Module ChE 311 Biochemical Engineering

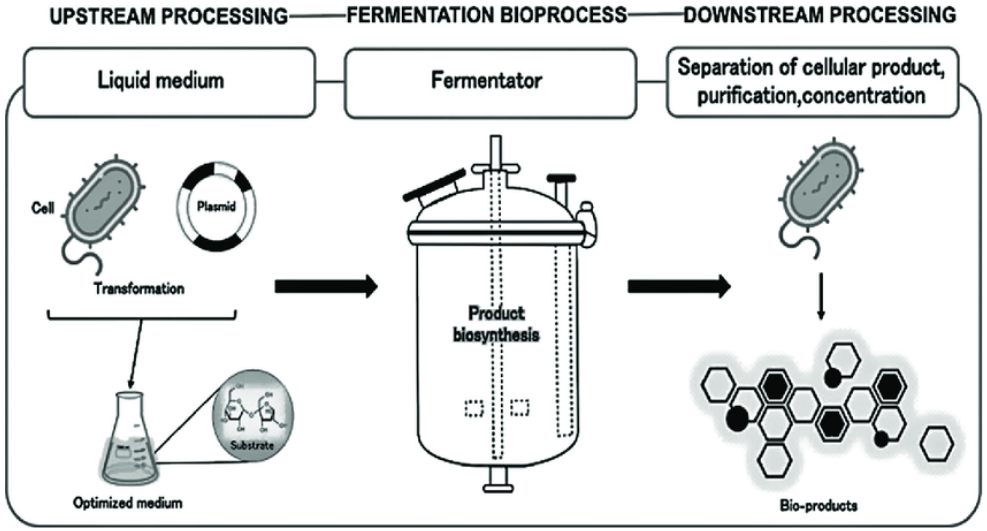
Downstream processing
Lecture 1 – DSP relevance and purification strategy

Simon Crelier, HES-SO Valais – Sion

simon.crelier@epfl.ch
+41 (0)27 606 86 65

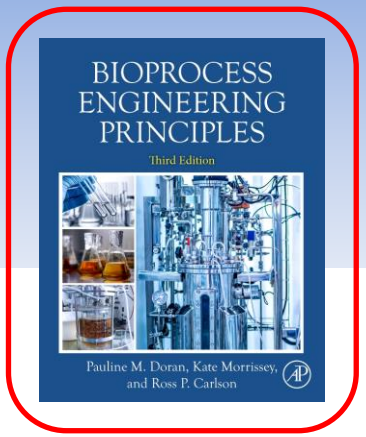
Downstream processing: module outline

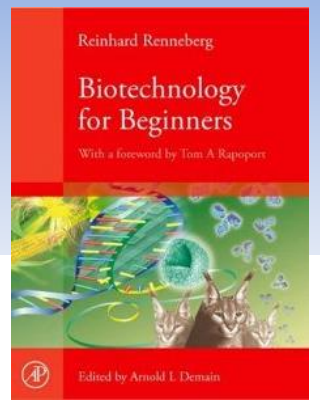
- **Lecture 1 – DSP: relevance and purification strategy**
- Lecture 2 – Liquid/solid separation and cell lysis
- Lecture 3 – Precipitation and crystallization
- Lecture 4 – Adsorption and chromatography
- Lecture 5 – Membrane techniques
- Lecture 6 – Recent trends and reserve time



Source: De la Fuente Salcido et al., 2019

Bibliography (1/2)





Biotechnology at large

- Reinhard Renneberg. Biotechnology for beginners. Academic Press, London, 2007
- Rolf D. Schmid. Pocket guide to biotechnology and genetic engineering. Wiley VCH, Weinheim, 2003

Bioprocess engineering

Best value for your money!
960 excellent pages for about CHF 100.-

- Pauline M. Doran, Kate Morrissey, Ross P. Carlsen. Bioprocess engineering principles. Academic Press, London, 3rd edition, 2024 (On sale August 15, 2024)
- Michael L. Shuler & Fikret Kargi. Bioprocess engineering. 2nd edition, Prentice Hall,Upper Saddle River, 2002
- Shigeo Katoh & Fumitake Yoshida. Biochemical – a textbook for engineers, chemists and biologists. Wiley VCH, Weinheim, 2009

Bibliography (2/2)

Great reference for industrial chromatographic purification! Price around CHF 130.-

Downstream processing

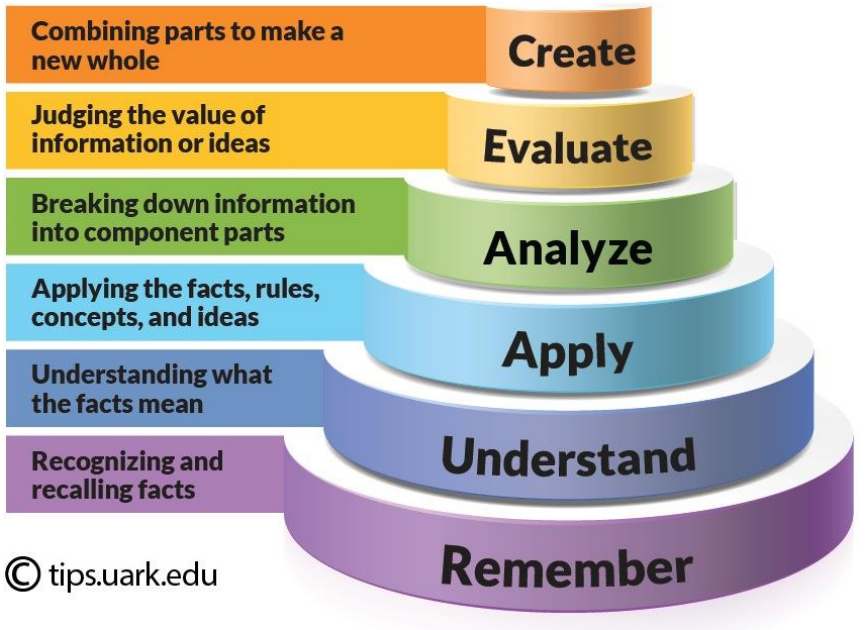
1. J. D. Seader & E. J. Henley. Separation process principles. 2nd edition, John Wiley & Sons, New York, 2006
2. R. G. Harrison, P. Todd, S. R. Rudge, D. P. Petrides. Bioseparations science and engineering. Oxford University Press, New York, 2003
3. P. A. Belter, E. L. Cussler, W.-S. Hu. Bioseparations – downstream processing for biotechnology. John Wiley & Sons, New York, 1988.
4. G. Carta & A. Jungbauer. Protein chromatography – process development and scale-up. 2nd edition, Wiley VCH, Weinheim, 2020
5. A. A. Shukla, M. R. Etzel, S. Gadam editors. Process scale bioseparations for the biopharmaceutical industry. CRC Press, Boca Raton, 2007

Before we get (really) started

- Although not limited to these molecules, downstream processing in biotechnology often has to deal with **proteins** (but not exclusively)
- Many approaches, techniques and equipments have proven their efficacy at laboratory scale (not all being fit for scale-up)
- Here are a few sites where useful information can be found:
  - EMBL
  - Wolfson Center for Applied Biology
  - Equipment suppliers
- This course will mostly deal with industrial aspects of protein purification, with some excursions into other types of (bio)molecules

Learning objectives for the DSP section

- Highlight the relevance of DSP in bioprocess developments
- Get acquainted with the different purification techniques, their corresponding unit operations, their scope and limitations
- Identifiy the similarities and specificities of biotech DSP as compared to chemical process development
- Be able to select a proper sequence of unit operations, leading to a satisfying degree of purification
- Calculate the size of a separation equipment for a given separation and a desired capacity



The Bloom taxonomy Source: University of Arkansas

Which purification techniques do you know?

