université PARIS-SACLAY | FACULTÉ DE PHARMACIE

Production of therapeutic interest biomolecules

Development of Drugs and Heath Products Master

TU08 Biotechnology

Inserm
Institut national de la santé et de la recherche médicale

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Agenda

- Introduction: Health and Biotechnologies
- Production process for therapeutic bioproducts: USP/DSP

- Example for Antibiotics production
- Example for Recombinant Proteins

Introduction: Biotechnology Concept

Pharmaceutical biotechnolgy: process using mirobial factories, plants or animals for the production of pharmaceutical products

Bioproduction is the production of biologics-based therapeutic drugs

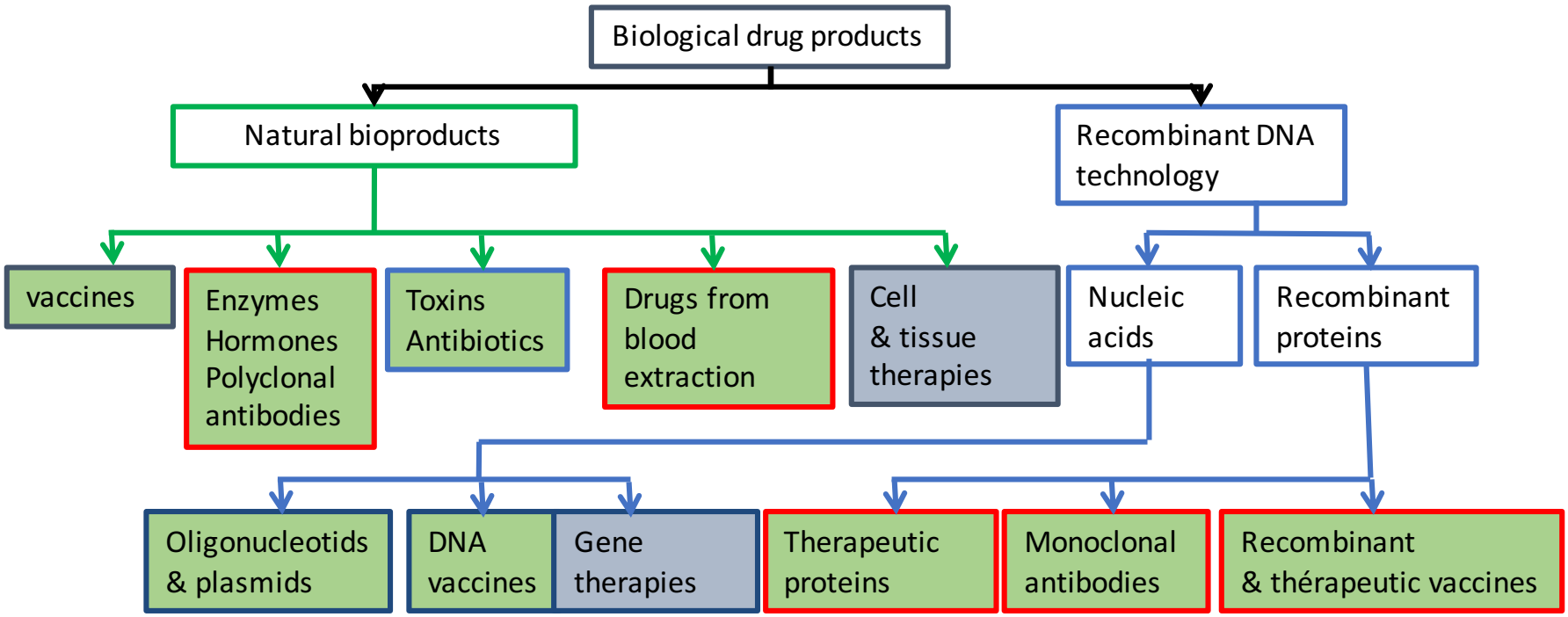
Biodrug:

- Drug substance produced by or extratcted from a **biological source**
- Well characterized & described **manufacturing and purification process**
- **Characterization** and **quality** assessment need a **combination** of physical, chemical and biological assays

EU definition of Biologic (Directive 2001/83/EC as amended, Annex 1 Active substance 3.2.1.1.b)

Biodrug classification

The CONCEPTION and the PRODUCTION are biotechnological process



Biodrugs origin

1- Extraction: human or animal tissues or fluids (Blood, urine, milk, cells…)

2- Cell substrates: procaryotic (bacteria) ou eucaroytic (fungi, insects, mammals…) →Culture production systems

- **Natural secretions: toxins ou bacterial enzymes (botulism toxin, tetanus toxin, streptokinase…)**
- **Recombinant secretions after genetic engineering**

3- From transgenic animals or plants

Biogrugs and micro-organisms

- **Proteins:**
  - Recombinant: hormones, antibodies and derivates, cytokines…
    - Bacteria, yeast, insect cells, mammal cells
  - Natural production: enzymes, hormones, antibodies, albumin, collagen…
    - Bacteria, yeats, fungi, mammal cells
- **Nucleic acids**
  - DNA vaccines: bacteria
  - Plasmids, oligonucleotides: bacteria
  - Viral vectors: viruses (gene therapy)
- **Antibiotics:** fungi, bacteria
- **Lipids, polysaccharids, organic complex molecules**: Yeast, bacteria
- **Vaccines**
  - Microbial: virus, bacteria
  - Recombinants: bacteria, yeast, mammal cells, insect cells

The production process: an overview

Bioproducts need complex industrial biological mastered process

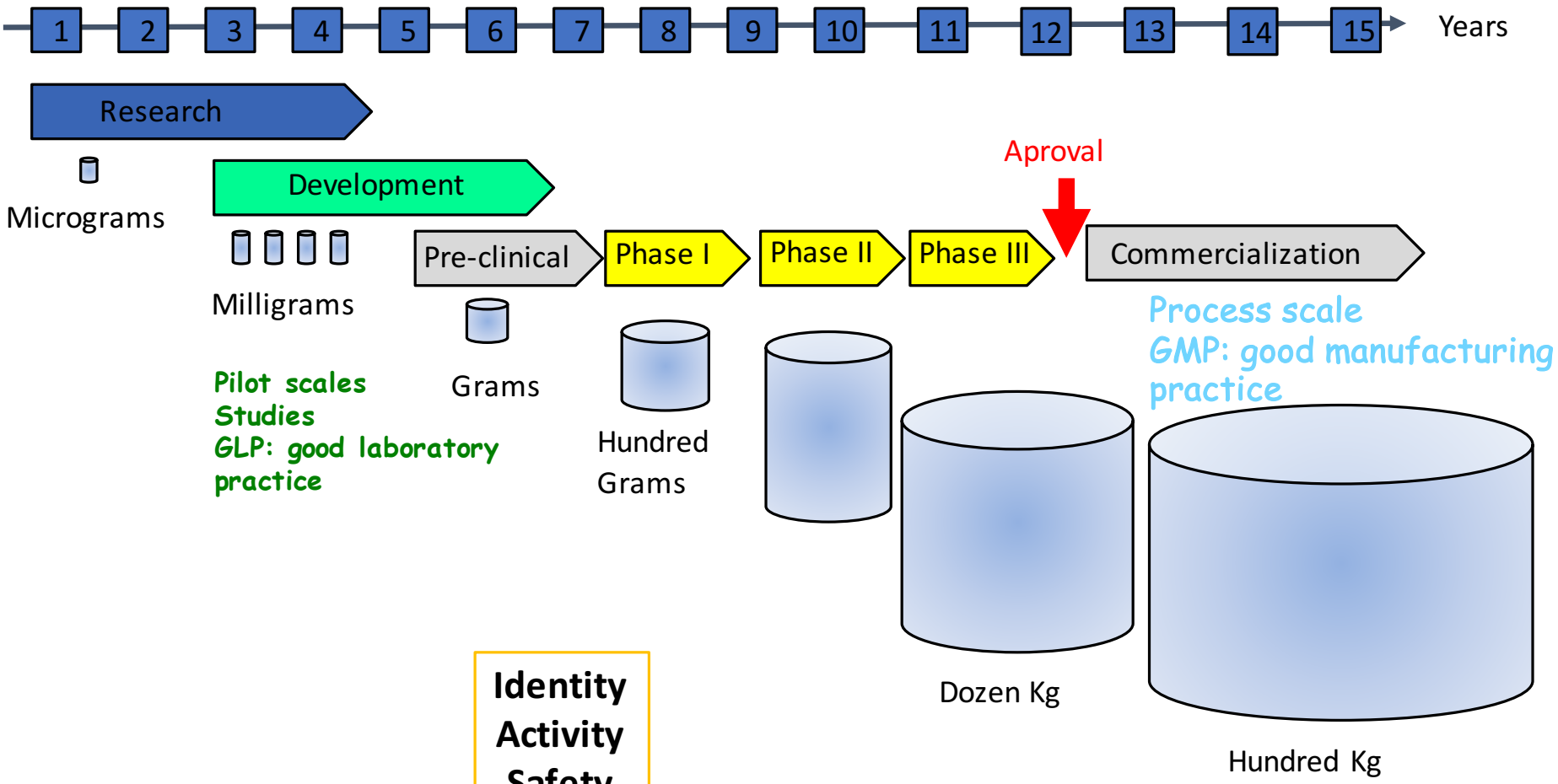
- ✓ Biological materials isolation
- ✓Modification
- ✓multiplication
  - ✓ Characterization & sequencing
  - ✓ Cloning
  - ✓ Expression

Genetic engineering

- ✓ Simple and robust process
- ✓Energy balance, raw materials, waste treatments
- ✓Regulatory environment

- ✓ Industrial production
- ✓ Purification and characterization
- ✓ Quality control
- ✓ Conditionning

From research to commercialization



General scheme of a production process

Upstream processing: biomass production
Initial culture
Expansion
Production

Extraction → Concentration/ Primary Purification → Purification Resolutive Chromatography

Downstream processing: Purification steps

Drug Substance

Formulation → Conditioning → Labelling Packaging Storage

Drug Product

Upstream processing (USP)

Productive cell-strain choice

Rusticity: adaptability to industrial conditions

Performances: growth speed, production speed, production yield…

Genetic stability

Bioreactors, required functions:

- **Confining**
- **Sterility**
- **Mixing**
- **Aeration + air filtration**
- **Nutrient and fluid supply**
- **Cleaning procedures**

