Module ChE-311 Biochemical Engineering

Downstream processing
Exercices Lecture 1
Selection of a purification strategy

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Exercise 1.1 Yield and purification factor

After cells were lysed and the lysate clarified, 100 L of a liquid with protein concentration 0.36 mg_{prot}/mL are obtained. The liquid features an enzyme activity of 2.2 U/ml. It is known that the specific activity of the pure target molecule (i.e. the enzyme) amounts to 40.0 U/mg_{prot}.

Purification is then carried out and finaly, 2.0 L of a fraction containing the target protein are obtained. Total protein concentration in this liquid is 1.11 mg_{prot}/mL , and the activity concentration is 43.2 U/mL.

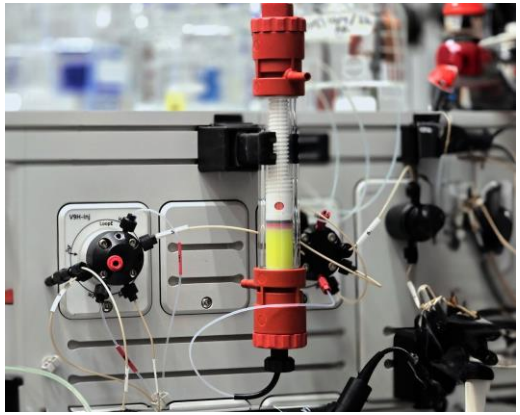
Please calculate:

1. The initial and final purity
2. The initial and final specific activity [U/mg_{prot}]
3. The purification yield
4. The purification factor

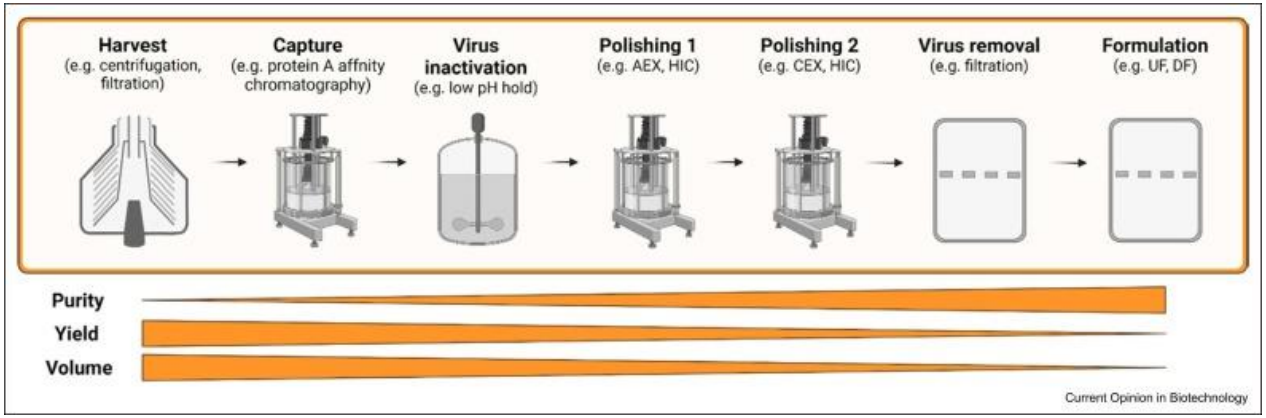
Exercise 1.2 Average yield for a series of steps

Starting from 500 L of clarified broth containing 0.336 U/mL, one obtains after 6 purification steps 2.35 kg dry product containing 92% protein and with a specific activity amounting to 48.9 U/g_{prot}.