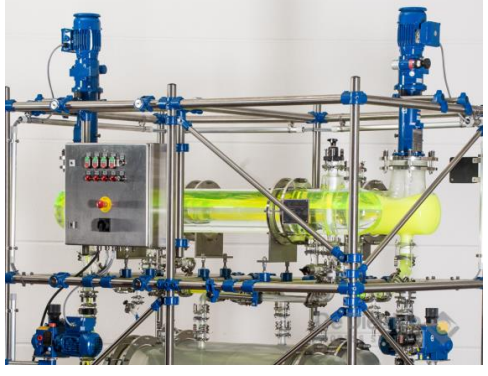
A few (more) keywords

Separation techniques are very similar in chemical- and bioprocesses …

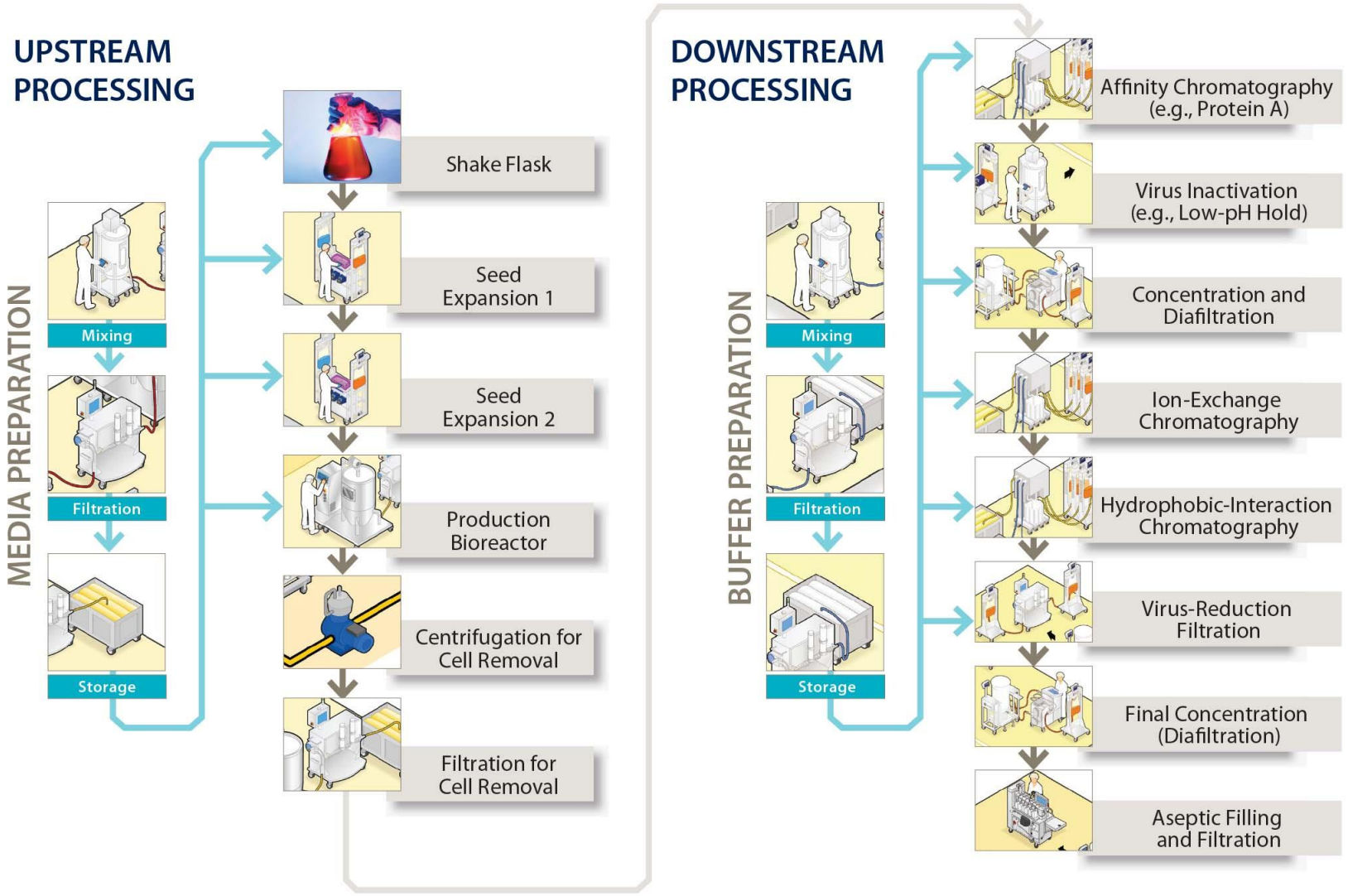
Why then a specific course on bioprocess DSP?

- The mixtures are (very) complex
- Biotechnology DSP deals mostly with aqueous media
- Product titres are often low (but steadily increasing)
- Target molecules are often labile and unstable
- These media are prone to contamination

The separation techniques used in bioprocess DSP are basically identical to the ones that are practiced in chemical, pharma or food engineering. However, they had to be adapted to the specific constraints of each domain.



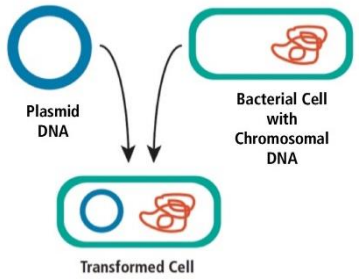
Bioprocesses: looking at the big picture …



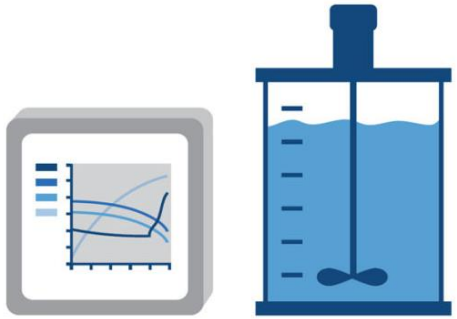
Process diagram for the production and purification of a monoclonal antibody © Bio Process International, 2015

DSP as part of a bioprocess

USP

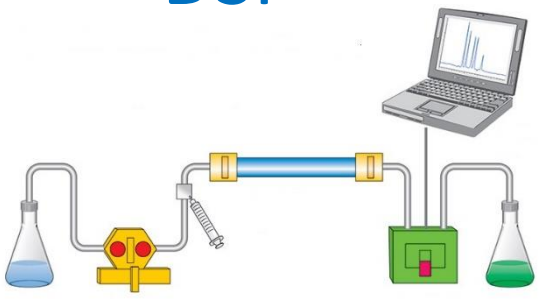


Source: https://blog.edvotek.com/



Source: Hamilton Company

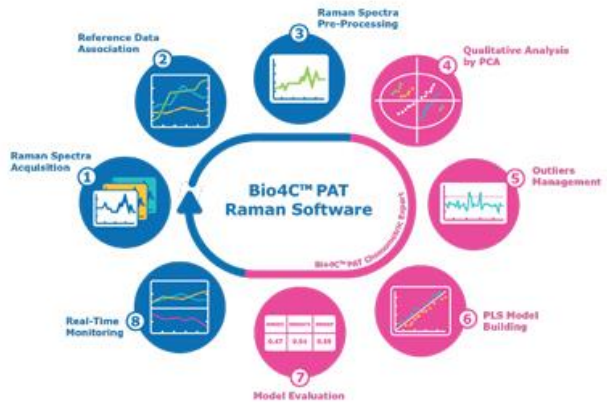
DSP



Source: www.cobaltresearchbv.com

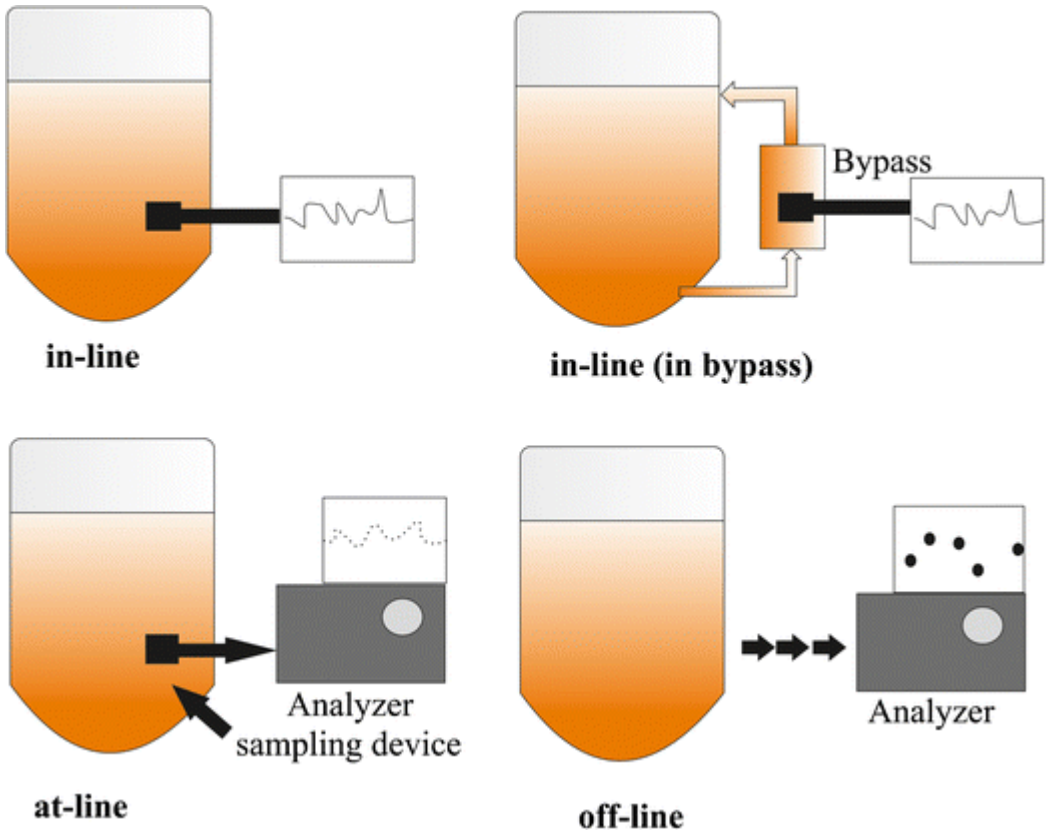
PAT

- Analytical and bioanalytical aspects are important elements in both fermentation and DSP
- They should be accounted for in process development
- PAT is an extension of (bio)analytical techniques that is being actively developed and implemented

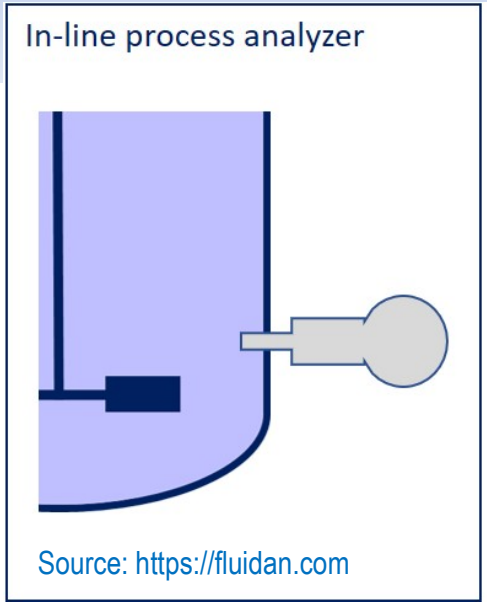


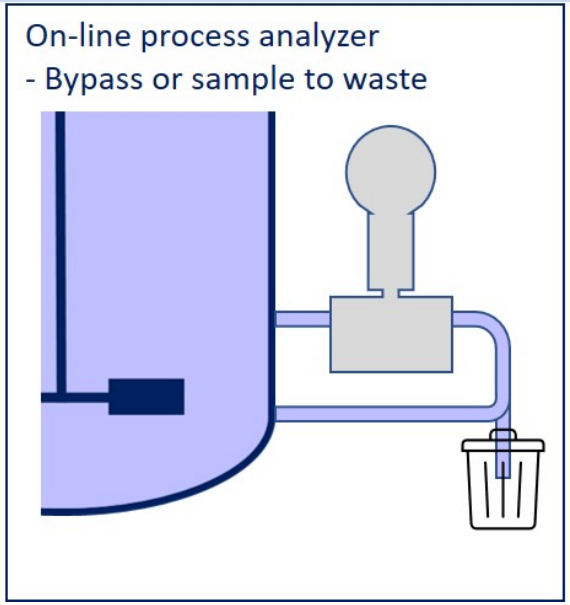
Source: Merck Millipore Sigma

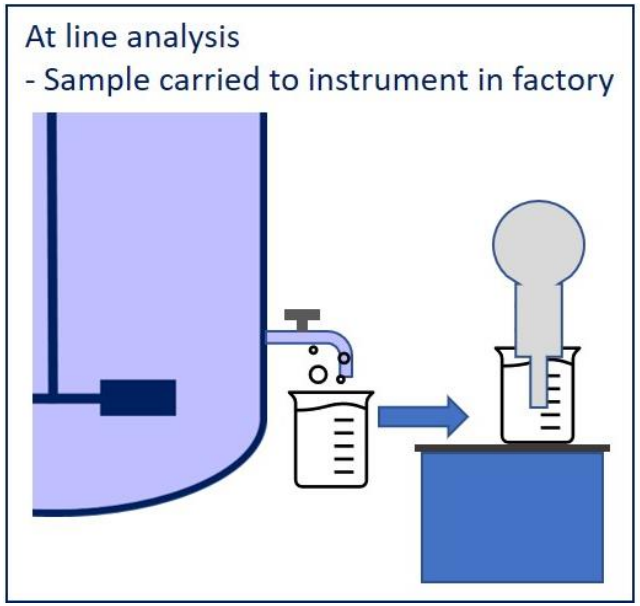
PAT: off-line, at-line, on-line, in-line …