Microbial Culture parameters

Physical, chemical:
pH, pO2, T°, osmotic pressure

Kinetics:
Dilution, nutrients, culture length…

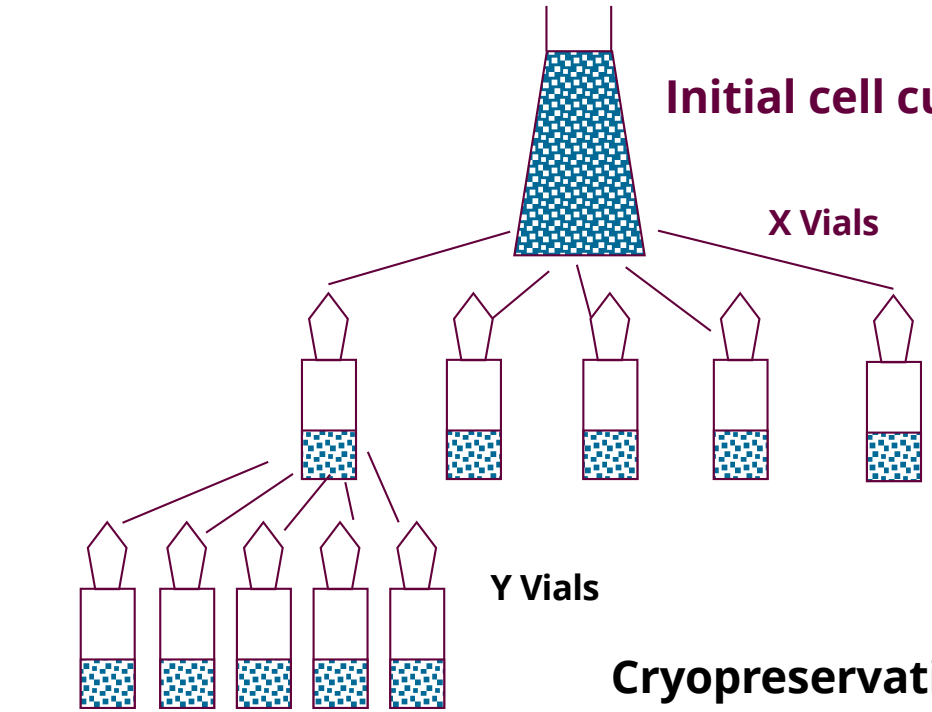
Nutrient composition :
Nitrogen, carbon, growth factors, lipids, oligoéléments (Mn, Fe, Co, Ni, Cu, Zn, Mo)…

Metabolit accumulation

Process modelisation and automation

BioBanking

Goal: to store and securize the well characterized inicial cell culture



Master Cell Bank (MCB)

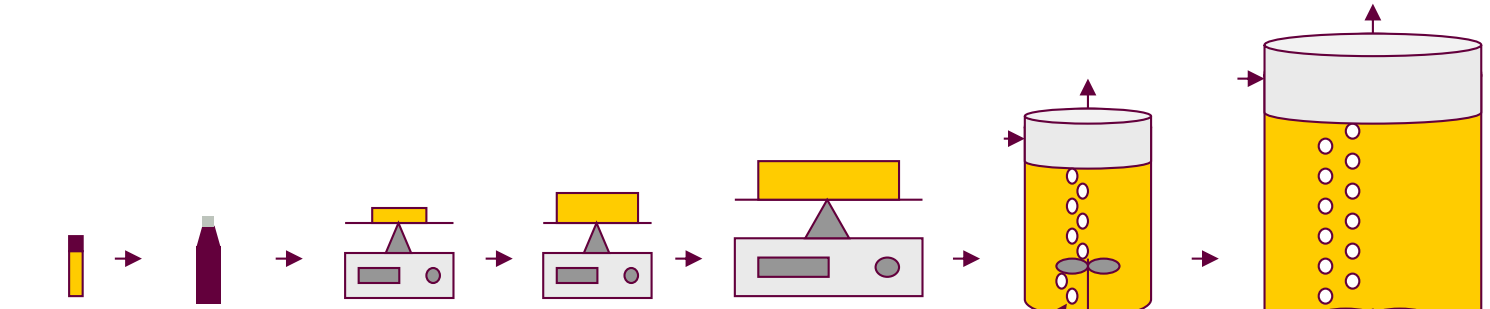
Working Cell Bank N°1 (WBC)

Cryopreservatives: PEG, DMSO…

Cell bank characterization tests
Culture purity
Species identity
DNA sequencing
Plasmid identity and integrity
Host strain identity
Viable count
Specific productivity
Plasmid retention
Restriction endonuclease mapping

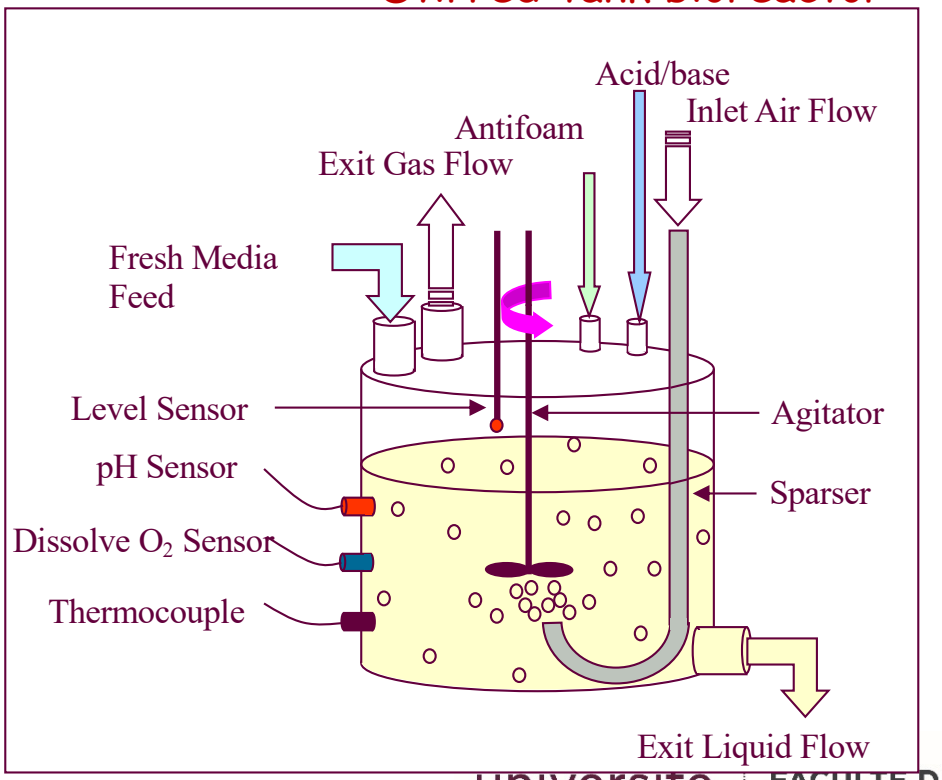
- ✓ **Repetability of cell growth**
- ✓ **Quality control procedures and cell bank viability**

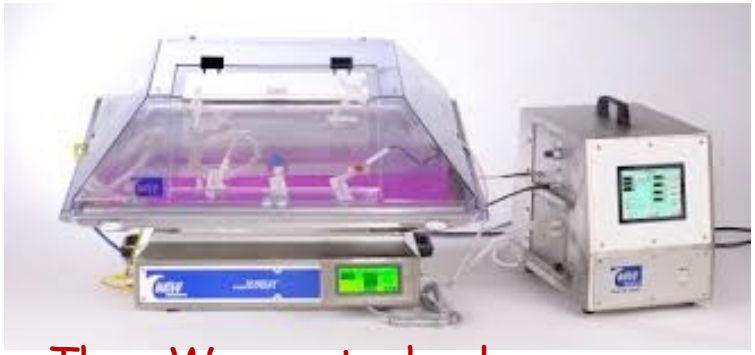
The scale-up



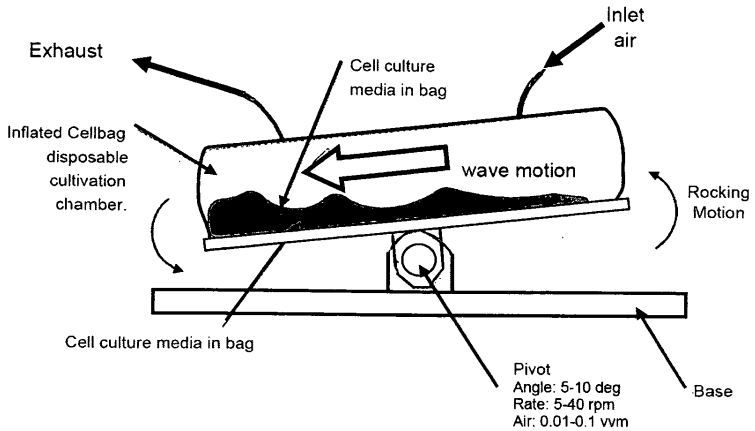
Thaw of the MCB: 40 million cells | **500ml Flask** | **2L Wavebag:** 10 billion cells | **20L Wavebag** | **200L Wavebag** | **3 000L Bioreactor** | **10 000L Bioreactor**

