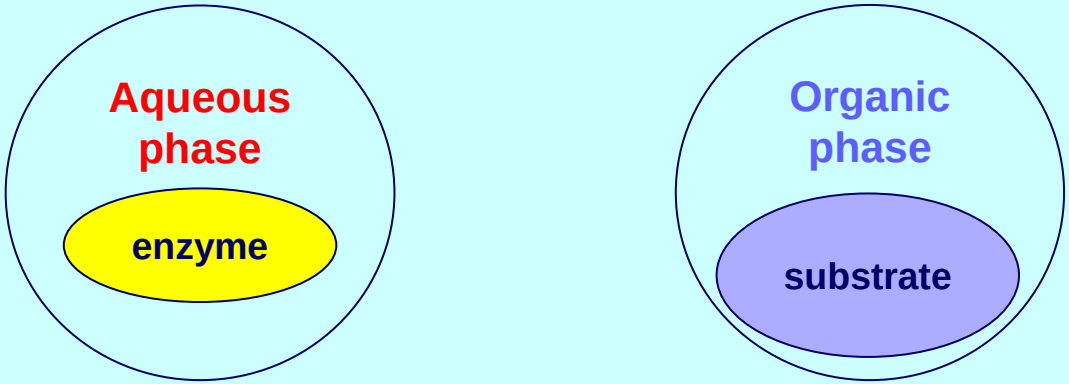
- Inverted micelles
- Dispersion of enzymes in non-polar solvents
- Possibility to convert water insoluble substances





Two-phase systems

Designed for the enzymatic conversion of water-insoluble substances



- **Mixing by shaking**
- **Transfer of substrate to aqueous phase**
- **Catalysis**
- **Return of product to organic phase**

The advantage of a two-phase system

- Reduced volume of the reaction mixture
- Easy separation of the product even from the non-immobilised enzyme

Examples of use

- Transformation of steroids
- Oxidation of hydrocarbons
- Conversion of glucose to ethanol
- Biosynthesis of tryptophan from indole and serine

Plant biotechnology

Plants - the most diverse source of natural substances

Products of multistep biosynthesis

Classical approach

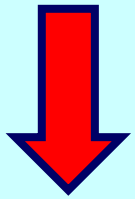
Biotechnology

Tissue cultures

Explant cultures grown as microorganisms

Basic thesis

Almost every somatic cell is totipotent



The crop will produce the same as the whole plant

Which plant cell is not totipotent?

Pollen

The crop will produce the same as the whole plant?

The plant is differentiated in time and space!

- **Metabolism of secondary metabolites occurs only during limited developmental stages**
- **Metabolites are only produced in certain tissues**
- **Processes are intricately regulated**

Do you know any examples of exceptions?

Transfer to explant culture - stress

Implications

- The metabolite is not produced
- Metabolite production is low
- Intermediate accumulates
- Something else is produced (activation of an alternative metabolic pathway)

Selection of a suitable "clone" is necessary

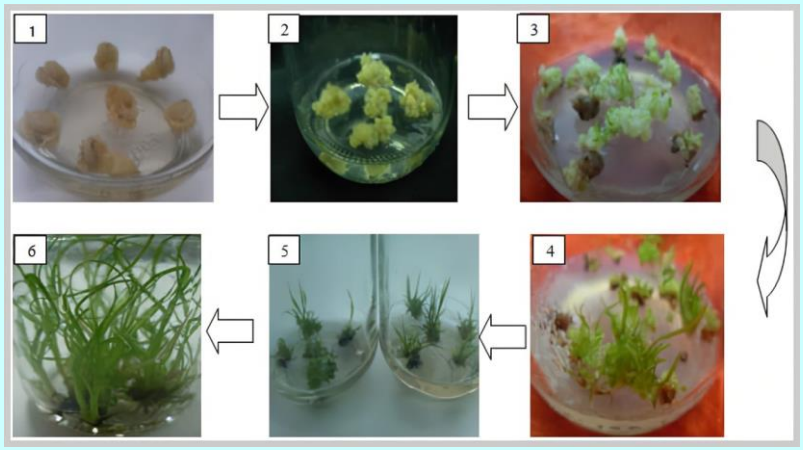
Types of cell cultures

Callus

- It is the default for both types
- Cut pieces of the plant on agar
- Irregular growth = callus
- Subculture and emergence of independence

Suspension

- Formed from callus after being affected by phytohormones and reduced cell adhesion



Suspension culture

Can't keep it for long

- **A total 5-10% of the inoculum is transferred to fresh medium**
- **Transfer after 2-4 weeks**
- **Growth faster than in agar (days x weeks)**

For longer preservation, callus culture is preferable

Types of cultivation

Batch

- Suspension of cells in flasks on a shaker or roller
- Laboratory only
- Technologically unsuitable for industry