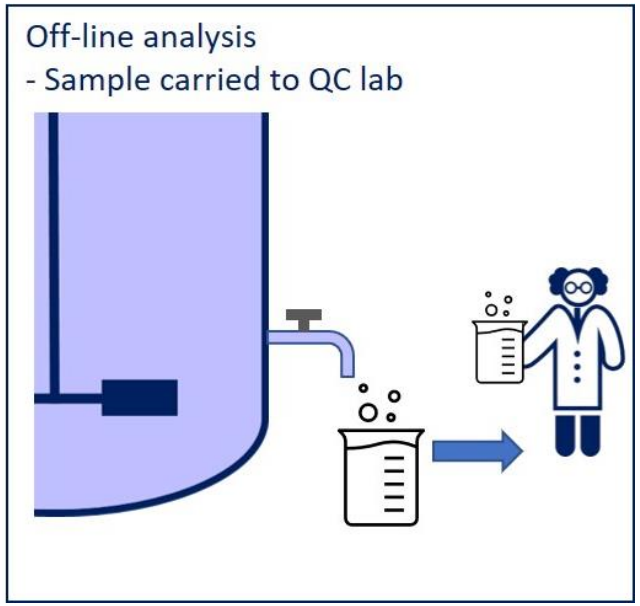


Source: J. Classen et al., Anal Bioanal Chem (2017) 409:651–666. DOI 10.1007/s00216-016-0068-xhttps://blog.edvotek.com/









Downstream processing in short

Source: Hochschule Biberach

DSP

Source: Roche

STARTING MATERIAL

- Complex, heterogenous mixtures
- Mostly aqueous
- Low product concentrations
- Large volumes to be handled
- Sensitive/labile molecules
- High risk of contamination

DSP SHOULD BE:

- Short
- Efficient
- «Gentle»
- Cheap

PRODUCT HAS TO BE:

- Pure
- Active
- Safe
- Well-formulated
- Stable

Altogether, is DSP really that important?

- DSP comes last in the sequence of production steps (if you include formulation)
- It should however not be neglected since it commonly represents 60 to 80% of the production costs
- This holds true at all productions scales, which vary tremendously
- This range may of course vary depending on the initial product titer, desired purity, product stability, mixture complexity, recovery yield etc …
- Hence, a sound selection of the purification strategy (number of steps, size of equipment, choice of technologies) is of great importance

Separation technique: expectations

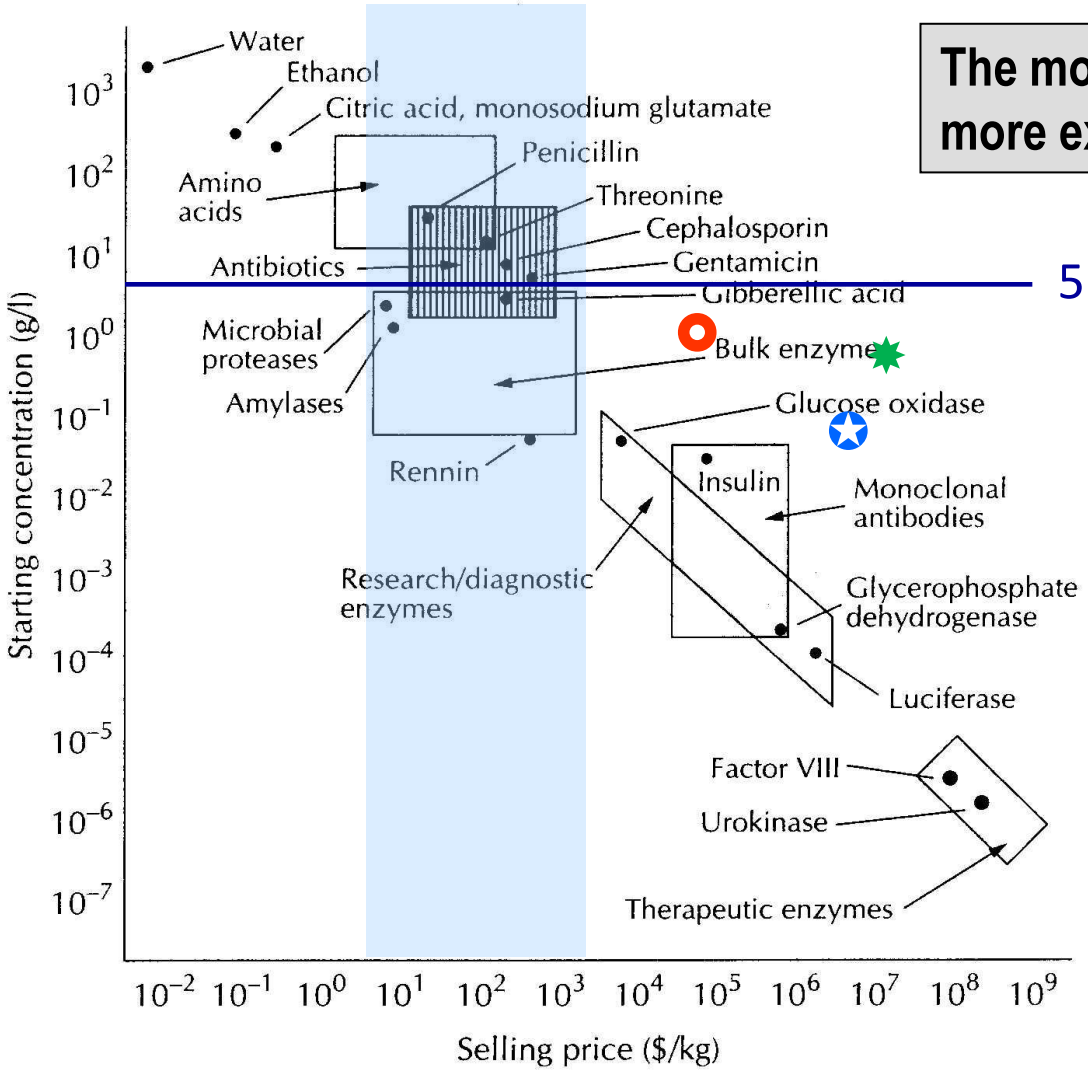
What should a good separation technique/technology be?

How do you assess its performance?

In the purification of monoclonal antibodies you end up using mostly three techniques. Which ones?

Why bother discussing all the rest?

A clear message from obsolete figures …



The more dilute the target molecule, the more expensive the purification process

5 g/l

Human Growth Hormone (1985)

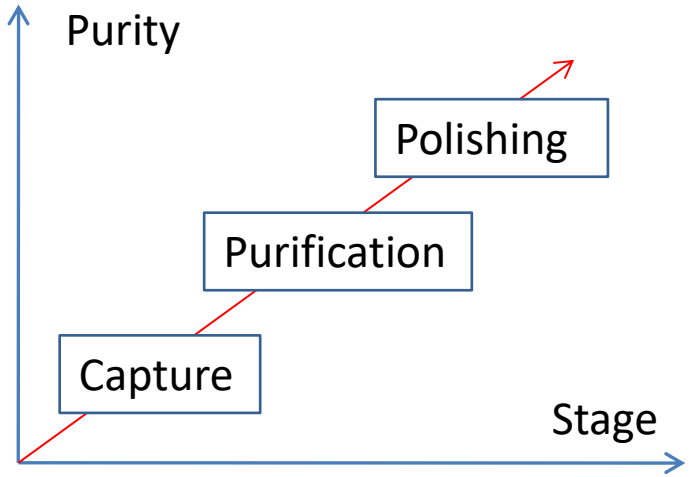
Tissue Plasminogen Activator (1987)

Hepatitis B Vaccine (1986)

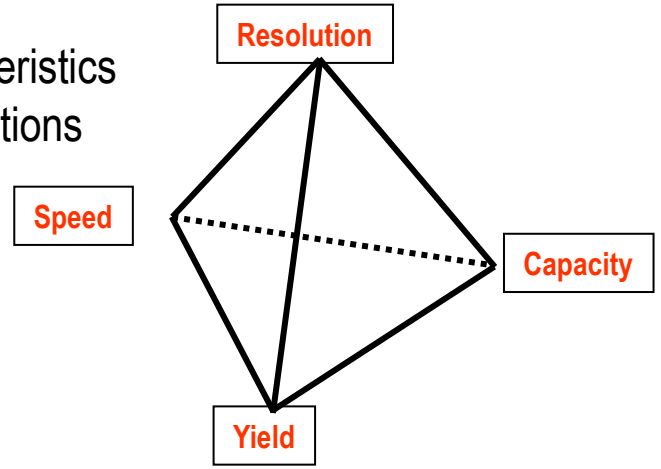
H. W. Blanch, D. S. Clark
Biochemical Engineering, Dekker, 1997

Original data compiled in: J. L. Dwyer, Bio/Technology 2 (11), pp 957-961, [1983]

Key elements in a nutshell



Technical characteristics of DSP unit operations

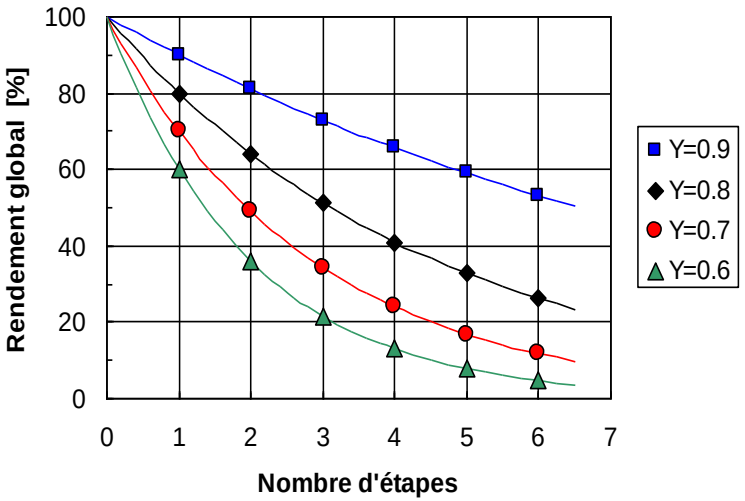


Physical methods

Sedimentation
Centrifugation
Filtration
Microfiltration
Ultrafiltration
Nanofiltration
Reverse osmosis
Electrodialysis
SEC

Thermal methods

L/L Extraction
ATPS
Precipitation
Adsorption
Chromatography
(IEX, AC, HIC)
Crystallization
Drying



Wanted: few steps with high recovery yields

An obsolete view of DSP?