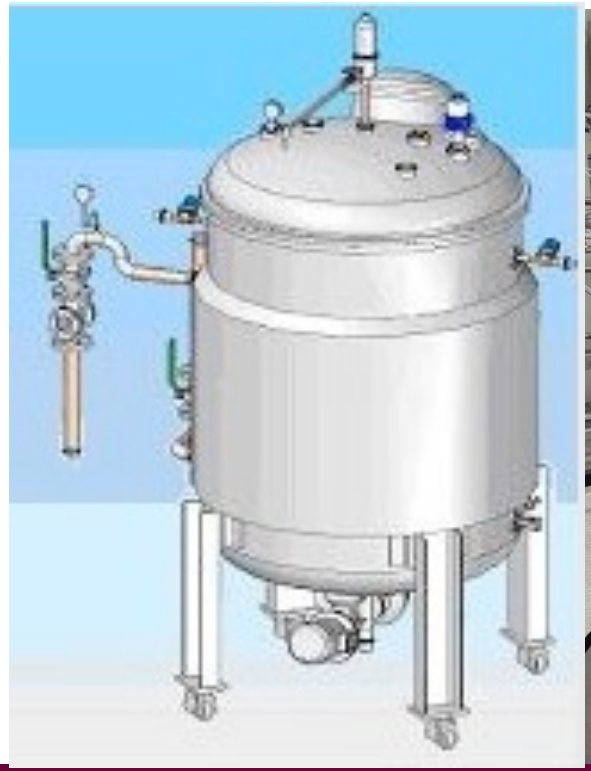
Stirred-tank bioreactor





The« Wave » technology





3 production modes

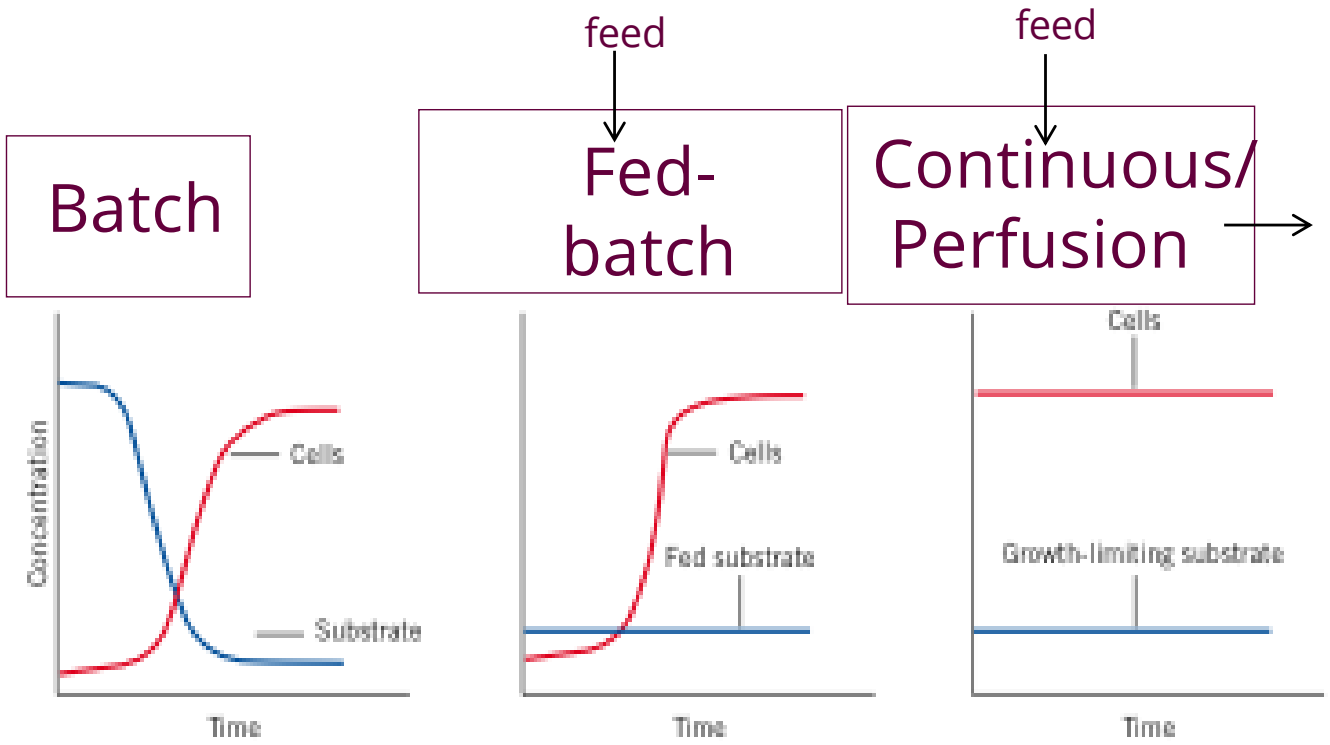


Fig. 95. Comparison of batch and perfusion concerning nutrients and waste products.

Continuous: removal of cells at the same rate

Perfusion: cells are retained or recycled

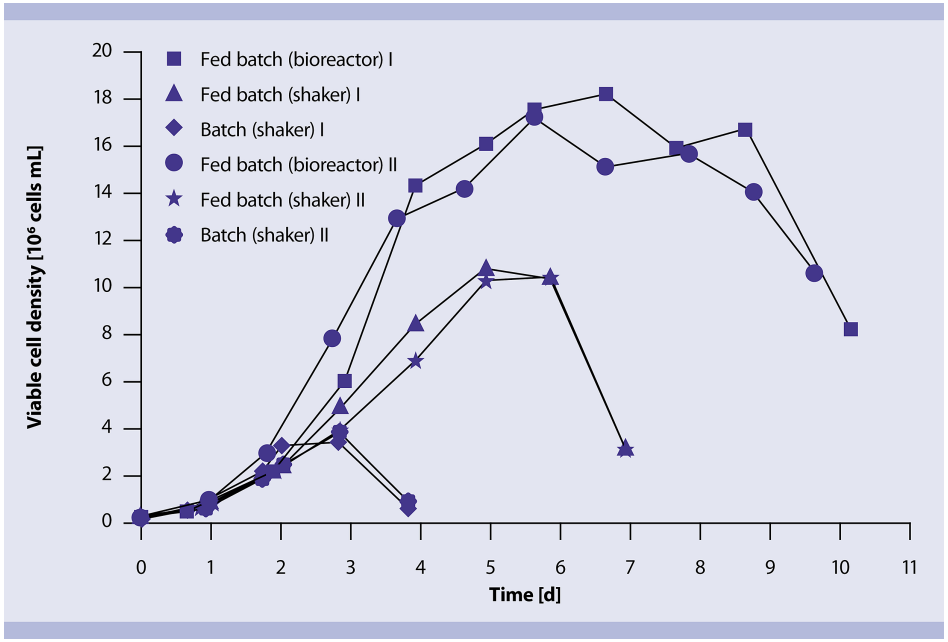
✓Variable medium composition
✓Toxic metabolite accumulation

✓Increase cell density
✓Easy automation
✓Bioproduct accumulation

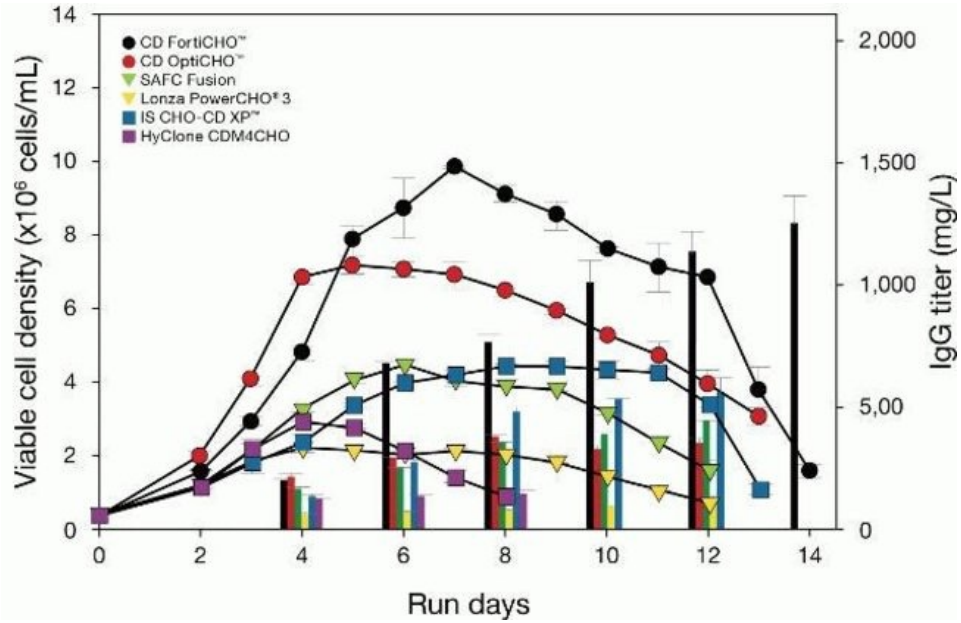
✓Metabolite elimination
✓Stable medium composition
✓Reduced degradation risk

Process development USP – cell growth optimization

Bioreactor comparison



Cell growth media comparison



Critical issues by regulatory authorities:

- ✓ **safety,**
- ✓ **genetic and phenotypic stability,**
- ✓ **monoclonality of the production cell line**

➔ Rigorous cell line development:
Full documentation
Full characterization of the final production cell line

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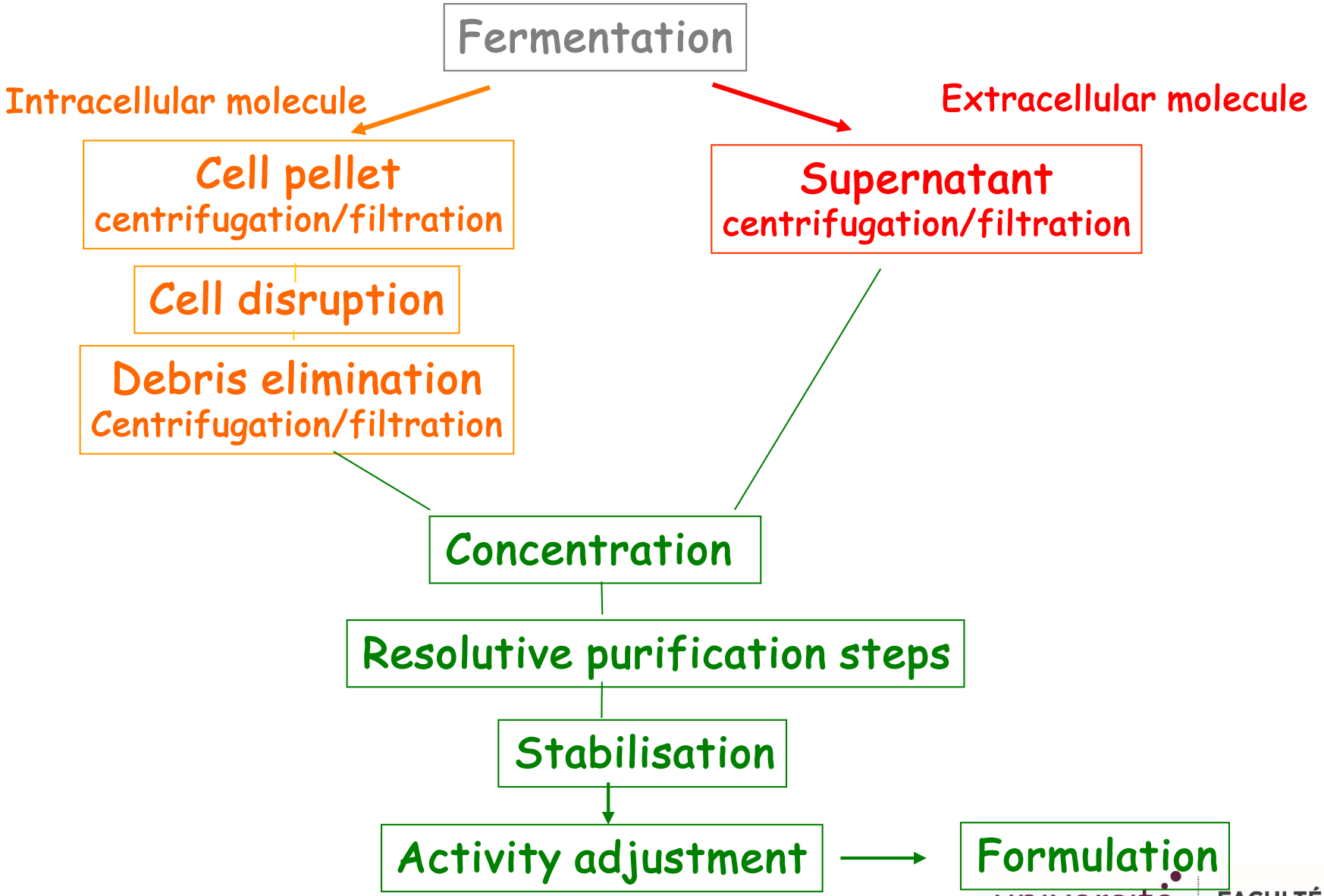
Downstream processing (DSP)