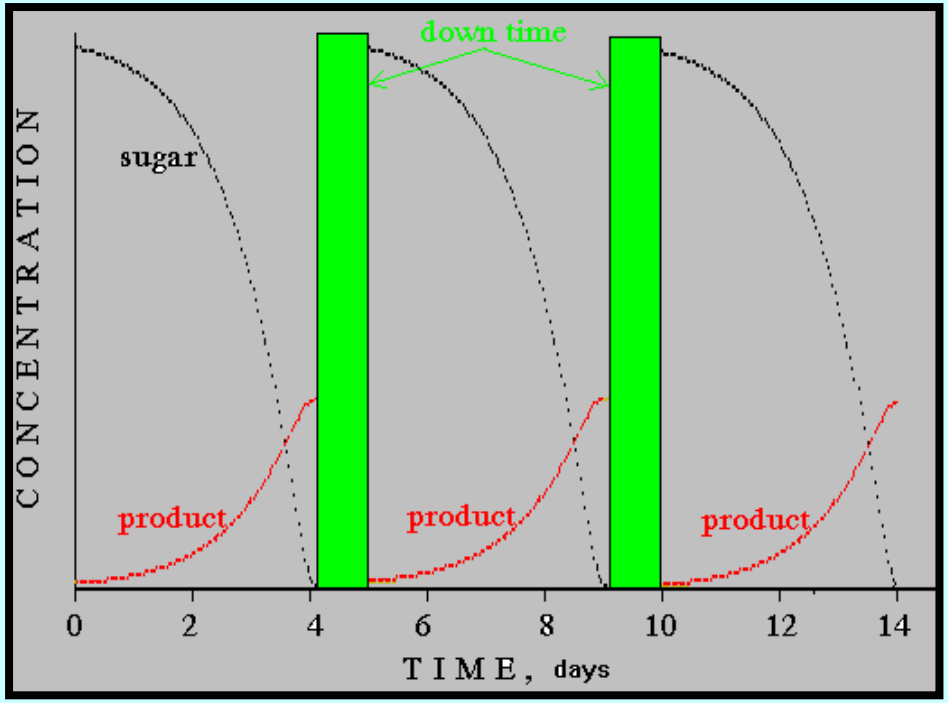
Continuous

- Long cultivation time
- Fragility of cells
- Changes in metabolic activation
- Formation of cell clusters
- Prolonged lag and death phases

Compromise solution

Periodic exchange of half the volumes of cultivated biomass and medium

- Before the end of the exponential growth phase



Fed batch cultivation

Immobilized plant cells

For large-scale production of plant substances

- They allow to obtain suspension cultures with high cell density
- Immobilization increases cell viability
- Can prolong biosynthetic activity

Forms of immobilisation

- Incorporation into polymer gels
- Binding to a rigid carrier
- Encapsulation of cells into defined structures