Textbook DSP

- Cell/medium separation
  - Sedimentation
  - Filtration
  - Centrifugation
- Cell lysis
  - Bead mill
  - HP homogenizer
  - Ultrasound
- L/L Extraction, Destillation
- Precipitation
- Adsorption
- Chromatography
- Membrane filtration
- Drying, etc …

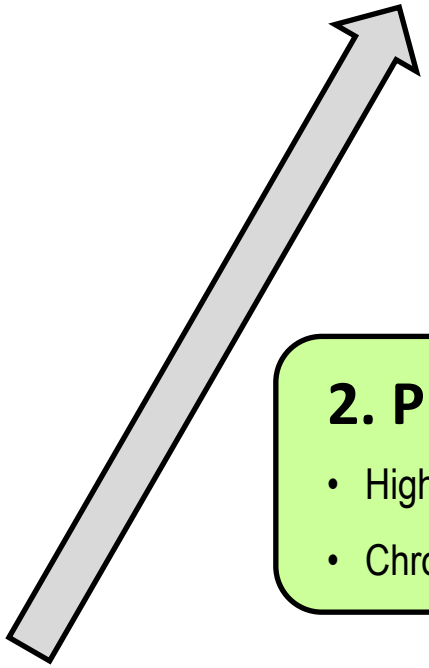
DSP in «modern» biopharma

- Centrifugation
- Micro, Ultra- and Diafiltration
- Chromatography

Are we teaching the right things?

Do we place our focus on the correct topic(s)?

Three steps, from fermentor to product (1/2)



3. POLISHING

- Aims at removing the last impurities (aggregates, fragments)
- Refolding and crystallisation very often used at this stage
- Drying, stabilization and formulation are also important

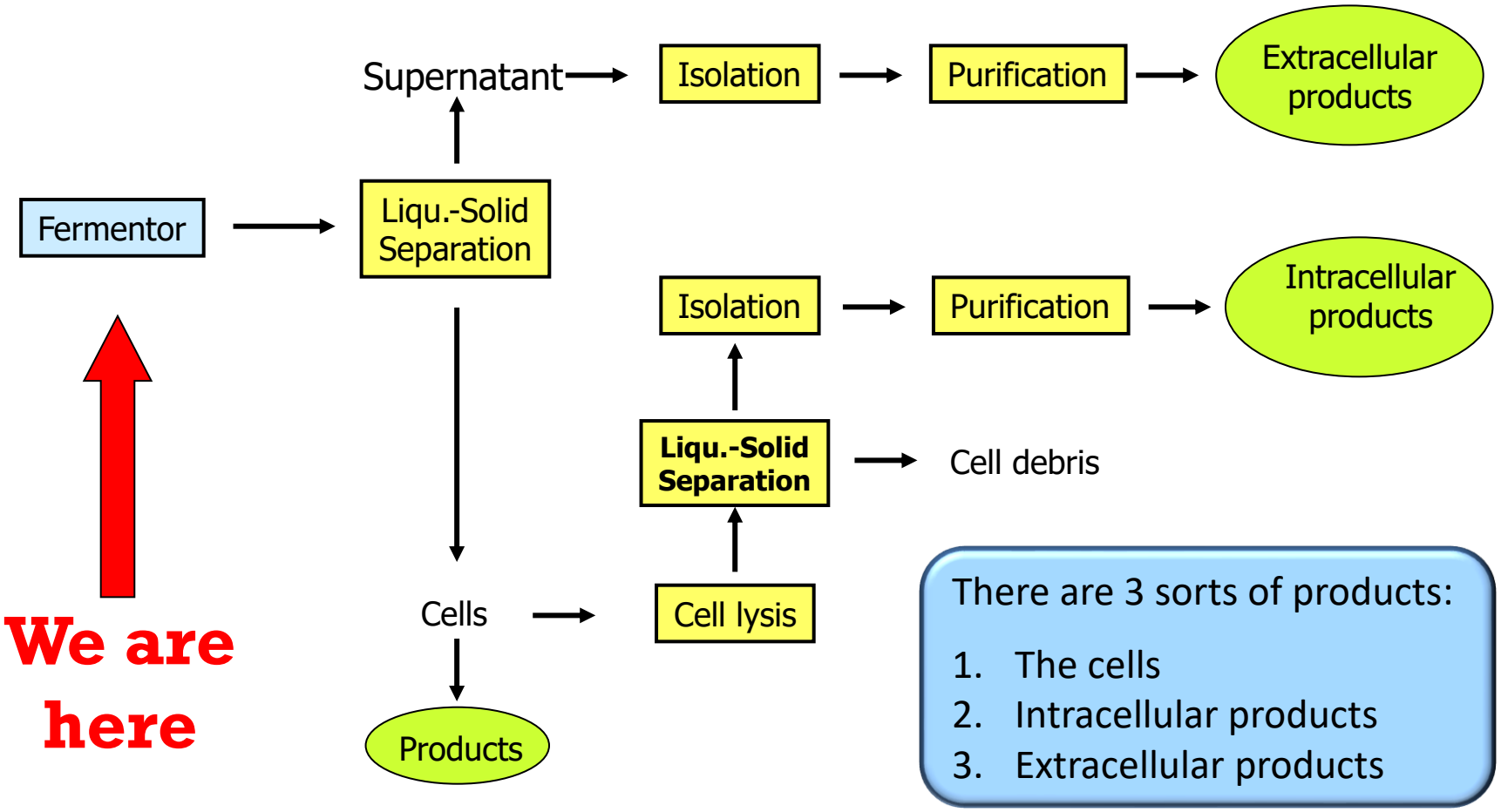
2. PURIFICATION

- High selectivity, removes impurities similar to product
- Chromatography is THE purification technique (but alternatives are being sought)

1. CAPTURE / ISOLATION

- Aims at reducing the volumes/quantities to be handled
- Not very specific, but significant increase in product concentration and purity
- Adsorption, precipitation, and liquid/liquid extraction are most often used

Common pathway for a purification protocol

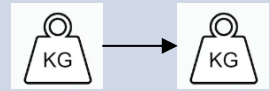


Points that need early addressing

1. Aspect and rheology of fermentation medium
2. Nature & concentration of target molecule
3. Physico-chemical properties of target molecule
4. Stability of target molecule
5. Foreseen application
6. Required purity level
7. Intra- or extracellular compound?
8. Nature and acceptable concentration of contaminants
9. Economic and material constraints

A few definitions (1/2)

The symbols used here are the ones recommended by IUPAC.
They may vary depending on the source, the author, the context …

What?	Symbol	Units	Conserved?
Mass	m	kg	Yes 
Mass concentration	ρ	$\frac{\mathrm{kg}}{\mathrm{m}^3}$	No
catalytic activity – amount of substrate converted per time with given amount of material	z	$\mathrm{kat} = \frac{\mathrm{mol}}{\mathrm{s}} = 60 \cdot 10^6\ \mathrm{U}$	Yes, if no enzyme **inactivation** occurs
Activity concentration – activity per volume of solution	b	$\frac{\mathrm{kat}}{\mathrm{m}^3} = 60 \frac{\mathrm{U}}{\mathrm{mL}}$	No
Specific activity – activity per mass of protein	$\tilde{z} = \mathrm{b}/\rho$	$\frac{\mathrm{kat}}{\mathrm{kg}} = 60 \frac{\mathrm{U}}{\mathrm{mg}}$	No

A few definitions (2/2)

- Purity P of product at stage n

$$P_n = \frac{m_{\mathrm{P},n}}{m_{\mathrm{P},n} + m_{imp,n}} = \frac{\tilde{z}_n}{\tilde{z}_{pure}} \qquad [-]$$

- Enrichment factor E (or purification factor F) from stage i to n

$$E_n = F_n = \frac{P_{\mathrm{P},n}}{P_{\mathrm{P,i}}} \qquad [-]$$

- Specific activity

$$\tilde{z} = \frac{z}{m_P + m_{imp}} \qquad \left[\frac{mol}{s\ kg}\right] = \left[\frac{kat}{kg}\right]$$

- Yield Y and loss L from stage m to stage n (typically only for target product)

$$Y_{m\to n} = 1 - \mathrm{L_{m\to n}} = \frac{m_n}{m_m}\ or\ \frac{z_n}{z_m} \qquad [-]$$

- Overall yield

$$Y_{tot} = \frac{m_{end}}{m_0} = \prod_{i=1}^{end} Y_{i-1\to i} \qquad [-]$$
$$= Y_{0\to 1} \cdot \ldots \cdot Y_{end-1\to end}$$

Required purity varies with application and drug dosage

Dose totale	Pureté	Exemple
Tests *in vitro*	95%	Diagnostic
100 mg	99%	Vaccin
1 g	99.9%	Erythropoïetine
3 g	99.99%	Superoxyde Dismutase
> 10 g	99.999%	Hormone de croissance humaine