Centrifuge

Filtration installatior

Chromatography

Example 1:

Antibiotic production

Main AB producers

Secondary metabolites: stationnary phase synthesis

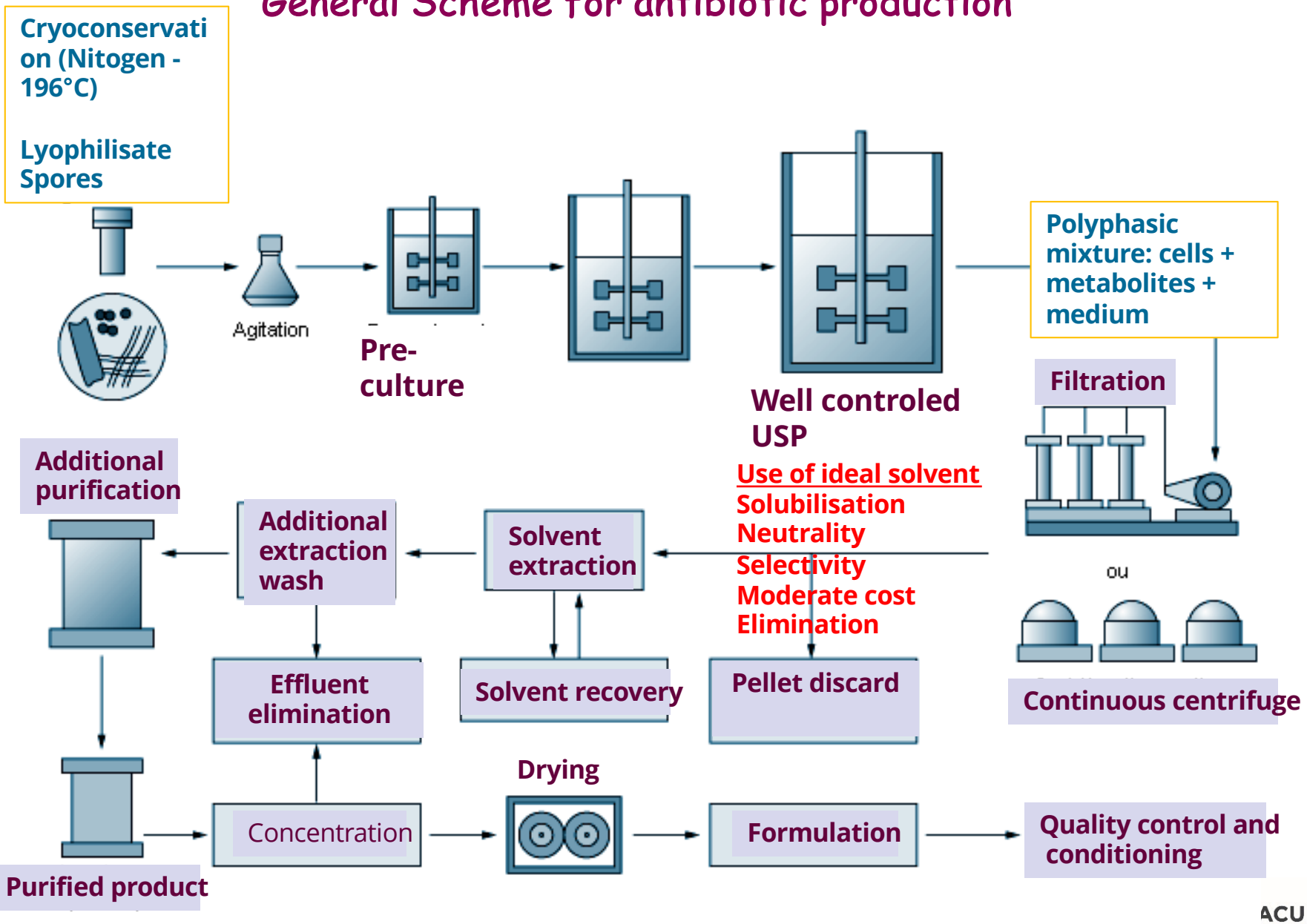
80% Bacteria, steptomyces

Bacillus

Fungi
Aspergillus, Penicillium, Cephalosporium, Heminthosporium, Fusidium

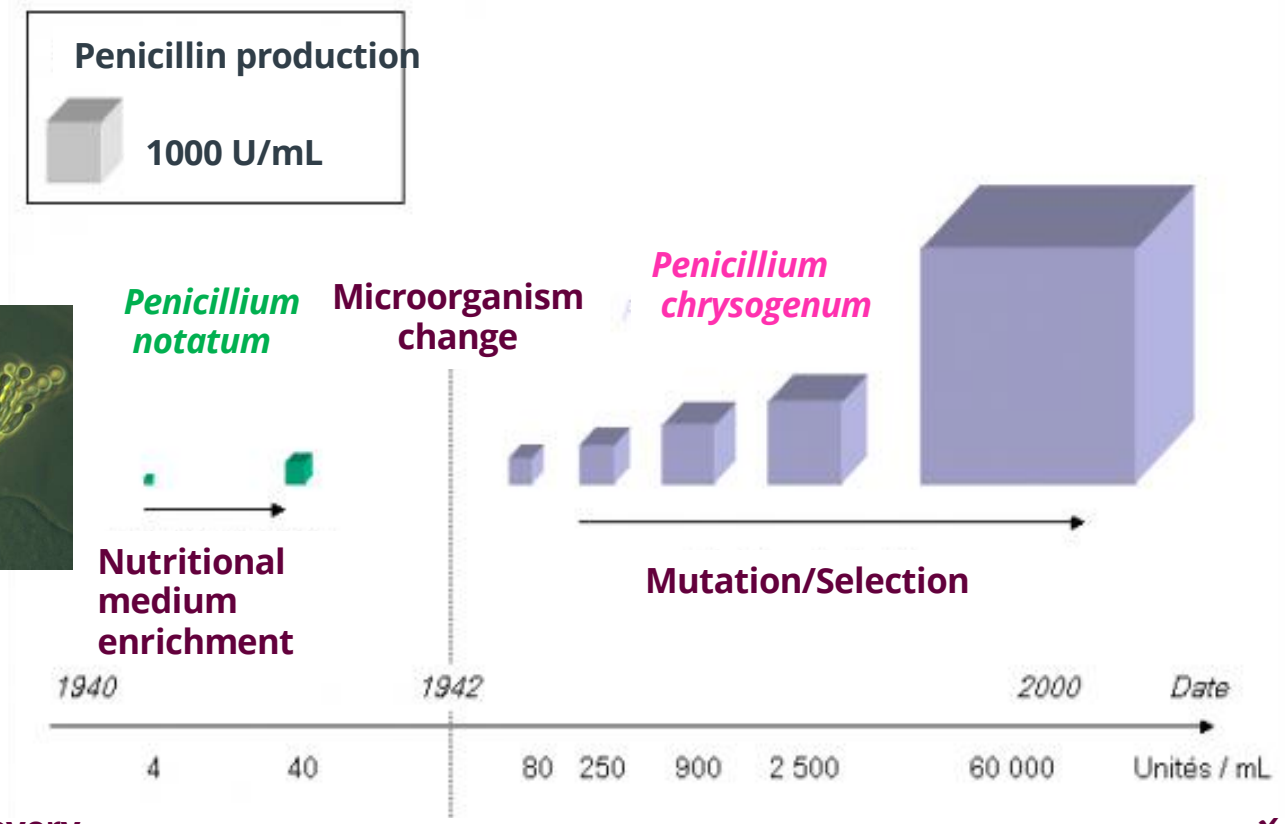
Classification	Antibiotique	Organisme producteur
Aminoglycosides	Streptomycine	*S. griseus*
	Spectinomycine	*S. spectabilis*
	Néomycine B	*S. fradiae*
Tétracyclines	Chlortétracycline	*S. aureofaciens*
	Oxytétracycline	*S. rimosus*
Polyènes	Nystatine	*S. noursei*
	Amphotéricine	*S. nodosus*
Macrolides	Spiramycine	*S. ambofaciens*
	Érythromycine	*S. erythreus*
	Rapamycine	*S. hygroscopicus*
	Natamycine	*S. natalensis*
	Avermectine	*S. avermitilis*
	Tylosine	*S. fradiae*
	Oléandomycine	*S. hygroscopicus*
Streptogramines	Pristinamycine	*S. pristinaespiralis*
	Virginiamycine	*S. virginiae*
Glycopeptides	Vancomycine	*S. orientalis*
Lincosamides	Lincomycine	*S. lincolnensis*
Autres	Chloramphénicol	*S. venezuela*

General Scheme for antibiotic production

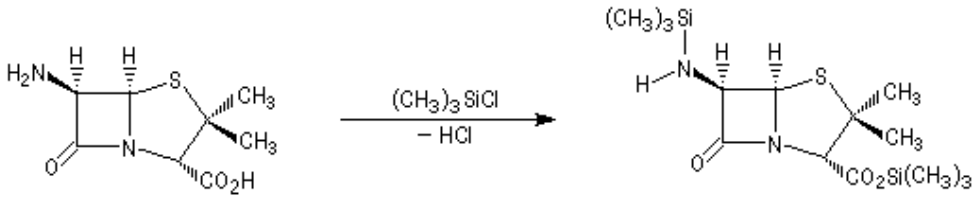


Source: Kroschwitz, 1992.