Advantages of explant cultures

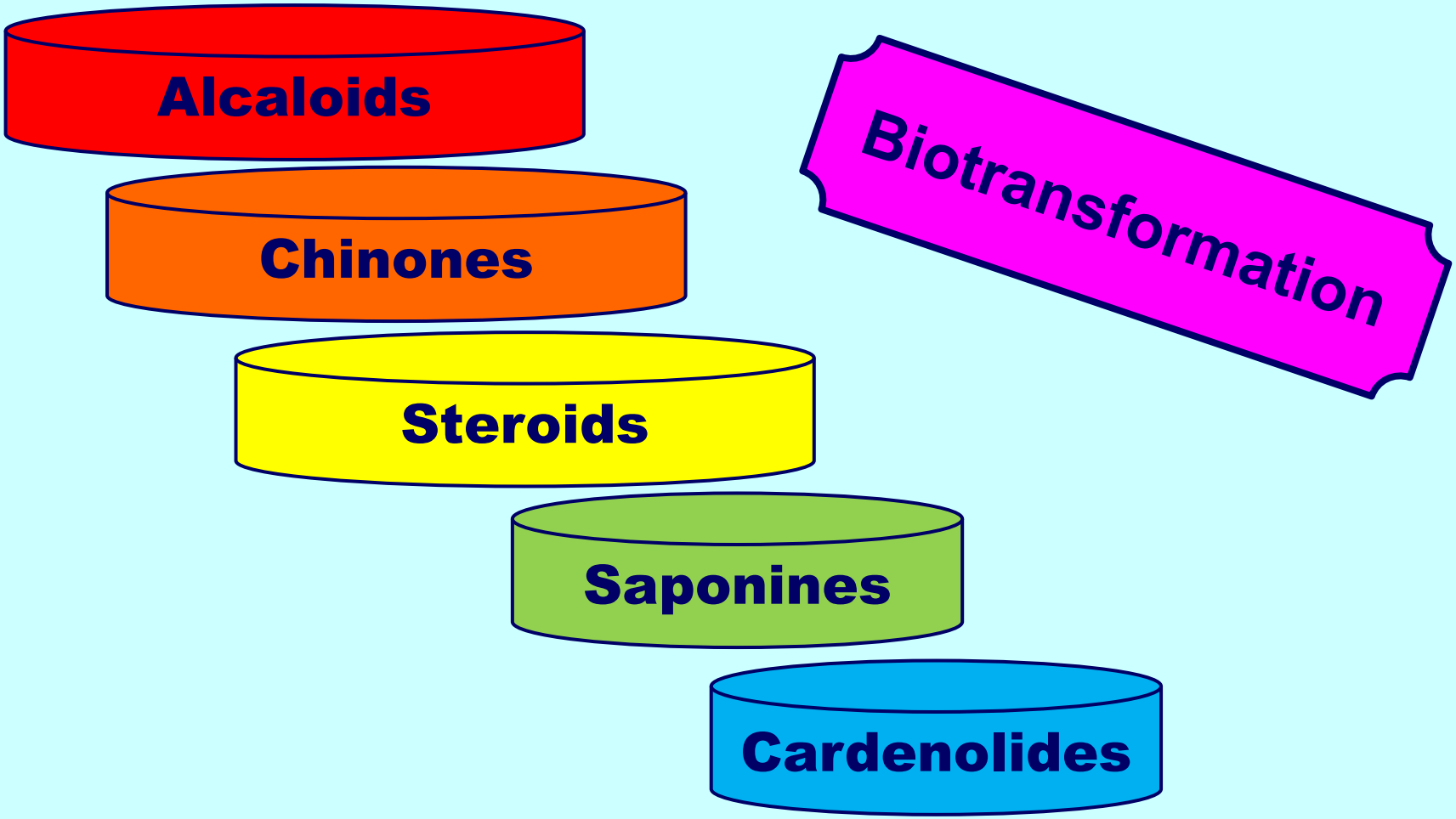
Try to derive them yourself

- **Controlled conditions independent of soil and climate**
- **Insect and pathogen free crops**
- **Continuous production of cells of different origin - tropics, mountains, rare, endangered**
- **Increased production after selection at the cellular level**
- **Uniform products**
- **Production of new substances not found in nature**

Plants are mainly useful to produce secondary metabolites indispensable for the pharmaceutical industry

The most frequent production

See separate lecture



Cultures of animal cells

Culture is established after mechanical or enzymatic tissue disintegration, followed by centrifugation and inoculation into a nutrient liquid medium with blood serum

In the culture bottle, the cells cover the bottom during growth

Proteolytic enzymes release and reinoculate the cells

Comparison of an animal cell and a microorganism

Feature	Microrganism	Animal cell
Complexity	Simple	Complex
Strength	Fixed	Fragile
Envelope	Cell wall	Plasmatic membrane
Metabolism	Independent	Part of organism
Nutrition	Glucose + ions	Complex
Cultivation	They can withstand intensive stirring in solutions	Mostly solid surfaces
Growth	Fast	Slow

Media for animal cells

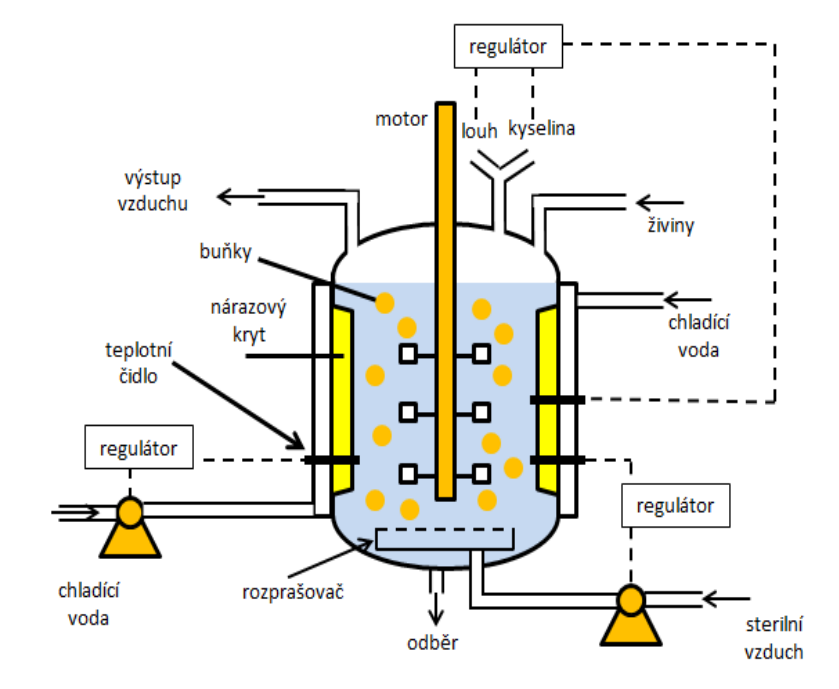
Glucose + ions + amino acids + 5-20% of blood serum

What is the blood serum needed for?

Serves to provide unidentified components necessary for cell nutrition

Cultivation conditions

- Strictly adhere to the pH
- Strictly adhere to temperature
- Strictly observe O_2 and CO_2 levels
- Regulate ion concentration (osmotic pressure)