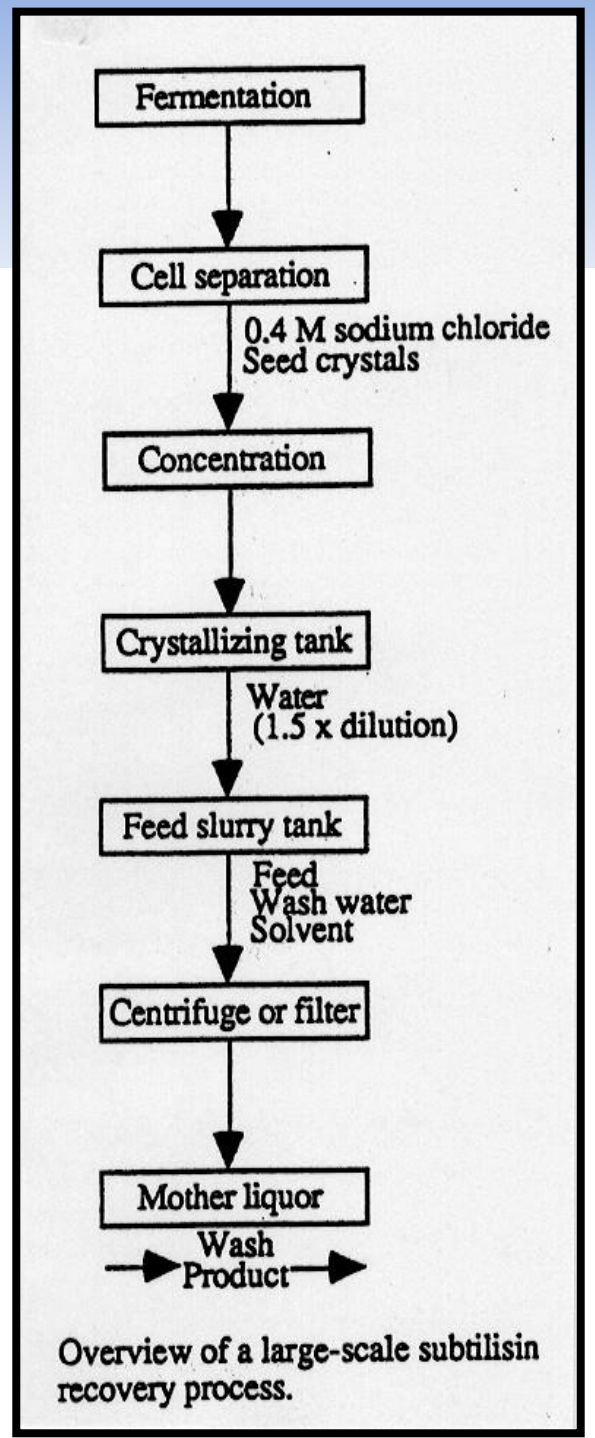
Certain illnesses or conditions require that drugs be taken over a lifetime (e.g. insulin for diabetics, or cyclosporin for transplanted patients). In such situations, tolerance for impurities is logically much lower than for a vaccine.

Superoxyde dismutase (SOD), EC 1.15.1.1: degrades the superoxide anion O_2^- into O_2 and H_2O_2. The enzyme is involved in the degradation of free radicals which are responsible for aging.

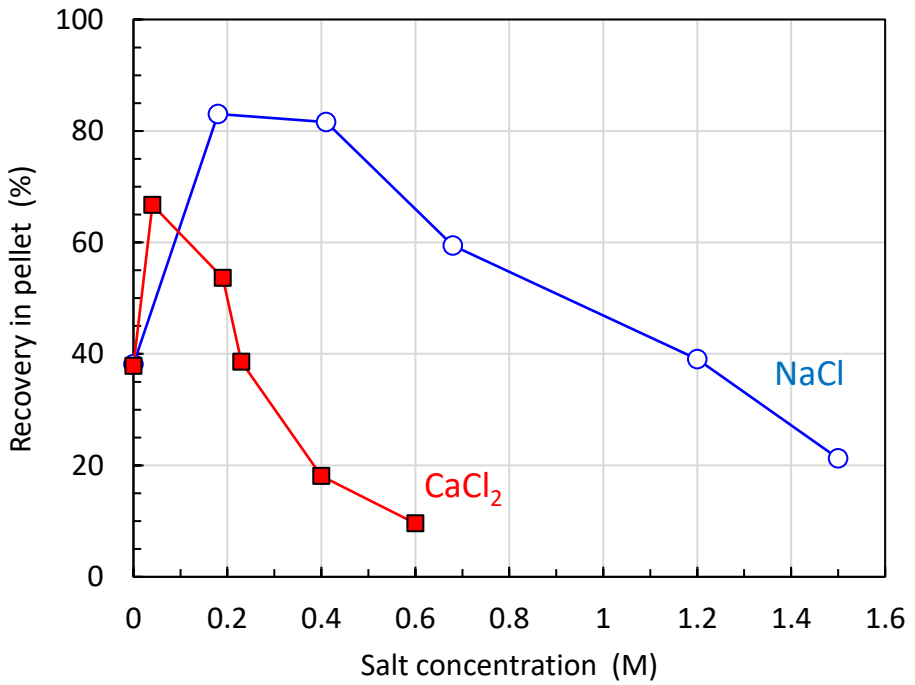
Summary

- The purification of fermentation products belongs to the process and strongly impacts production costs
- The selection of a separation strategy has to be based on both technical and economic criteria
- Approaches featuring a minimal number of steps with high recovery yields should be favoured (have a Plan B ready)
- Maximizing the mass recovery yield is often done at the expense of the activity recovery yield (always watch for the impact of purification techniques on the product)

Example 1: Subtilisin (EC 3.4.21.62)



Overview of a large-scale subtilisin recovery process.



These graphs illustrate two concepts we have been discussing earlier. Which ones?

United States Patent [19]
Becker et al.

[11] **Patent Number: 5,041,377**
[45] **Date of Patent: Aug. 20, 1991**

[54] **SUBTILISIN CRYSTALLIZATION PROCESS**

[75] Inventors: **Todd Becker**, Burlingame; **Virgil B. Lawlis, Jr.**, San Mateo, both of Calif.

[73] Assignee: **Genencor International Inc.**, South San Francisco, Calif.