- **Penicillin example**



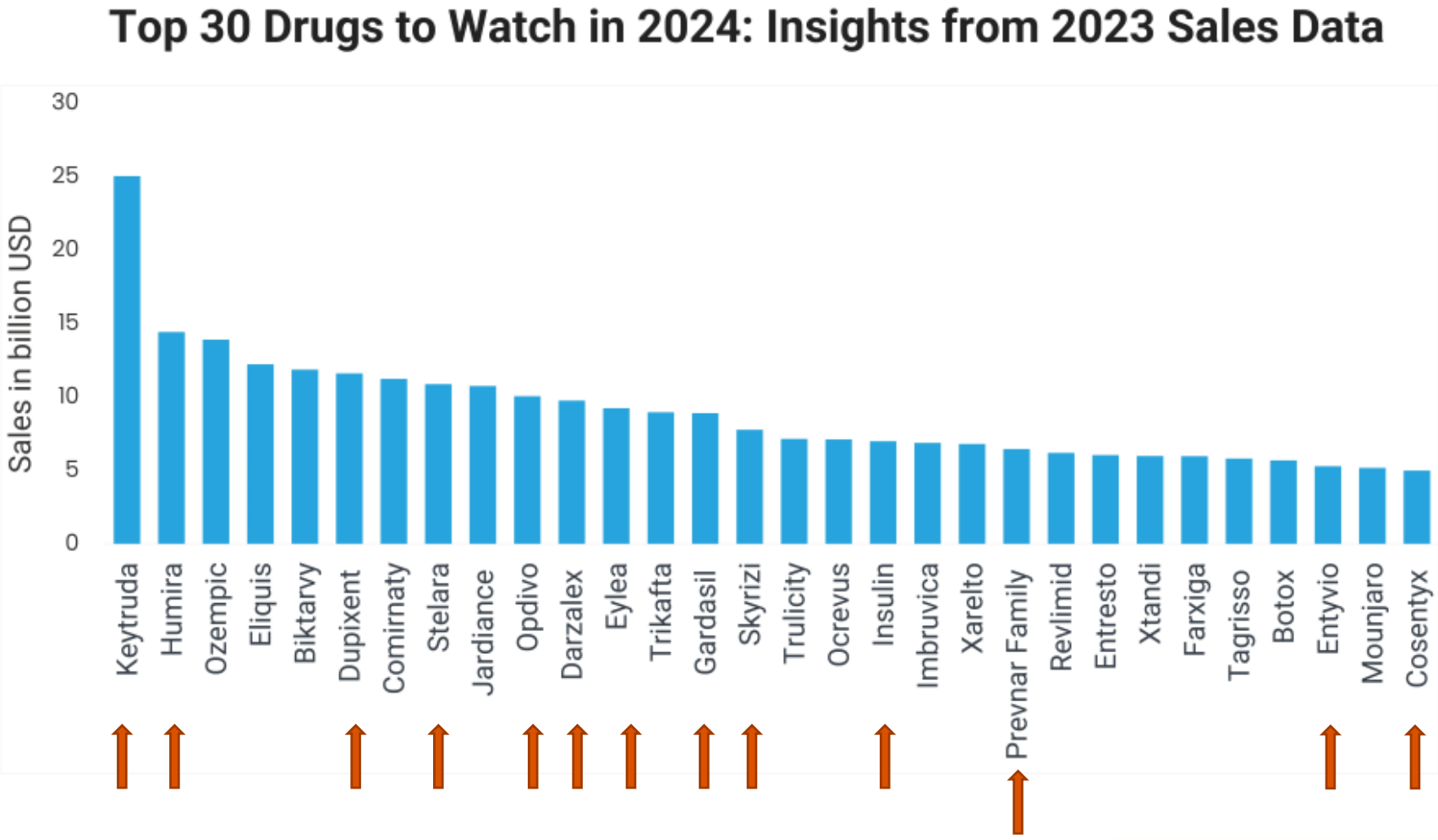
Discovery
1897 (E Duchesne)
1928 (A. Fleming)



- **Production pH≈7**
- **Penicillin excretion at early stationnary phase;**
- **Organic solvent extraction**
- ✓ **Aqueous phase**
- ✓ **Penicillin cristallisation**
- ✓ **Hémisynthesis: transformation into derivates AB: ampicillin amoxicillin…**

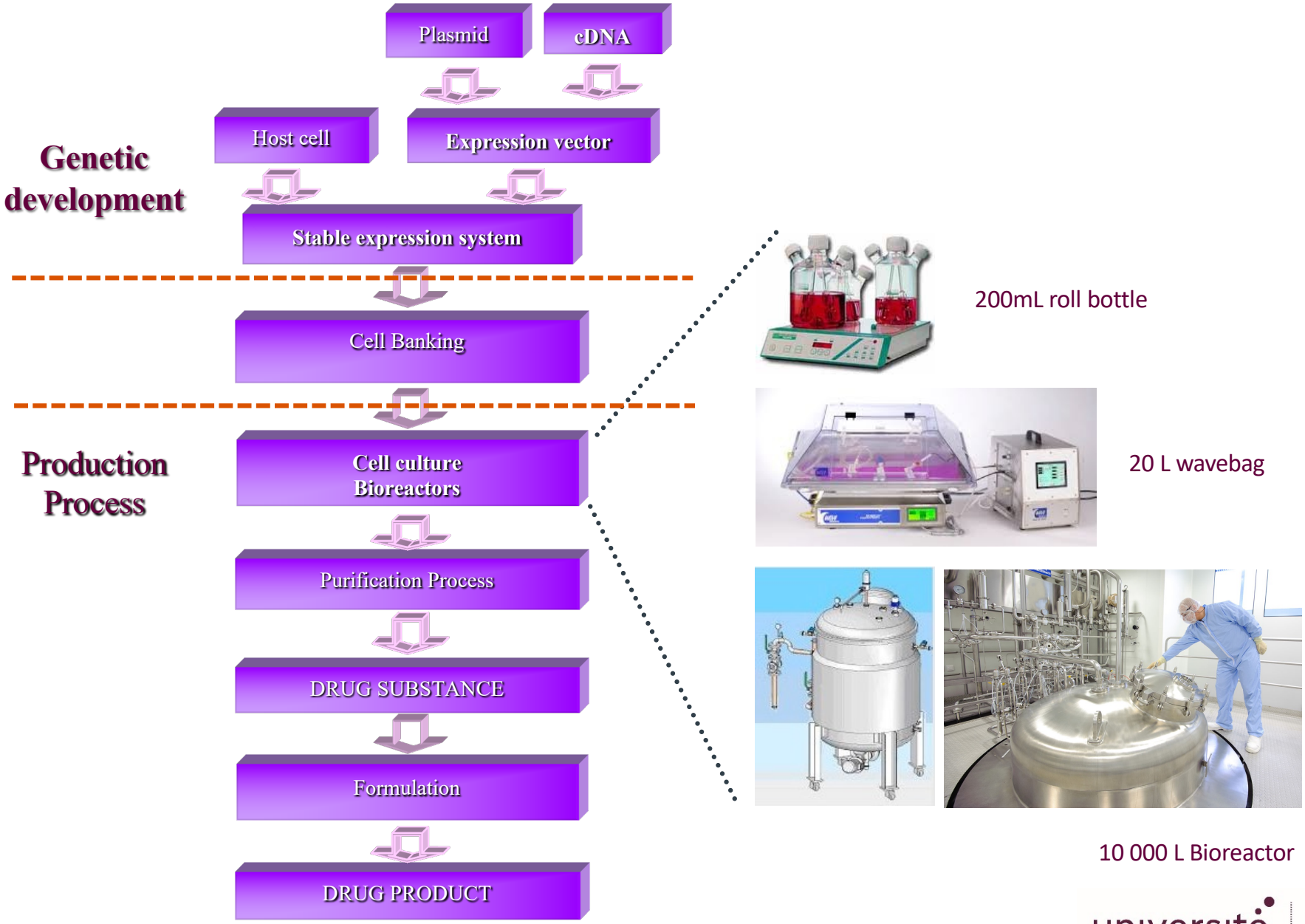
Example 2:

Recombinant protein production

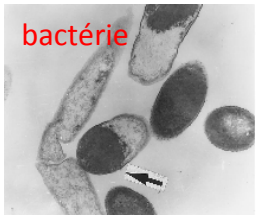


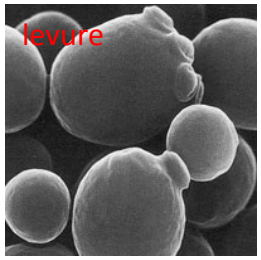
https://xtalks.com/top-30-drugs-to-watch-in-2024-insights-from-2023-sales-data-3908/

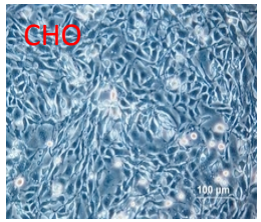
Recombinant Protein production, an overview



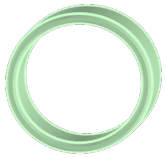
Recombinant Protein technology, the cell line development







cDNA sequence

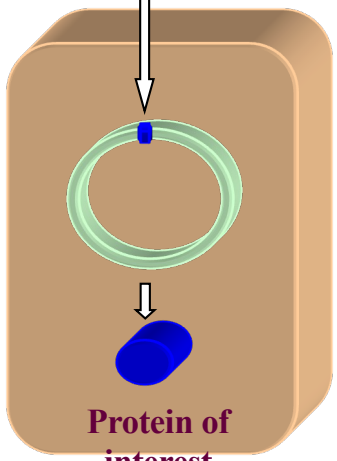


plasmid

Insertion

Expression vector

Transfection



Genetically modified organism = Host cell

Objective: to generate THE producing cell

- ✓ Expression vector optimization
- ✓ Targetting the insersion site

- ✓ Transfected cells selection
- ✓ High producers selection
- ✓ Cell line genetic optimization
- ✓ Culture condition optimization

- GMO choice
- Protein expression level
- Protein properties

Industrial production (cell factory)

	E. Coli	Yeast	Mammalian	Insect
Proteolytic clivage	**?**	**?**	**yes**	**yes**
Glycosylation	**No**	**?**	**yes**	**?**
Secretion	**?**	**yes**	**yes**	**yes**
Folding	**?**	**?**	**yes**	**yes**
Phosphorylation	**No**	**?**	**yes**	**?**
Acetylation	**No**	**yes**	**yes**	**?**
Amidation	**No**	**yes**	**yes**	**yes**
% P / total	**>50%**	**1%**	**<1%**	**>30%**
MM, quantity	**60-70 kDa 100g/L**	**30kDa 10g/L**	**<300kDa 1-5g/L**	**60kDa 200g/L**