1. Calculate the average yield of each purification step



Source: www.ibioic.com



Source: S.P. Schwaminger et al., Current Opinion in Biotechnology 7, 102768 (2022). https://doi.org/10.1016/j.copbio.2022.102768

Exercise 1.3 a

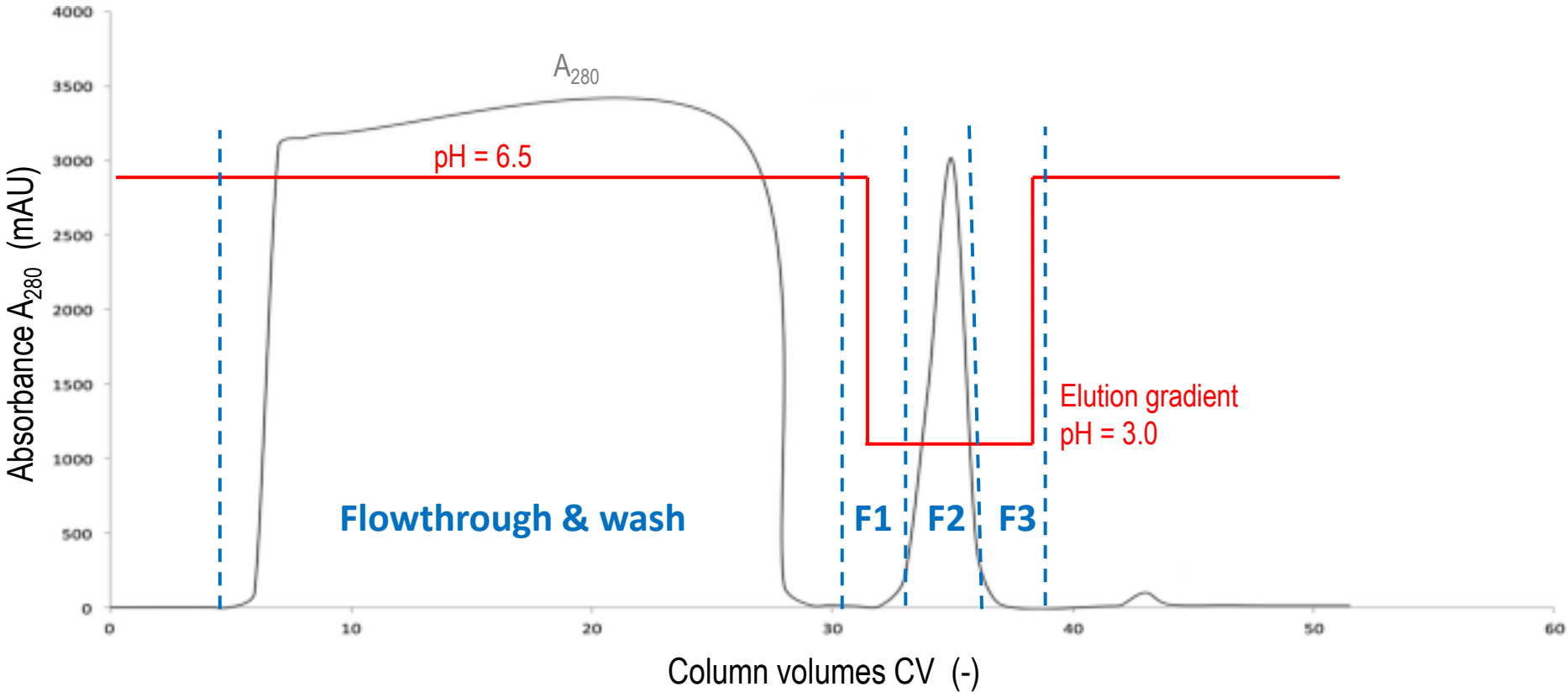
Yield, mass balance and purification factor

You are developing an affinity chromatography (AC) step for the purification of an enzyme (pI 4.6, 465 kDa, tetrameric structure). The process is performed at pH 6.5 in a citrate buffer, using a column with 8 mm int. diameter and 200 mm bed height. In this setup, you are loading a 20 mL sample with a protein concentration of 8.6 g/L and a total activity of 223 U. In the flowthrough (FT) and wash (W), you collect a volume of 27 mL with a protein concentration of 1.7 g/L and no activity. During the elution step you collect three fractions (F1, F2, F3), each with 4 mL volume. Their composition is given on next slide, together with the corresponding chromatogram.