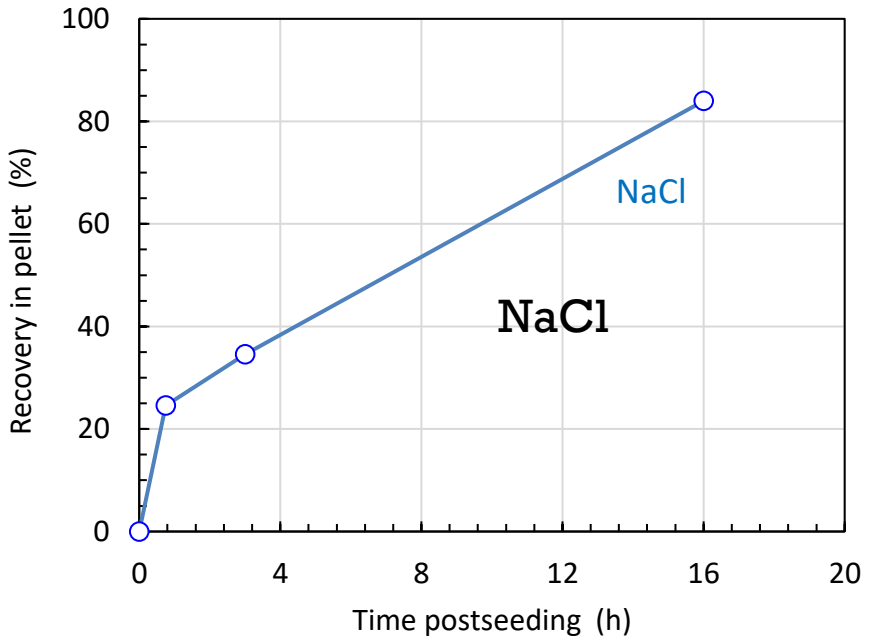
[21] Appl. No.: **611,967**

[22] Filed: **Nov. 13, 1990**

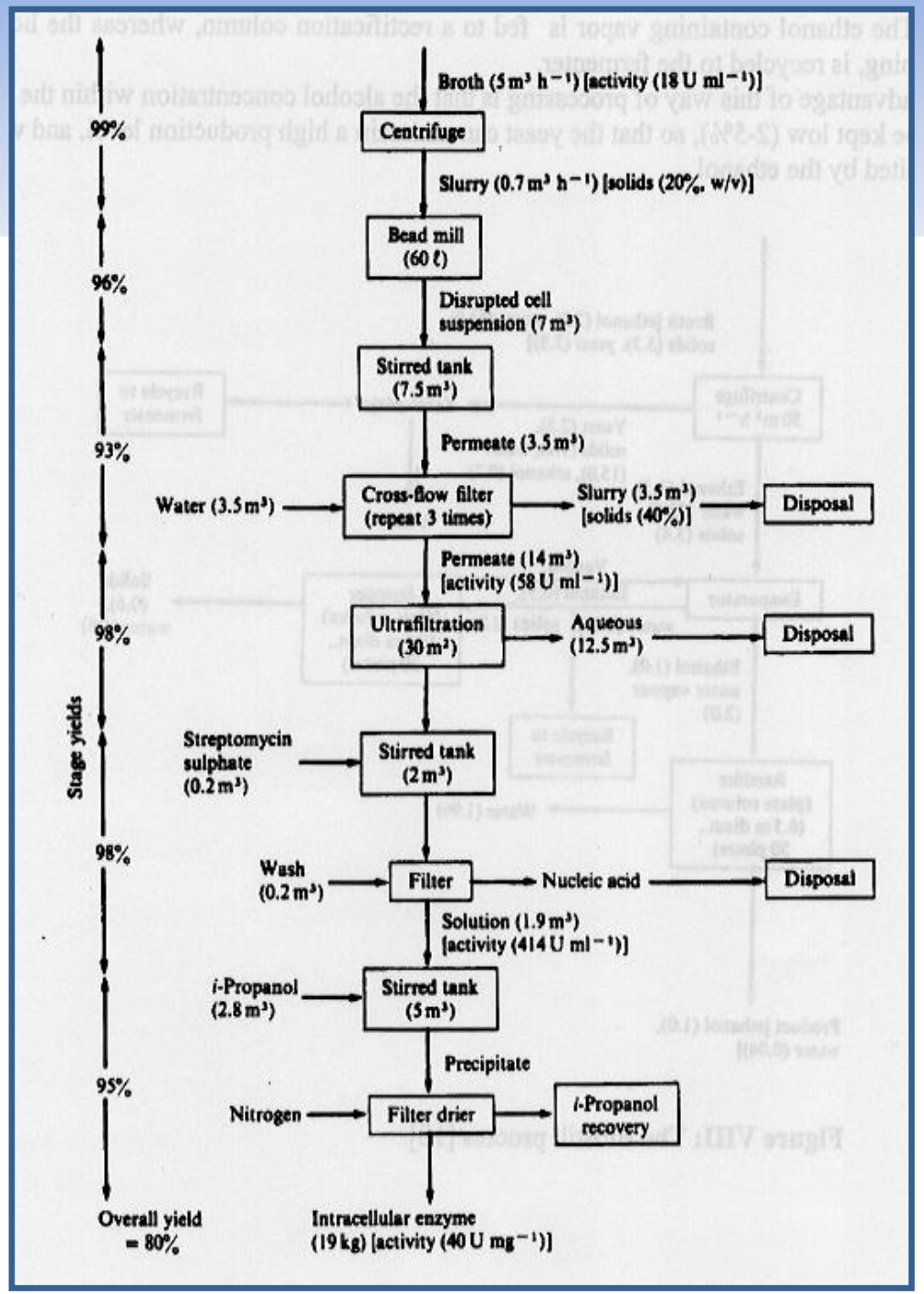
Related U.S. Application Data

[63] Continuation of Ser. No. 538,597, Jun. 13, 1990, which is a continuation of Ser. No. 169,990, Mar. 18, 1988, abandoned.

Abstracts, 97:2861h (1982) "Saturated Enzyme Solutions".
Albert L. Lehninger, *Biochemistry, First Edition, N.Y.: Worth Publishers, Inc., 1970, pp. 133–134.*
Northrup et al., *Crystalline Enzymes*, (1948) Columbia Vine Press, New York, N.Y., pp. 253–254.
Tauber et al., *Chemistry and Technology of Enzymes*, (1949) Wiley and Sons, New York, N.Y., pp. 137–140.
Scopes, Robert K., "Crystallization of Proteins", *Protein Purification: Principles and Practice*, Second Edition, New York, Springer–Verlag, 1987, pp. 296–299, and pp. 256–259.
Lehninger, Albert L., "Separation Procedures Based on Solubility Differences", *Biochemistry*, Second Edition, N.Y., Worth Publishers, Inc., 1975, pp. 160–163.



Example 2: Intracellular enzyme



What is particularly striking in this purification process?

.....

.....

.....

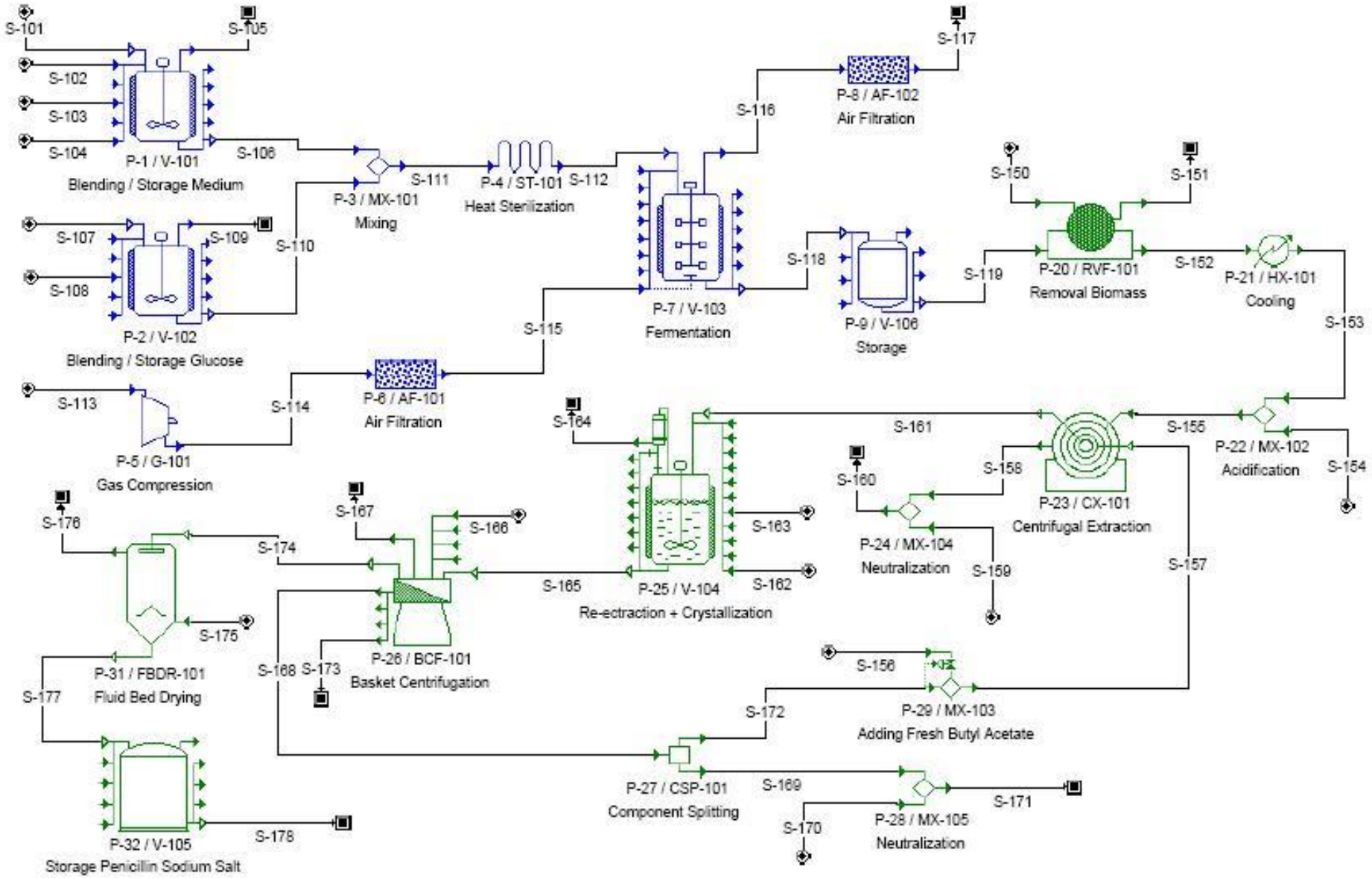
.....

.....

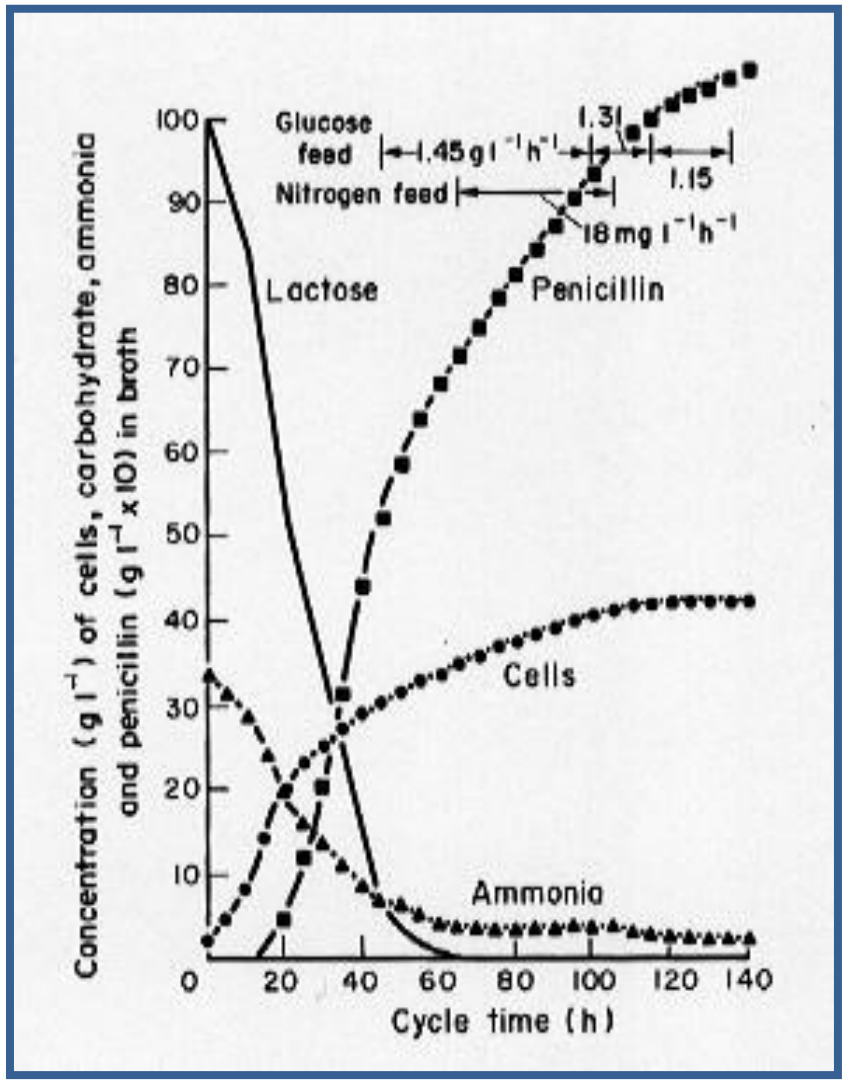
.....

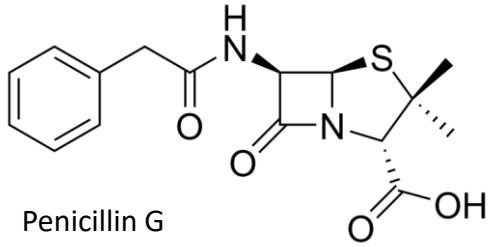
.....