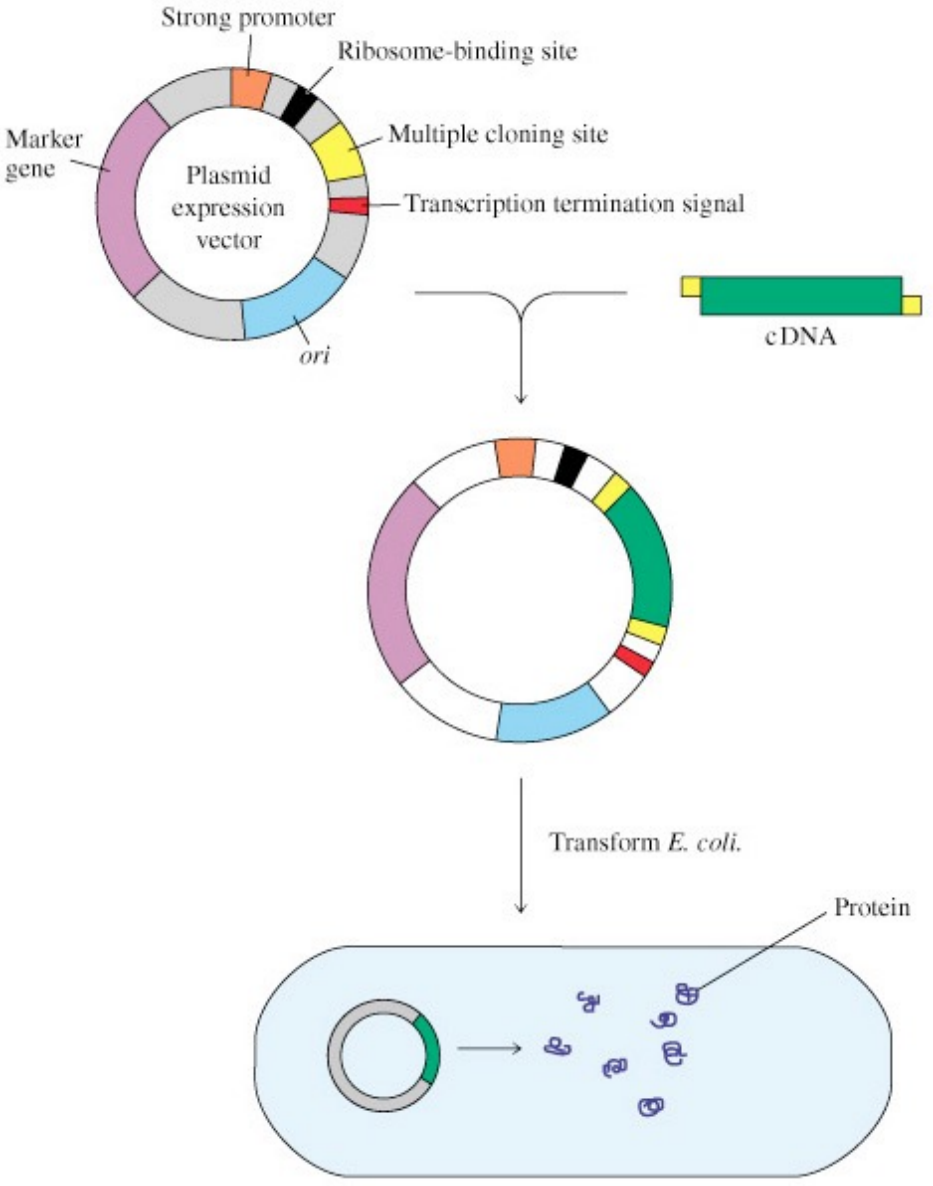
Protein	System	Production level
Hirudin	*S. cerevisiae* (Y)	60 mg/L
	H. polymorpha (Y)	–
Interferon α-2b	*H. polymorpha* (Y)	120 mg/L
Hepatitis B vaccine	*H. polymorpha* (Y)	–
Angiostatin	*P. pastoris* (Y)	108 mg/L
Anti-HBs Fab	*P. pastoris* (Y)	50 mg/L
Human serum albumin	*K. lactis* (Y)	3 g/L
	S. cerevisiae (Y)	3 g/L
	P. pastoris (Y)	10 g/L
Human interleukin 6	*A. niger* (F)	150 mg/L
Human apolipoprotein AI	CHO cells (M)	80 mg/mL
Insulin precursor	*P. pastoris* (Y)	3 g/L
	S. cerevisiae (Y)	98 mg/L
Human tPA	CHO cells (M)	34 mg/L
Human gonadotropin	CHO cells (M)	3 g/L
Erythropoietin (epoetin α)	CHO cells (M)	–
Monoclonal Ab	NSO cells (M)	3 g/L
HPV vaccine (Cervarix™)	Insect cells	–
Human proapolipoprotein AI	Insect cells	80 mg/L
Clotting factor VIIa	BHK cells (M)	–

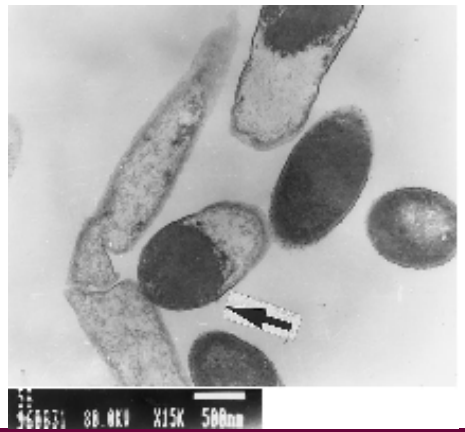
Current Opinion in Biotechnology 2012, 23:965–971

Protein production in Bacteria (*E.coli)*

Intra cellular production

- ✓ Few folding proteins
- ✓ No post-tranpost-translational modification
- ✓ Reducing environment





Inclusion bodies =protein precipitation

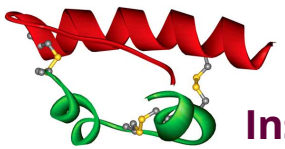
PROS

- Fast and inexpensive growth
- Well-known genetic system, many improved strains
- Controllable gene expression
- Many expression vectors available, ease of transfection into host bacteria
- Good protein production yields (>10 g/L culture)
- Cytoplasmic inclusion bodies: purification ease

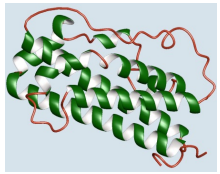
CONS

- No post-translational modification
- Inclusion body: insoluble, poorly folded protein
- Presence of bacterial endotoxins: the purification process must include elimination and control steps

A few therapeutic proteins produced in *E Coli*:

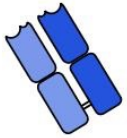


Insulin
16kDa



Growth hormone
22kDa

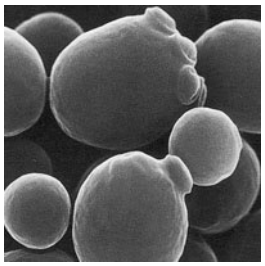
Interferons
20 kDa



Antibody fragment
25 kDa

Fab

Protein production in Yeast



Saccaromyces cerevisiae

Pichia pastoris

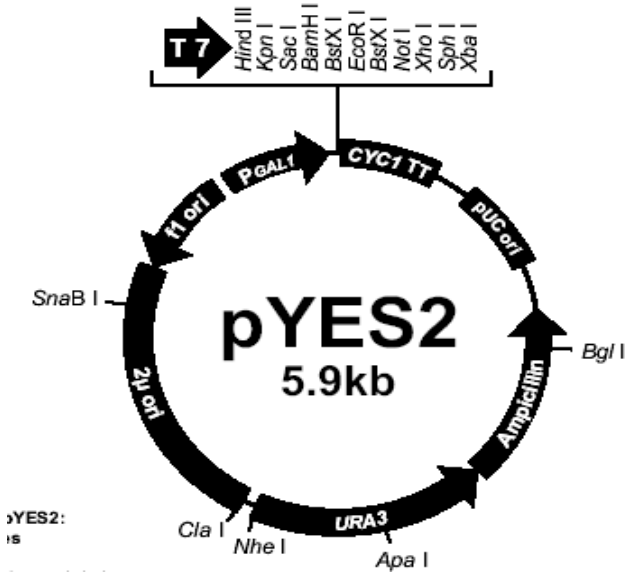
PROS

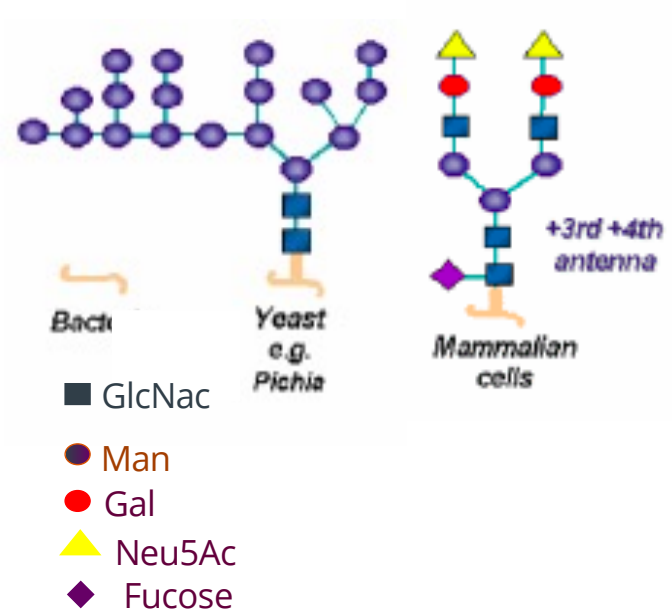
- ➢Small, eukaryotic genome and well characterized
- ➢ Absence of endotoxin
- ➢ Inexpensive fermentation
- ➢ Good yields (grams per liter of culture)
- ➢ Simple post-translational modifications
- ➢Secretion of the protein of interest

INCONVENIENTS

- ➢**Hypermannosylations**
- ➢**Folding**

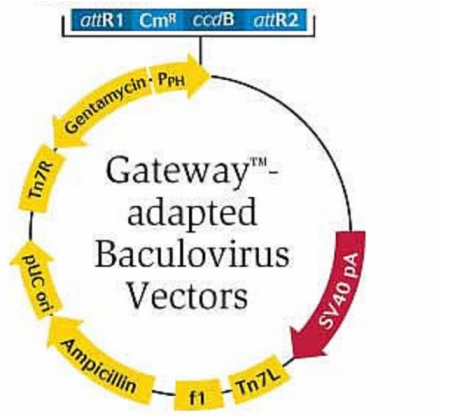
Glyco-engineerinf for humanization





Proteins produced in yeast:
Insulin
GM-CSF (growth factor)
Vaccine anti-HBV
Vaccine anti-HPV
Glucagon