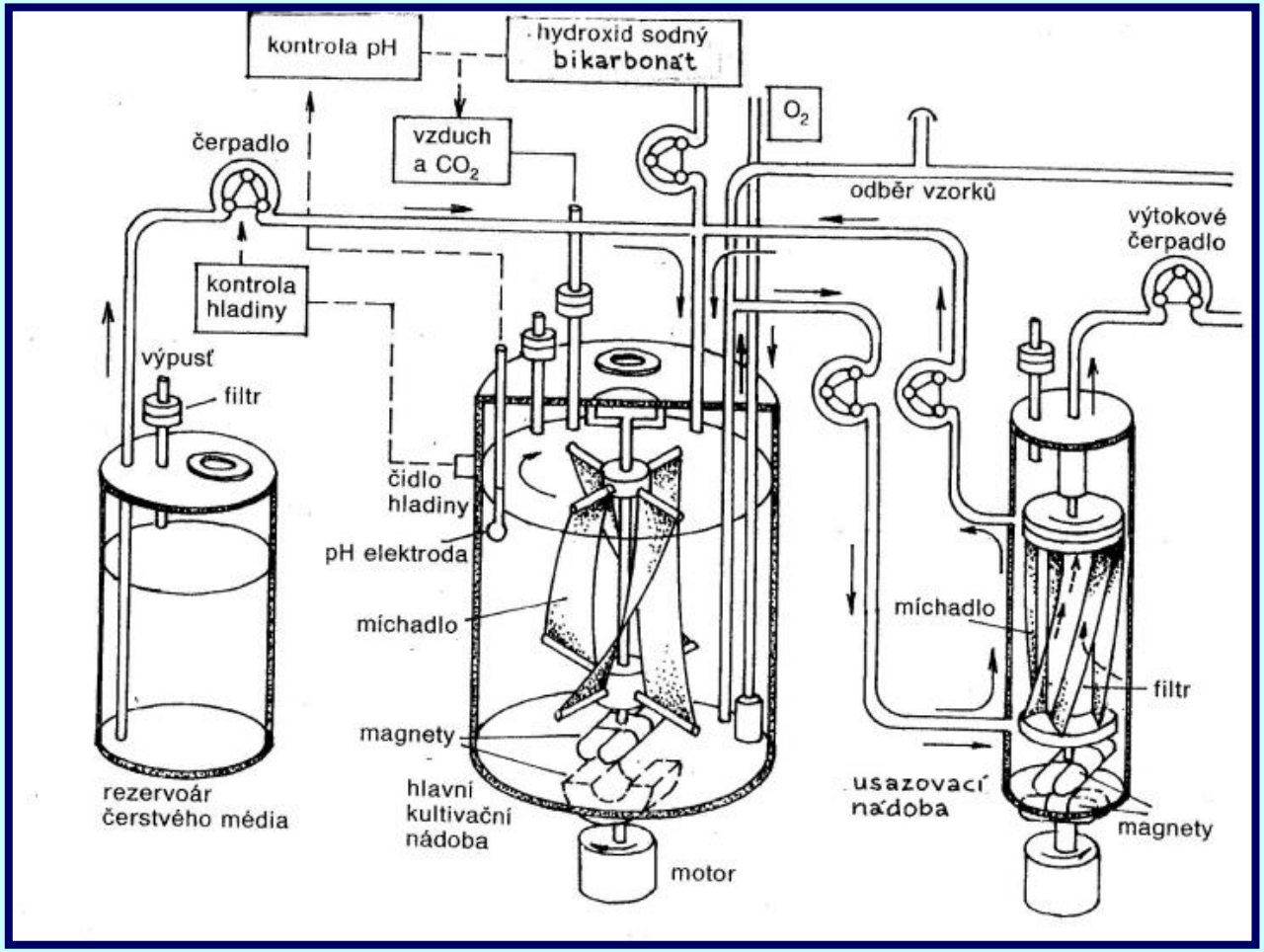
Perfusion bioreactor

Designed for large-scale cultivation of mammalian cells

- **Developed by modifying fermenters for submerged cultivation of microorganisms**
- **Requires absolute sterility - must be heat sterilized**
- **Usually up to 100 litres to fit in an autoclave**

Large-scale production is achieved by using a series of bioreactors

Schematic of the perfusion bioreactor



from Vodrážka (1991): Biotechnologie, skripta VŠCHT, Praha

Do all animal cells require a solid surface?

No, blood cells, lymphoid tissue cells and most cancer and other transformed cells (hybridoma) can be grown in suspension

How to increase the carrier area

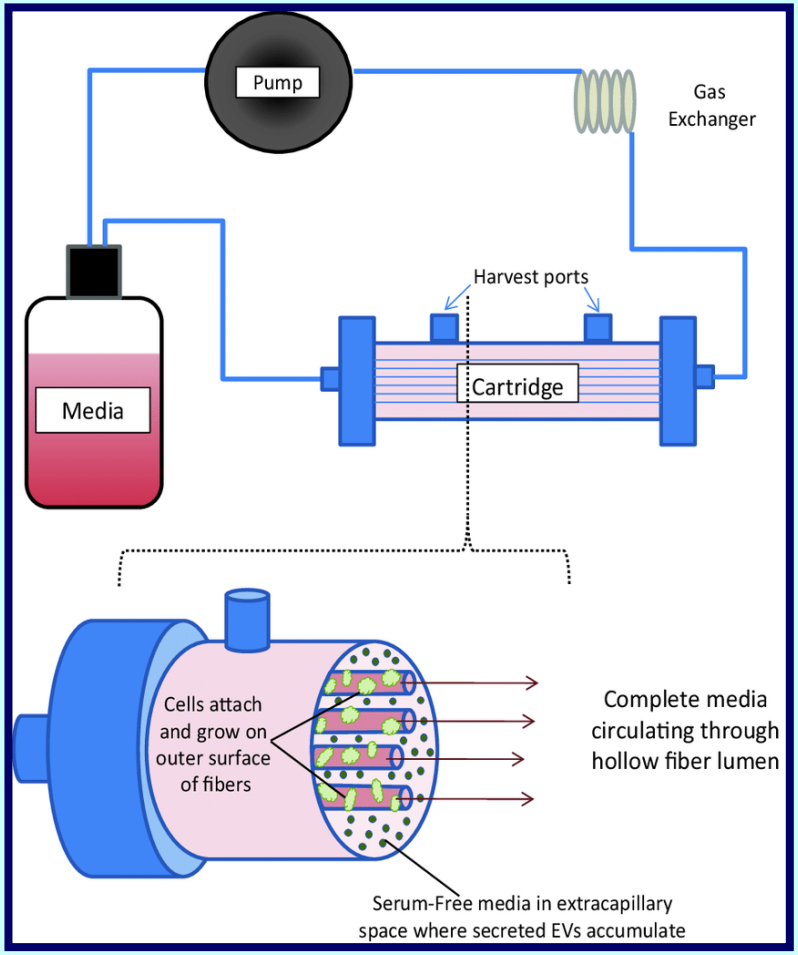
The surface of the bioreactor walls does not provide sufficient surface area in relation to the culture volume