Process Flow Diagram: Penicillin

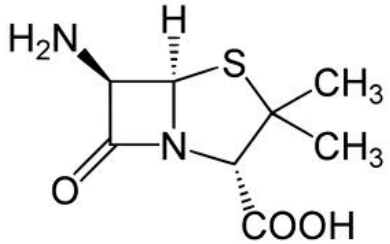


Example 3: Penicillin G and 6-APA





Penicillin G



6-Amino Penicillanic Acid (6-APA)

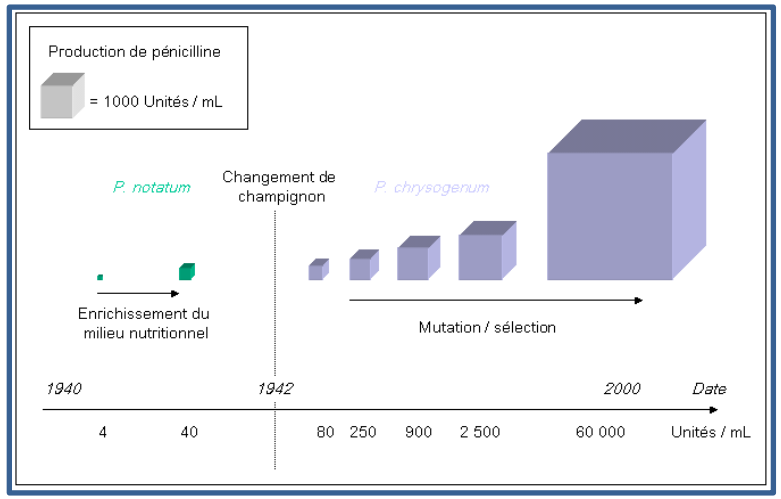
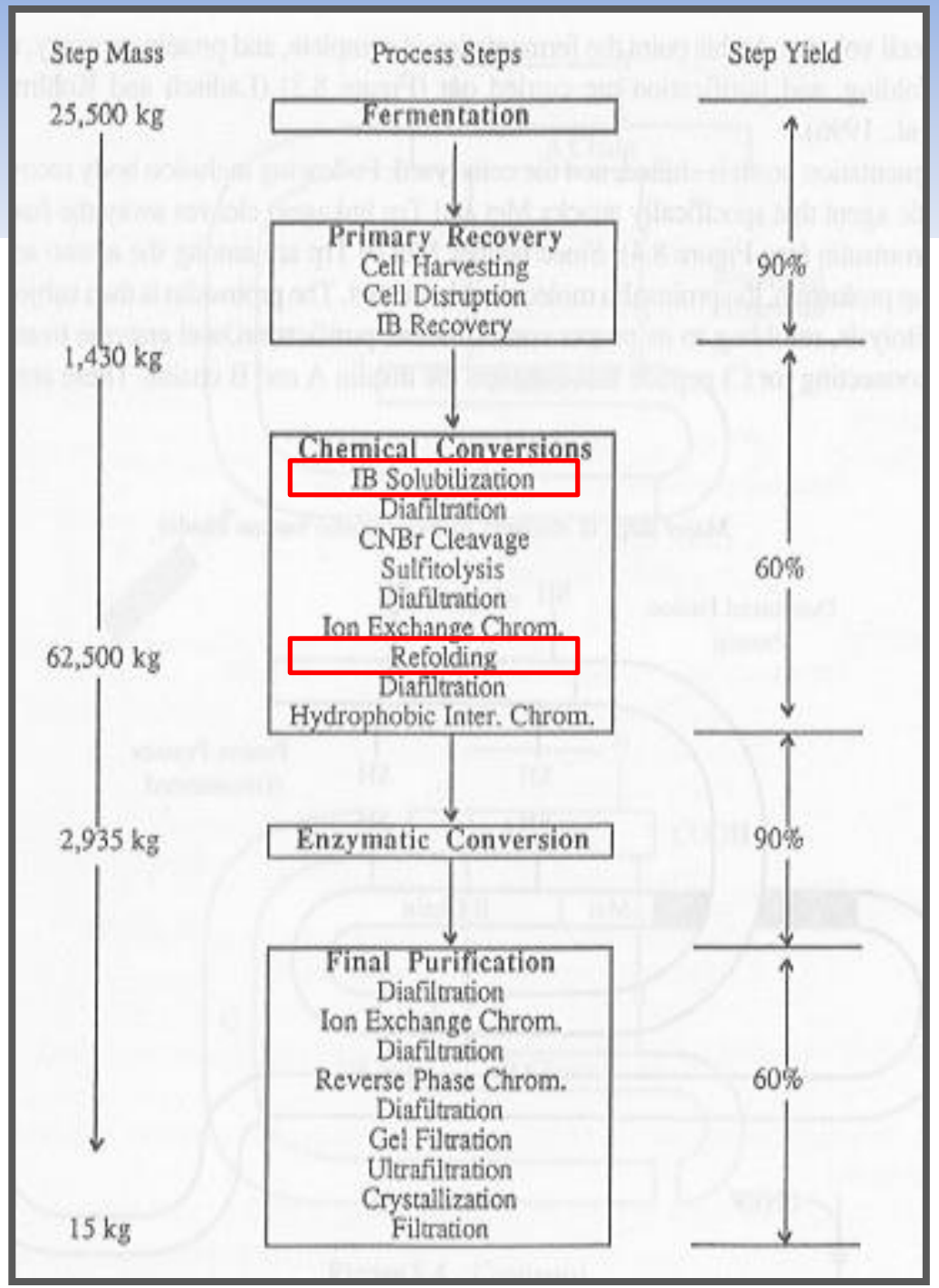
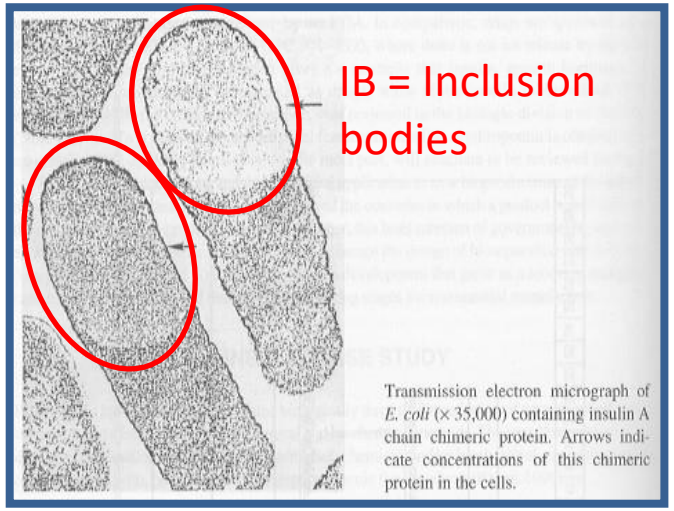


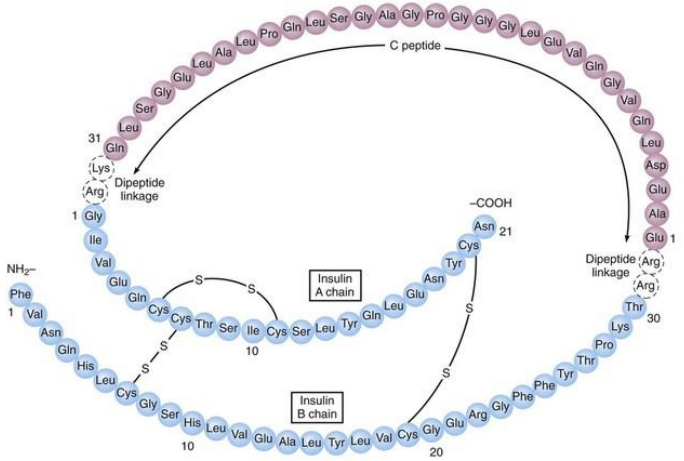
Table 11 Typical Yields in Penicillin Purification

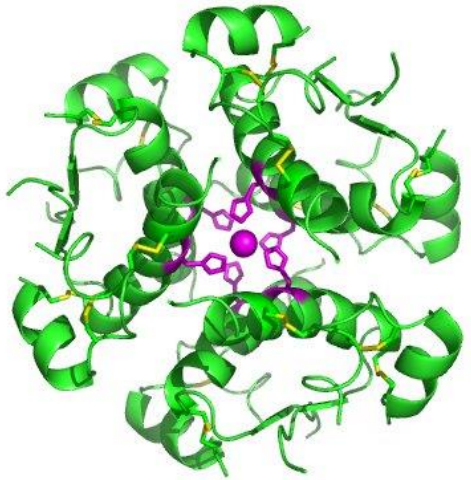
Step	*Yield (approx.)*
Holding	95% but losses can be large if broth is not rapidly chilled or if microbial contamination occurs
Filtration	90–95% based on 'filtrate' assay. 5% of the 'loss' is accounted for by insoluble solids in the broth. Other losses are due to degradation and leakage to drain
Solvent extraction	
single stage	80–90%
lead-trail	92–96%
Aqueous (back) extraction	95–97%
Crystallization	95%
Drying	95%
Overall	~78%

Example 4: Insulin









Last pieces of advice

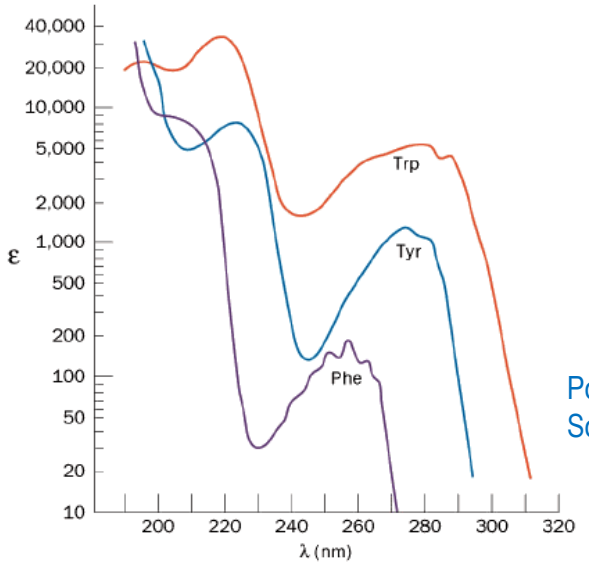
1. Make sure you have a robust analytical support (see dedicated next few slides)
2. Choose orthogonal separation techniques
3. Select methods which make best use of the largest differences between target molecule and impurities
4. Start with the technique that leads to the biggest volume reduction
5. The most expensive technique should be used last
6. What works in the lab does not always work on the line
7. Integrate DSP as early as possible in the development
8. KISS (Keep It Stupid Simple) – if you can