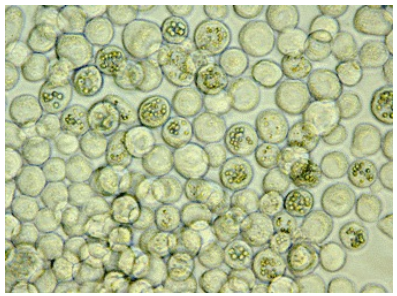
Protein production in insect cells



Baculovirus: insect cell virus

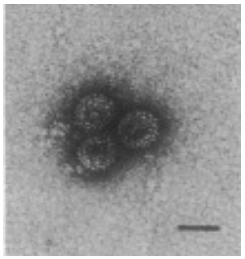
➔ Use of recombinant baculovirus (bearing the cDNA sequence of interest) to infect insect cell suspension

➔ Protein synthesis



➢**CERVARIX® (EMA, 2007 and FDA, 2009) bivalent vaccine bivalent against papillomavirus: prévention of cervical cancer**

HPV-16 L1 protein + HPV-18 L1 protein



Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic

R. KIRNBAUER*, F. BOOY†, N. CHENG†, D. R. LOWY*, AND J. T. SCHILLER*

Proc. Natl. Acad. Sci. USA, 1992

➢**PROVENGE® (FDA, 2010)**

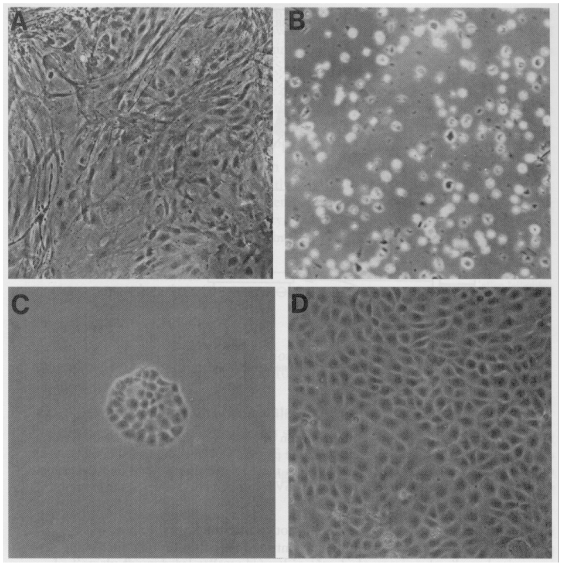
Autologous product for prostate cancer cell therapy: PSA produced in *S. frugiperda*

➢**FluBlok® (FDA, 2011)**

Recombinant influenza vaccine➔ Time and cost-reduced compared to egg production protocol

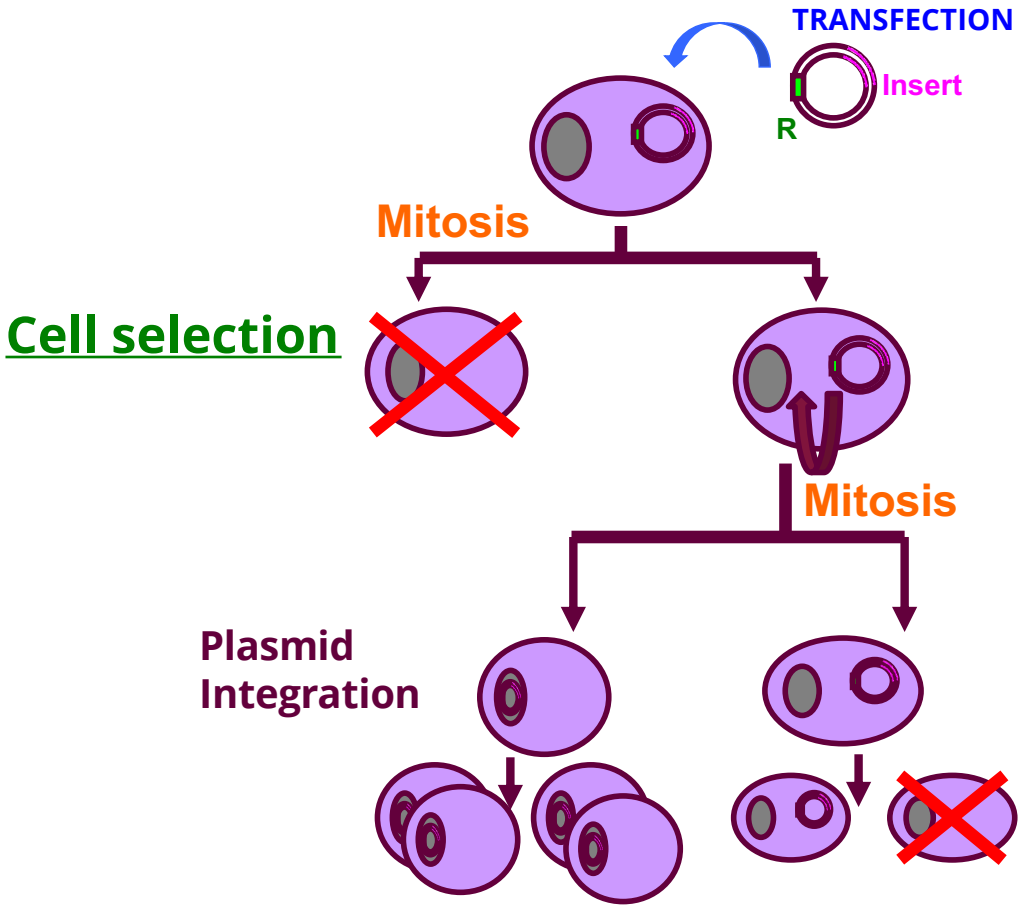
Protein production in mammalian cells

Vector integration in the cell genome



A Non transfected cells

B-D Transfected cells with selection pressure



➔ **Stable cell line for protein production**

Secretion of the protein

Tight quality control of the cell-line

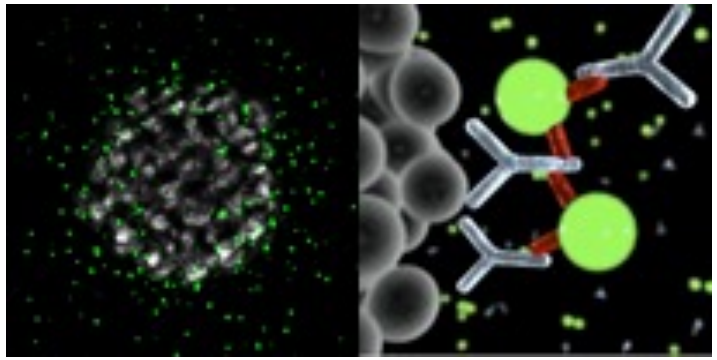
Protein production in mammalian cells

ECACC : European Colection of Cell Culture www.ecacc.org.uk

Cell line		Culture medium	Culture conditions
BHK21	Syrian hamster kidney cells	GMEM + 2mM Glutamine + 5% Tryptose phosphate broth + 10% Fœtal veal serum (FVS)	2-9 10^5 cells/ml 37° C, 5% CO2 Suspension Agitation
CHO	Chineese hamster ovarian cells	Ham F12 + 2mM Glutamine + 10% FVS	0.3 10^5 Cells/ml 37° C, 5% CO2 Adherence, suspension
NSO	Murine myelome No IgG secretions	RPMI + 2mM Glutamine + 10% FVS Selection in HAT medium	3-9 10^5 cells/ml 37° C, 5% CO2 suspension

Best producing clones selection

http://www.genetix.com



CHO → Transfection → selection → **Clonality**

Clonality → Cell growth
Serum-free medium

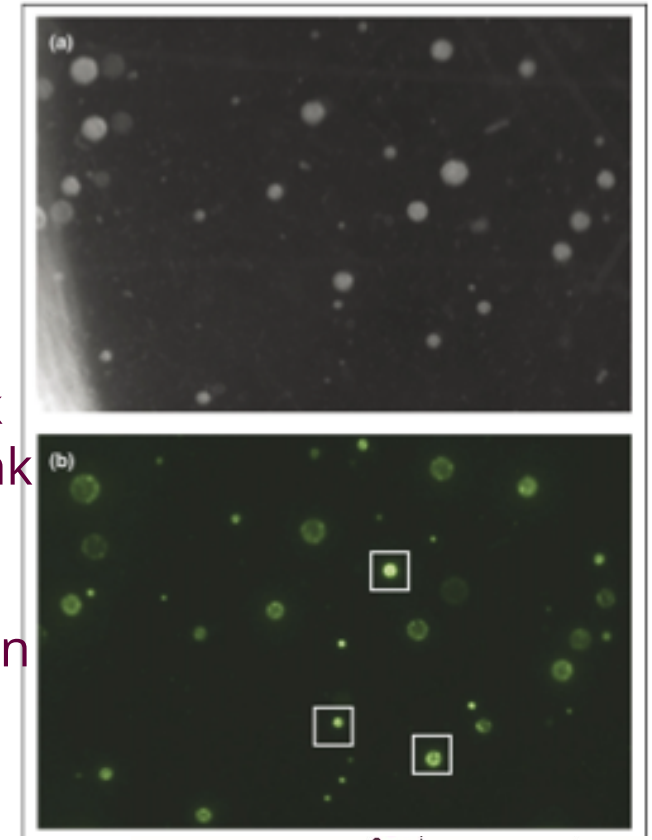
Cell growth → Clones selection

Clones selection → Clone characterization :

- ✓ Transgene copy number (qPCR)
- ✓ Cell identity
- ✓ Cell viability
- ✓ Cell purity…

Clone characterization → ✓ Master Cell Bank
✓ Working Cell Bank

Clone characterization → ✓ End of Production



Browne et al. Trends in Biotechnology. 2007

Quality control of the producing cell cultures

Table 2 Overview of tests performed and results obtained

	MCB	WCB	End of production cells	Bulk harvest
Identity				
Isoenzyme analysis	HEK 293	–	–	–
RAPD	HEK 293	HEK 293	HEK 293	–
Microbiological tests				
Sterility	Complies	Complies	Complies	Complies
Mycoplasma	Negative	Negative	Negative	Negative
Viral tests				
In vitro assay for the detection of adventitious viruses (28 d, three detector cell lines: Vero, MRC5 and 293)	Negative	–	Negative	Negative
In vivo assay for the detection of adventitious viruses (adult and suckling mice, embryonated eggs)	Negative	–	Negative	Negative
QF-PERT	Negative	–	Negative	Negative
PCR screen for human viruses (HIV 1/2, HTLV 1/2, CMV, EBV, HHV 6/7/8, HBV, HCV, B19, HPV, HPyV)	Negative	–	–	–
PCR screen for AAV-2	Negative	Negative	Negative	Negative
TEM	No virus detected	–	No virus detected	No virus detected
MMV infectivity assay	Negative	–	–	–
In vitro bovine virus screen (BVDV, BAV, BRSV, BPV, REO3, BTV, RV)	Negative	–	–	–
PCR screen for bovine polyoma virus	Negative	–	–	–
In vitro porcine virus screen (PPV, PAV, TGE, HEV)	Negative	–	–	–