- **Porous polymers**
- **Tubes or hollow fibres**
- **Beads from 50 µm to several hundred µm in diameter - microcarriers**

Microcarrier based on

- **Glucose polymers**
- **Polystyrene, polyacrylamide, epoxy**
- **Gelatine, dextran coated**
- **Glass, coated with dextran or synthetic polymer**

Hollow fiber bioreactor



A three-dimensional system containing hollow tubes = semi-permeable capillary membranes assembled in parallel rows usually located in a carbonate tube.

Cells on the surface of the capillaries

Medium flows through the interior

hollow fiber bioreactor - Bing images

Use of animal cells

Mostly to produce vaccines

- The first human vaccine produced by culturing mammalian cells was the poliovirus vaccine, 1954

Other examples of vaccines

- Smallpox
- Chickenpox
- Yellow fever
- Influenza
- Herpes simplex II

Study the cells that produce SARS-CoV-2 vaccines

Cell crossing

Any crossing of genetically different individuals resulting in offspring with a genotype different from their parents

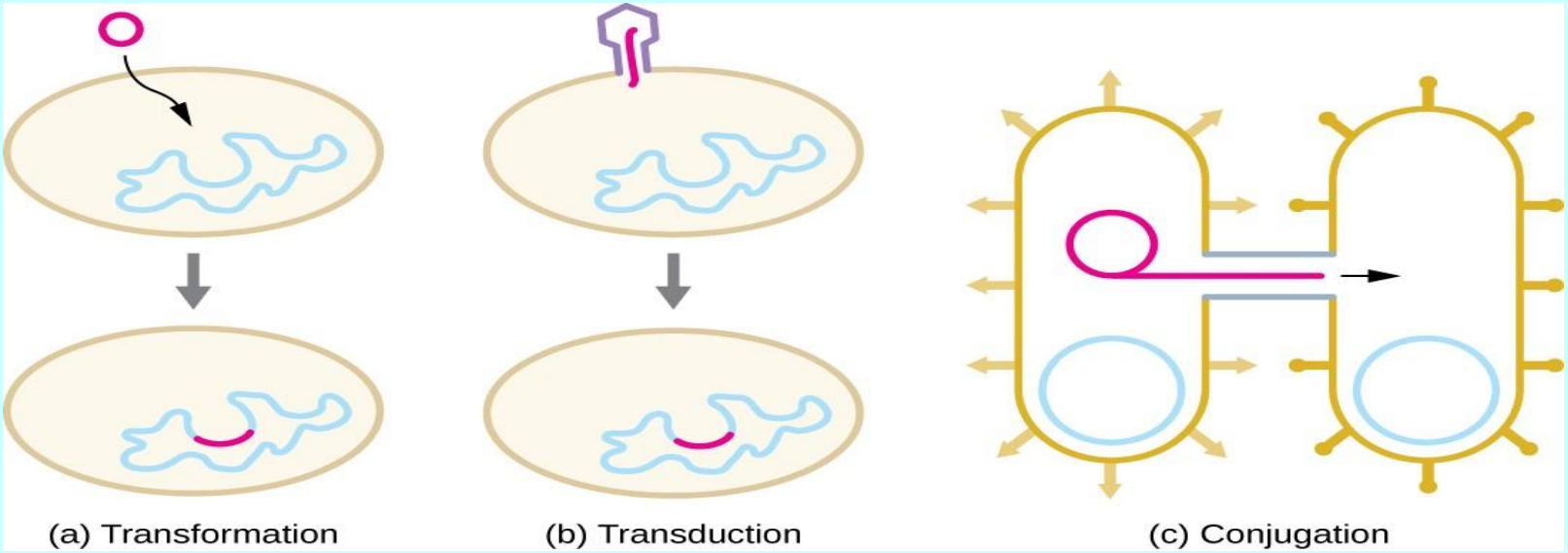
Breeding of livestock and plants

Somatic cell fusion

Crossing → gene recombination

In nature it occurs naturally in 3 ways

transformation **transduction** **conjugation**



https://courses.lumenlearning.com/cuny-kbcc-microbiologyhd/chapter/how-asexual-prokaryotes-achieve-genetic-diversity/

Which of the following processes is most similar to cell fusion?