Basic analytics to monitor protein purification processes (1/3)

Total protein concentration

https://bitesizebio.com/23824/top-5-protein-quantification-assays/

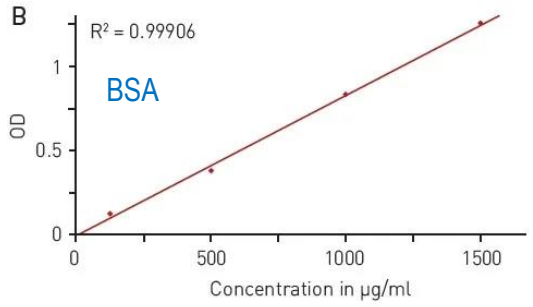
1. Measuring absorbance at 280 nm

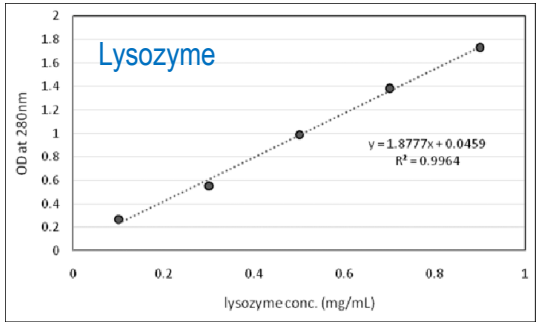


Source: www.chegg.com

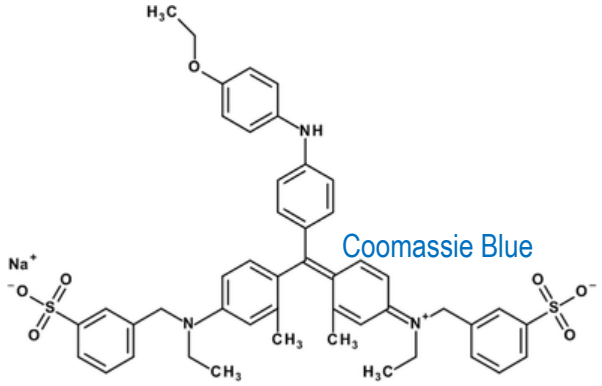
Polystyrene cuvettes
Source: https://www.universalmedicalinc.com/

What could go wrong using these polystyrene cuvettes to measure protein absorbance at 280 nm?



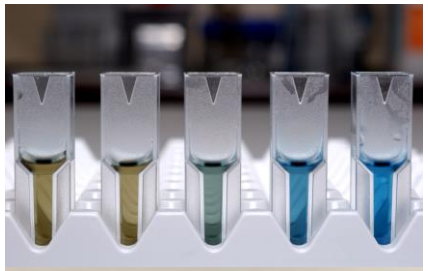


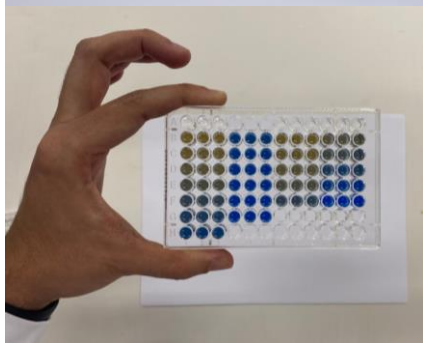
2. Bradford assay

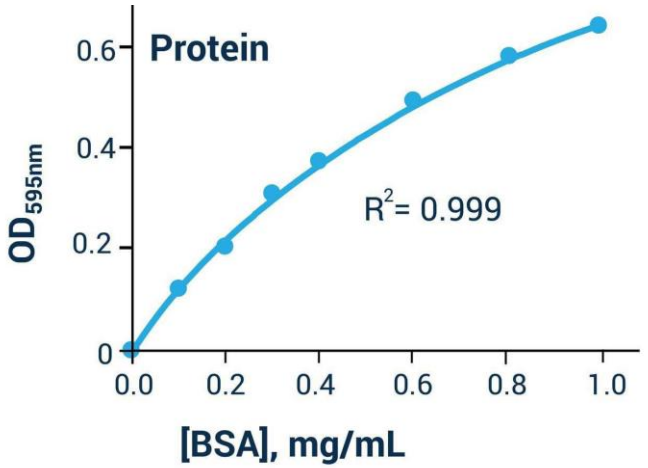


Arginine
Histidine
Lysine
Phenylalanine
Tyrosine
Tryptophan

AA-Dye Complex
λ = 595 nm







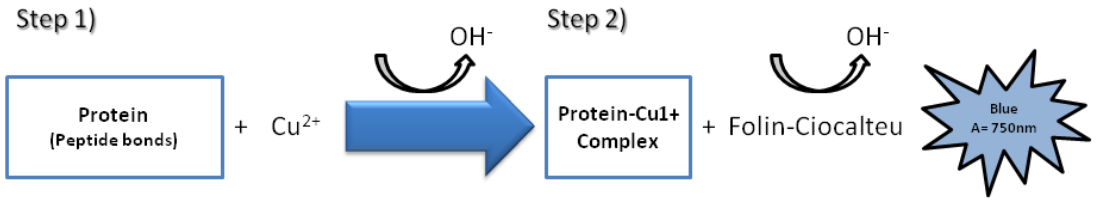
A calibration curve does not need to be a straight line !!!

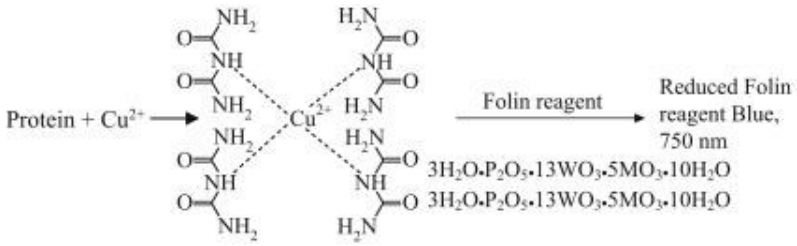
Basic analytics to monitor protein purification processes (2/3)

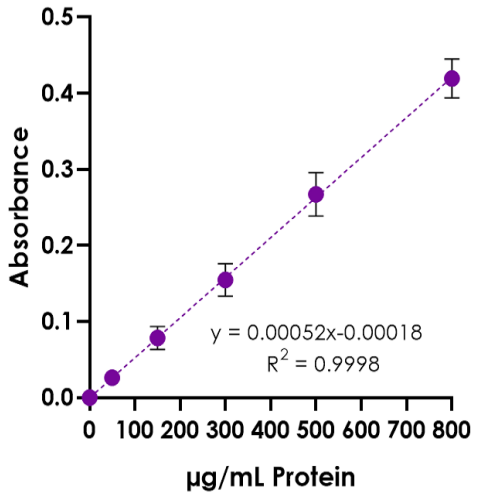
Total protein concentration

https://bitesizebio.com/23824/top-5-protein-quantification-assays/

3. Lowry assay

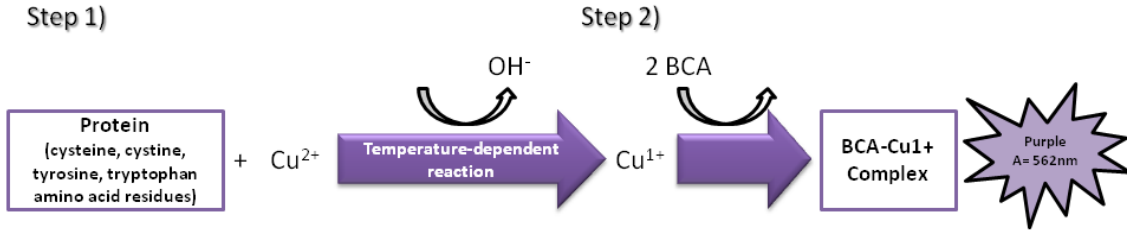




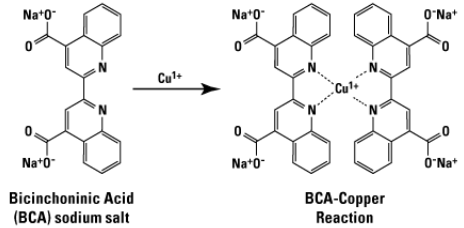


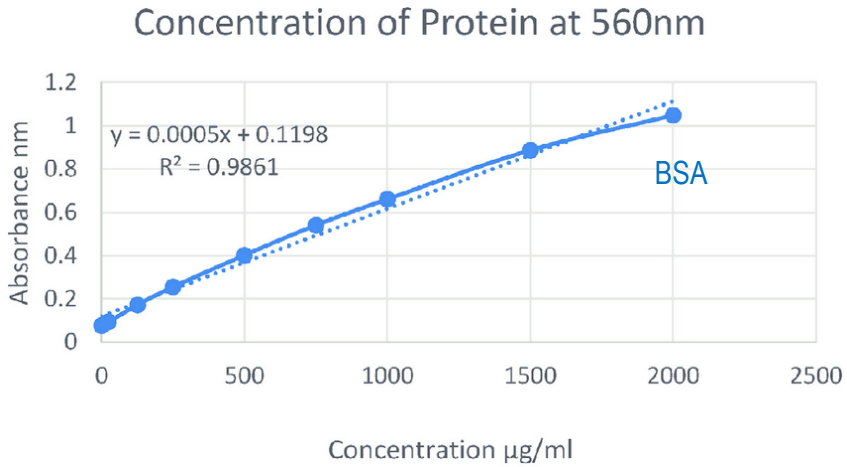
For a detailed presentation of these techniques, check the Thermo Scientific Pierce Protein Assay Handbook poster on the Cyberlearn Platform

4. BCA (bicinchoninic acid) assay



Two … well, even three things are wrong with this calibration curve. Can you tell?





Source: www.thermofisher.com

Basic analytics to monitor protein purification processes (3/3)

Concentration of the target molecule

- Besides total protein concentrations, product titre needs to be determined
- To this effect, a specific type of assay is required
- What can you propose, depending on the nature of the product?

This question is the topic of exercise 1.9, with specific examples of model compounds