1. Calculate global mass and activity balances for this chromatographic step
2. Calculate the enzyme recovery yield
3. Calculate the purification factor for the enzyme
4. Now consider collecting and keeping fraction F2 only.
    a. Would the enzyme yield drop significantly?
    b. What would be the impact on the purification factor?

Exercise 1.3 b

Yield, mass balance and purification factor



Fraction	Protein conc. (mg/mL)	Activity conc. / (U/L)
F1	7.8	3 450
F2	12.1	50 900
F3	10.5	2 110

Exercise 1.4

What happened?

The specific activity of an enzyme (expressed in U/g_{prot}) has increased steadily over the first three steps of its purification before starting to decrease.

The electrophoretic analyses however show that the amountof contaminants decreases continuously over the whole process. Further, the final fraction is almost completely devoid of contaminants and the protein can be considered pure.

1. What do you think happened?

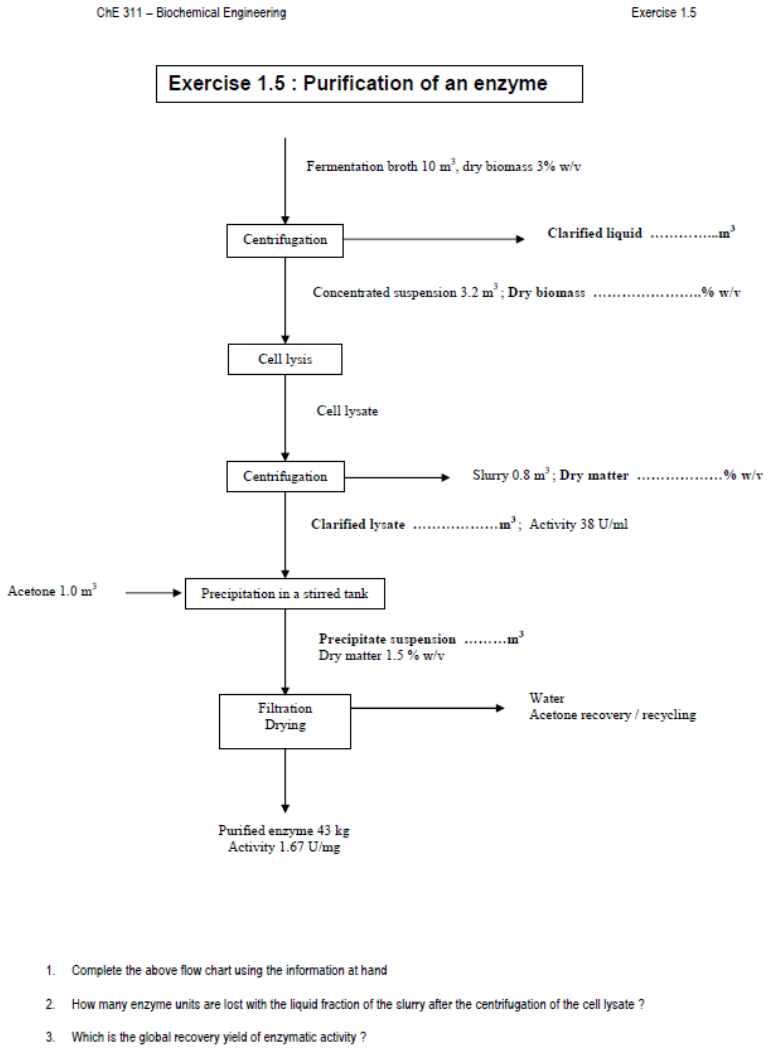
2. What would you propose to reduce the loss of specific activity?

Exercise 1.5 Mass, volume and activity balances

Please complete the flowsheet that describes the purification steps of an industrial enzyme.

Taking into account mass and activity balances, determine the overall purification yield.