Cell identity

Microbiological tests

Viral security

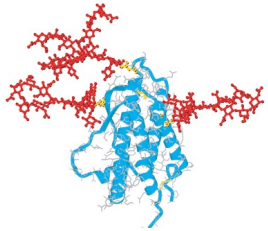
MCB, master cell bank; WCB, working cell bank; EPC, end of production cells; BH, bulk harvest; RAPD, random amplified polymorphic DNA; QF-PERT, quantitative fluorescent product-enhanced reverse transcriptase; PCR, polymerase chain reaction; HEK, human embryonic kidney; HIV, human immunodeficiency virus; HTLV, human T-cell lymphotropic virus; CMV, cytomegalovirus; EBV, Epstein–Barr virus, HHV, human herpes virus; HBV, hepatitis B virus; HCV, hepatitis C virus; B19, human parvovirus B19; HPV, human papilloma virus; HPyV, human polyoma viruses JC and BK; AAV, adeno-associated virus; TEM, transmission electron microscopy; MMV, mouse minute virus; BVDV, bovine viral diarrhoea virus; BAV, bovine adenovirus; BRSV, bovine respiratory syncytial virus; BPV, bovine parvovirus; REO, reovirus; BTV, bluetongue virus; RV, rabies virus; PPV, porcine parvovirus; PAV, porcine adenovirus; TGE, transmissible gastroenteritis virus; HEV, haemagglutinating encephalitis virus.
– indicates tests have not been performed.

 Casademunt et al., European Journal of Haematology. 2012; 89 (165–176)

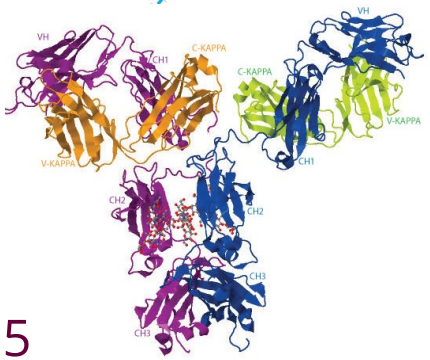
Production in mammalian cell lines

✅ Full antibodies and High molecular weigth proteins
✅ Optimal glycosylation
✅ Best fuctionnal activity
✅ Optimized expression vectors

❌ Culture media costs
❌ Time consuming procedures : construction of the cell line, cell banking, characterizatiosn…
❌ Weak production yields,
❌ Viral security (human cell lines)

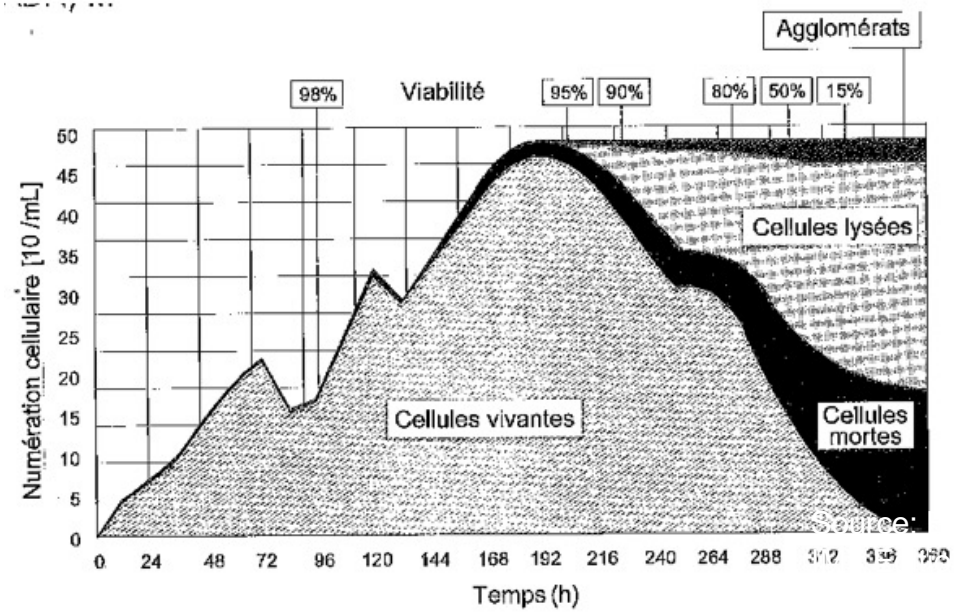


Erythropoïétin (**EPO**)
165 aa, MM : 30,6 kDa ; 40% glycosylation



Full antibodies (mAb): 1200 aa
MM:150kDa, 1 glycosylation site

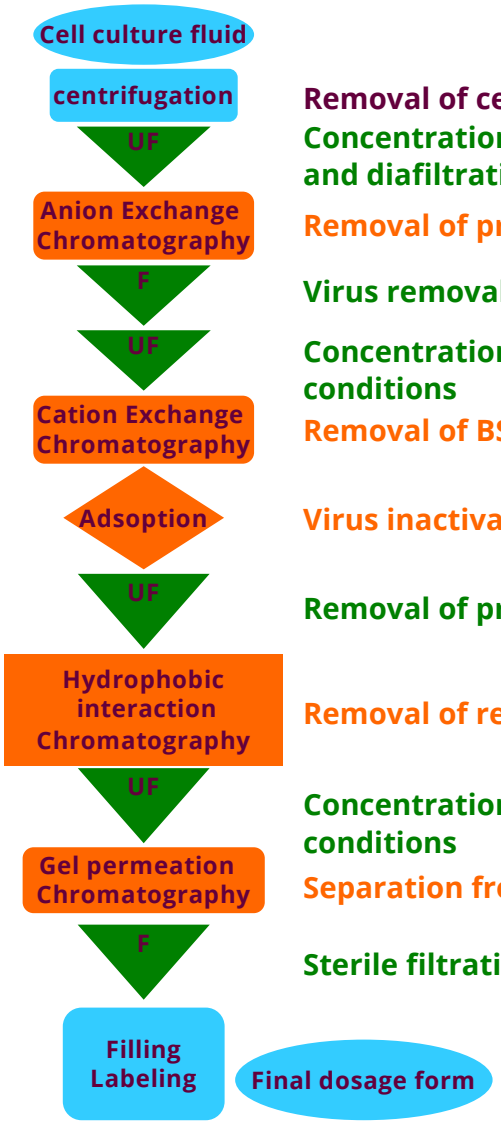
What substances to eliminate during the purification process?



- ✓ Host impurities:
  - Cell debris, nucleic acids, lipids
  - Host-cell proteins (HCP)
- ✓ Microorganisms:
  - Viral particles, Bacteria,
  - Pyrogen substances
- ✓ Process-related impurities:
  - Remaining buffer, chromatography resin, Metals, polymers
  - «extractibles and leachables »…
- ✓ Product-related impurities:
  - Aggregated, truncated, unfolded…protein of interest forms

Industrial production of interferon alpha

28 kDa, glycosylated



Steps that ensure impurities elimination

Steps that ensure or contribute to viral security

Therapeutic protein characterization, A battery of validation tests

- **Identity / structure**

✓ Primary structure (AA composition)

✓ Secondary structure

✓ Glycosylation analysis

✓ Physical parameters: Molecular weigth, isoelectic point…

- **Purity:**

✓ Host cell impurities (DNA, proteins, lipids…)

✓ Fabrication process imurities: leachates and extractables

✓ Product-related impurities: unfolded, truncated , aggregated, chemically degradated mAbs

- **Activity:**

✓ Target binding (affinity measurement)

✓ *In vitro* assays

✓ *In vivo* assays

- **Security:**

Virus particles or genome

Endotoxin detection

Which level?

Cell culture → Raw materials → Production/ Purification steps → Purified product → Drug product

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Conclusion

Pre-clinical trials

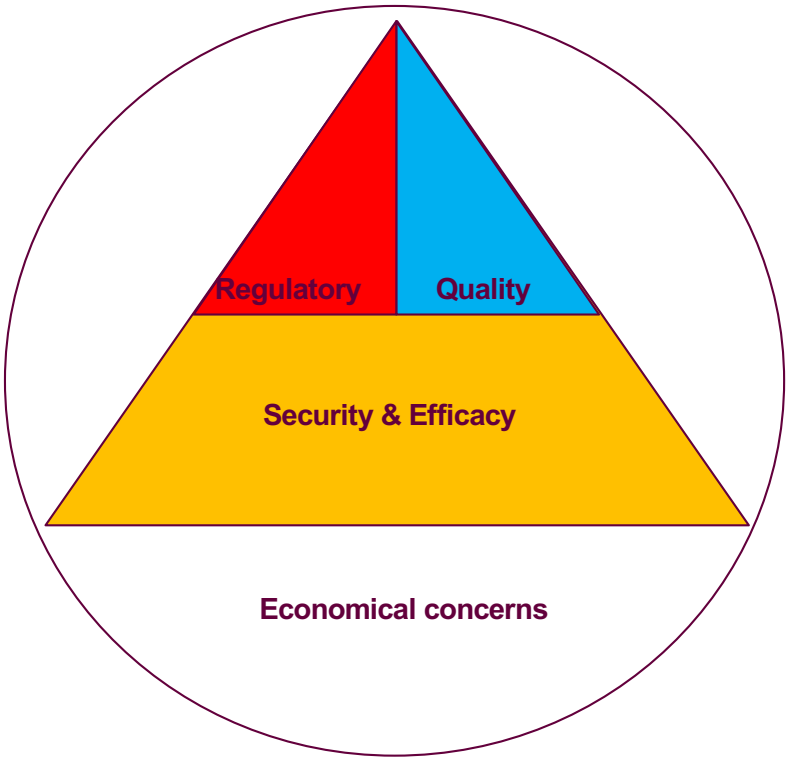
Clinical trials: I To IV

Good Laboratory Practice

Good Clinical Practice

Good Manufacturing Practice

« Good Distribution Practice »



Bioproduct production is under pharmaceutical regulatory concepts that are adapted to living material use

Define critical points at early development stages (MCB conception):
Process complexity
Quality controls
Equipment costs, Staff costs, time consuming

« DEVELOPING A PROCESS WITH THE END IN MIND »