Conjugation

Reflection

The fusion of two cells creates new genotypes

Animal cells contain billions of nucleotides

(human 3 x 10^9 nucleotides)

How many new genotypes will be created if the genotype of the parent cells differs by e.g. 10 nucleotides?

2 cells, 10 nucleotides

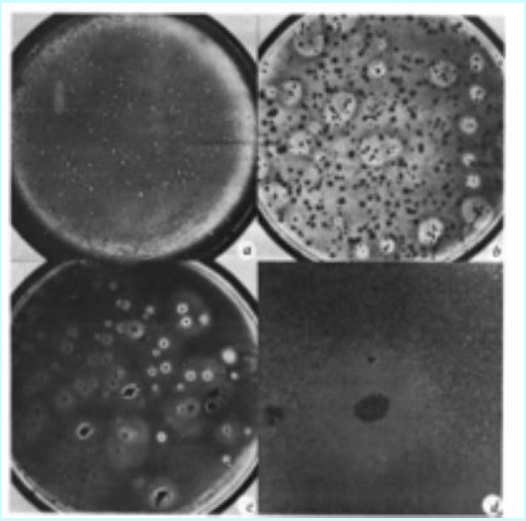
2^{10}

1 024 new combination

Hybridoma

Hybrid cells resulting from the fusion of somatic and tumour cells

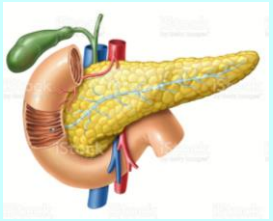
Köhler and Milstein, 1975



https://hybridoma.com/kohler-and-milstein/

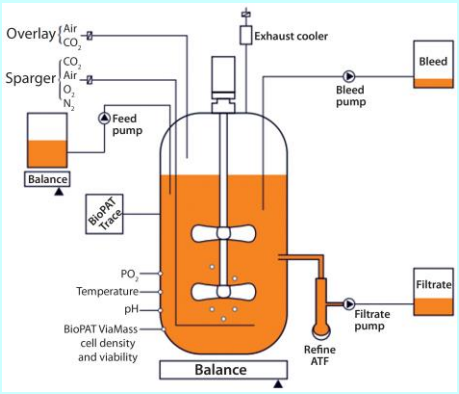
Example of hybridoma

Human pancreatic cell



Tumour cell

Hybridoma



Insulin

Not used because of more economically viable solutions

Use of hybridoma

Technology has revolutionized immunodiagnostics

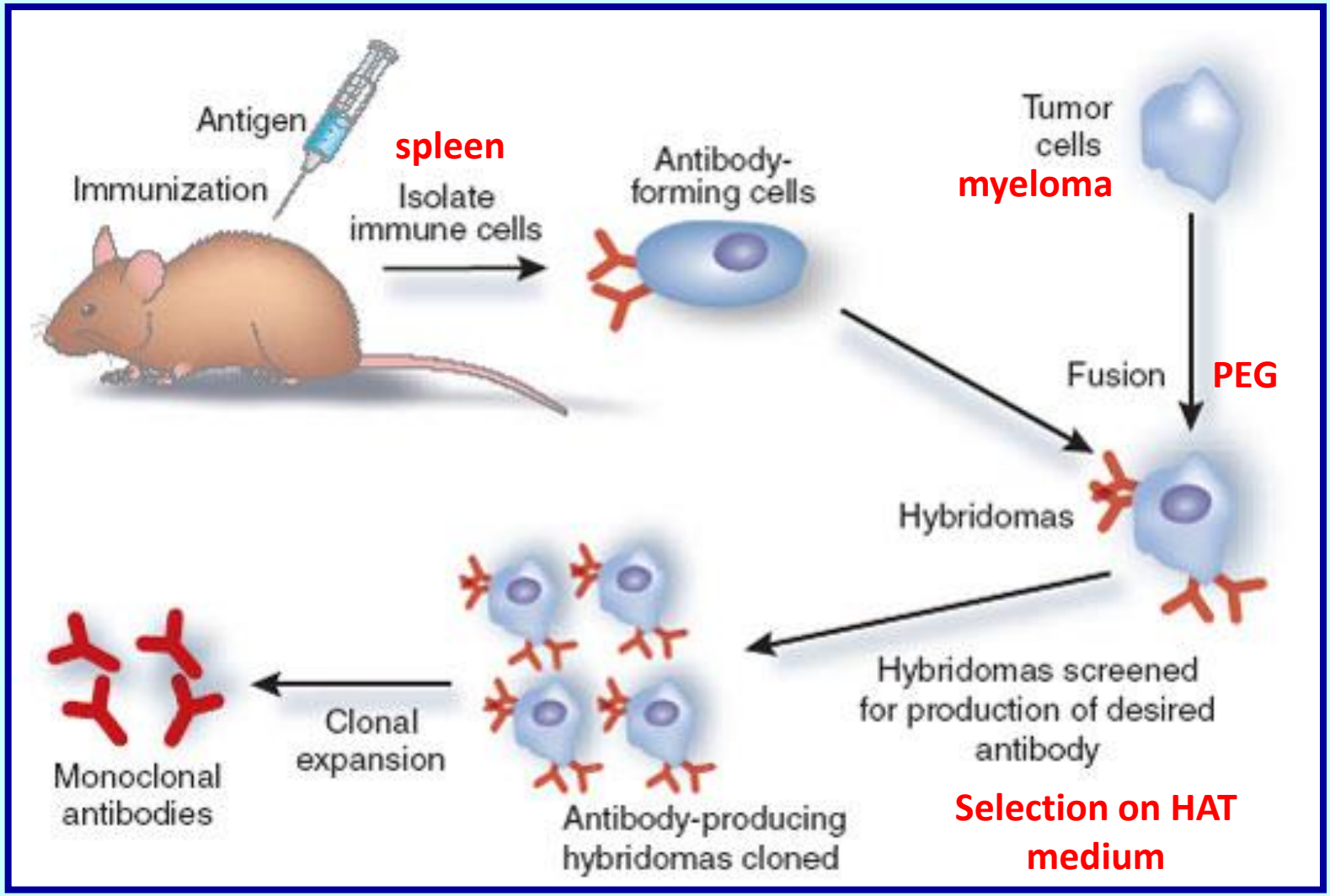
- **Analytical methods, research (protein structure)**
- **Diagnostic methods - pathogens, hormones, interferons, ... (ELISA)**
- **Cancer specific diagnostics and therapy (tumour specific antibodies)**
- **Immunotoxins**
- **Vaccines, antiviral antibodies**