PS: The diagram is shown in a separate document to be found in the Moodle (only a miniature version is shown on the right)



ChE 311 – Biochemical Engineering Exercise 1.5

Exercise 1.5 : Purification of an enzyme

Fermentation broth 10 m^3, dry biomass 3% w/v

Centrifugation → **Clarified liquid** ……..m^3

Concentrated suspension 3.2 m^3 ; **Dry biomass** ……% w/v

Cell lysis

Cell lysate

Centrifugation → Slurry 0.8 m^3 ; **Dry matter** ……% w/v

Clarified lysate ……m^3; Activity 38 U/ml

Acetone 1.0 m^3 → Precipitation in a stirred tank

Precipitate suspension ……m^3
Dry matter 1.5 % w/v

Filtration
Drying → Water
Acetone recovery / recycling

Purified enzyme 43 kg
Activity 1.67 U/mg

1. Complete the above flow chart using the information at hand
2. How many enzyme units are lost with the liquid fraction of the slurry after the centrifugation of the cell lysate ?
3. Which is the global recovery yield of enzymatic activity ?

Exercise 1.6 Selection of a proper DSP sequence

As a development engineer you are asked to purify a recombinant enzyme from a culture of *Escherichia coli* at industrial scale.

Choosing from the list of techniques below,please select a logical sequence of 6 purification steps that will take you from the content of the bioreactor to a reasonably pure form of your enzyme (many possible answers). Draw the corresponding flow sheet.

NB: it is worth mentioning that *E. coli* is (usually) not able to excrete the product into the surrounding medium. Also, certain methods can be used repeatedly.

Purification techniques: Ion exchange chromatography – Sedimentation – Liquid-liquid extraction – Precipitation – Ultrafiltration – Bead mill - Refolding - Diafiltration - Crystallization – High pressure homogenization – Gel electrophoresis – Centrifugation - Size exclusion chromatography – Electrodialysis – Destillation - Absorption

Exercise 1.7 Purification of erythromycin A

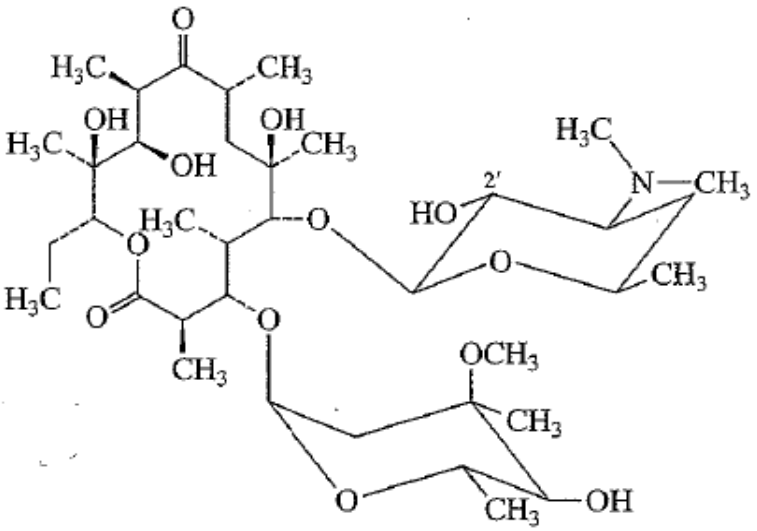
You have been given the assignment to purify the antibiotic erythromycin A from a *S. erythraeus* fermentation broth. You have at your disposal the *Merck Index*, which has the information on erythromycin shown on the next page.

1. On the graph next page, identify the two sugar moieties present in the erythromycin A molecule, i.e. cladinose and desosamine.
2. The total synthesis of erythromycin A has been achieved, e.g. by R.B. Woodward et al., JACS 103, 3215 (1981). Why then decide to produce the antibiotic by fermentation?
3. Starting with the content of your bioreactor at the end of the fermentation, propose two or three first steps (unit operations) you could use for the purification of erythromycin.