Production of monoclonal antibodies

From hybrid cells resulting from the fusion of a B-lymphocyte and a tumour cell

- **The lymphocyte produces an antibody**
- **The tumour cell ensures immortality**

Preparation of monoclonal antibodies



Advantages of the technology

- The antigen for immunization does not have to be completely pure, but we can always obtain a clone that produces the desired antibody
- The method is gentle on experimental animals
- Frozen hybridoma has an indefinite shelf life, no need to repeat the preparation

Hybridoma selection

Grow on selective HAT medium

- Contains hypoxanthine, ametopterin, thymineRequires cells with functional hypoxanthine-guanine phosphoribosyltransferase (HGPRT+)

- Myeloma cells don't grow because

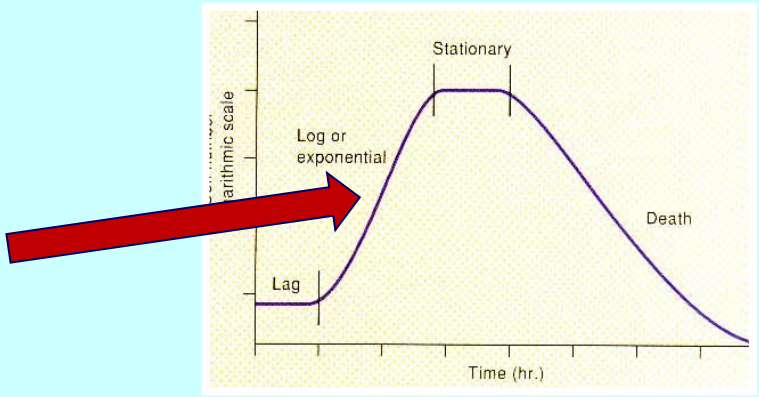
are HGPRT$^-$

- Lymphocytes don't grow because

do not proliferate in the culture

Hybridoma cultivation

- Keeping in an immediately usable state – passaging
- Multiplication - to obtain inoculum, for preservation in frozen state
- Cultivation for production
- Recloning of an existing line



In the exponential growth phase (between 1-2 days after inoculation) the generation time is 12-20 hours

Hybridomas are stored indefinitely in liquid nitrogen, DMSO as a protectant

Problems

The critical parameter is the stability of the culture

- **The ability to produce is lost in 50-70% of the original cells**
- **Regular monitoring of production is necessary**

Hybridoma cultures are contaminated with bacteria, yeasts, fungi and mycoplasmas

- **For example, the PCR method can be used to verify**
- **Remove of the infected culture!**

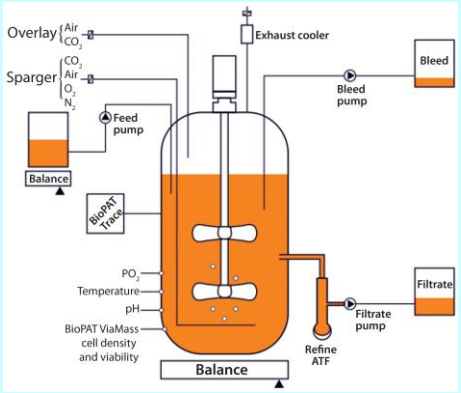
Possibilities of antibody production in hybridoma

In vivo

- In 2-10 ml of ascites 5mg antibodies/ml
- Non-humane but easy

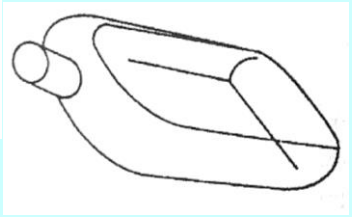
In vitro

- Submersion
- In medium10-100µg/ml



Stationary

- For small amounts



Encapsulated hybridoma

How to prepare?

- Calcium alginate encapsulation
- Particles 3-5mm in size
- Liquefaction with chelating agents

The resulting homogeneous suspension consists of hybridoma enclosed by a polylysine membrane

- Suspension in growth medium in a bioreactor
- Cells grow to high densities
- Antibodies remain inside the capsules and concentrate up to 10 g/l
- Extraction of antibodies by gentle homogenization

Preparation of MP by genetic engineering methods

- The immunoglobulin coding sequence is cloned into an expression vector
- The host cell is a mammalian cell
- Transfectants behave as hybridoma

Repetition - types of antibodies

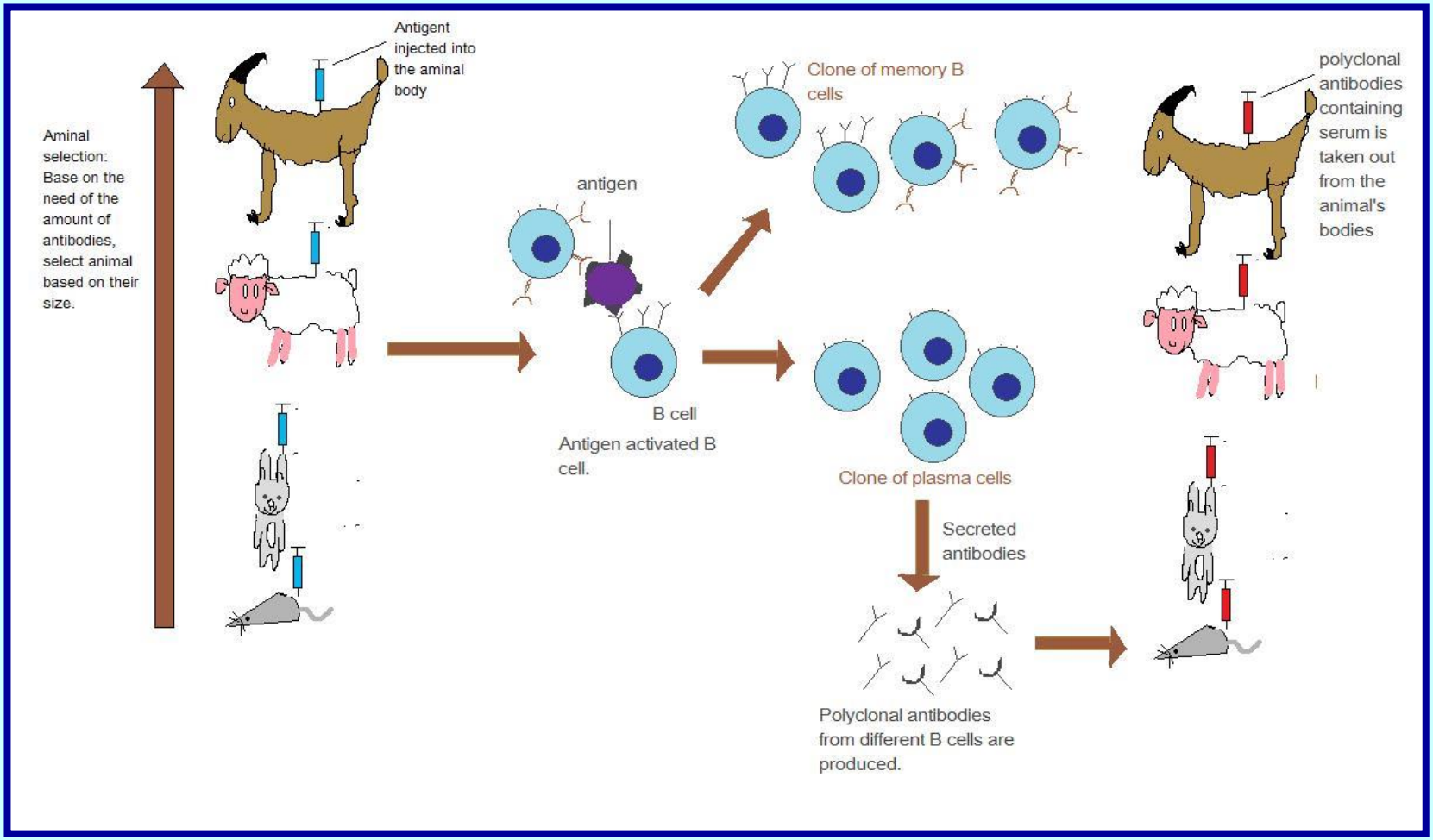
Monoclonal

- The only type of immunoglobulin molecule produced by a single clone of B-lymphocytes
- Cannot be produced directly in the animal body
- Produced in hybridoma cells

Polyclonal

- Heterogeneous mixture of multiple antibodies obtained by immunization of an animal (rabbit)
- They are the product of different clones of B-lymphocytes

How to prepare polyclonal antibodies



Summary

1) Industrial microbiology
2) Enzyme engineering
3) Immobilized enzymes
4) Plant biotechnology
5) Animal cell culture
6) Cell crossing, hybridoma