Streptomyces erythraeus, a.k.a. *Saccharopolyspora erythraea*
Source: https://bacdive.dsmz.de

Source: www.medicinedirect.co.uk

3720. Erythromycin. Erythromycin A; Abomacetin; Ak-Mycin; Aknin; E-Base; EMU; E-Mycin; Eritrocina; Ery Derm; Erymax; Ery-Tab; Erythromast 36; Erythromid; ERYC; Erycen; Erycin; Erycinum; Ermysin; Ilotycin; Inderm; Retcin; Staticin; Stiemycin; Torlamicina. $C_{37}H_{67}NO_{13}$; mol wt 733.94. C 60.55%, H 9.20%, N 1.91%, O 28.34%. Antibiotic substance produced by a strain of *Streptomyces erythreus* (Waksman) Waksman & Henrici, found in a soil sample from the Philippine Archipelago. Isoln: McGuire *et al., Antibiot. & Chemother.* **2,** 281 (1952); Bunch, McGuire, U.S. pat. **2,653,899** (1953 to Lilly); Clark, Jr., U.S. pat. **2,823,203** (1958 to Abbott). Properties: Flynn *et al., J. Am. Chem. Soc.* **76,** 3121 (1954). Solubility data: Weiss *et al., Antibiot. & Chemother.* **7,** 374 (1957). Structure: Wiley *et al., J. Am. Chem. Soc.* **79,** 6062 (1957). Configuration: Hofheinz, Grisebach, *Ber.* **96,** 2867 (1963); Harris *et al., Tetrahedron Letters* **1965,** 679. There are three erythromycins produced during fermentation, designated A, B, and C; A is the major and most important component. Erythromycins A and B contain the same sugar moieties, desosamine, *q.v.,* and cladinose (3-*O*-methylmycarose). They differ in position 12 of the aglycone, erythronolide, A having an hydroxyl substituent. Component C contains desosamine and the same aglycone present in A but differs by the presence of mycarose, *q.v.,* instead of cladinose. Structure of B: P. F. Wiley *et al., J. Am. Chem. Soc.* **79,** 6070 (1957); of C: *eidem, ibid.* 6074. Synthesis of the aglycone, erythronolide B: E. J. Corey *et al., ibid.* **100,** 4618, 4620 (1978); of erythronolide A: *eidem, ibid.* **101,** 7131 (1979). Asymmetric total synthesis of erythromycin A: R. B. Woodward *et al., ibid.* **103,** 3215 (1981). NMR spectrum of A: D. J. Ager, C. K. Sood, *Magn. Reson. Chem.* **25,** 948 (1987). Biosynthesis: Martin, Goldstein, *Progr. Antimicrob. Anticancer Chemother., Proc. 6th Int. Congr. Chemother.* **II,** 1112 (1970); Martin *et al., Tetrahedron,* **31,** 1985 (1975). Cloning and expression of clustered biosynthetic genes: R. Stanzak *et al., Biotechnology* **4,** 229 (1986). *Reviews:* T. J. Perun in *Drug Action and Drug Resistance in Bacteria* **1,** S. Mitsuhashi, Ed. (University Park Press, Baltimore, 1977) pp 123-152; Oleinick in *Antibiotics,* vol. **3,** J. W. Corcoran, F. E. Hahn, Eds. (Springer-Verlag, New York, 1975) pp 396-419; *Infection* **10,** Suppl. 2, S61-S118 (1982). Comprehensive description: W. L. Koch, *Anal. Profiles Drug Subs.* **8,** 159-177 (1979).



Erythromycin A

Hydrated crystals from water, mp 135-140°, resolidifies with second mp 190–193°. Melting point taken after drying at 56° and 8 mm. $[\alpha]_D^{25}$ − 78° (c = 1.99 in ethanol). uv max (pH 6.3): 280 nm (ϵ 50). pKa_1 8.8. Basic reaction. Readily forms salts with acids. Soly in water: ~2 mg/ml. Freely sol in alcohols, acetone, chloroform, acetonitrile, ethyl acetate. Moderately sol in ether, ethylene dichloride, amyl acetate.

Ethylsuccinate, $C_{43}H_{75}O_{16}$, *Anamycin, Arpimycin, Durapaediat, E.E.S., E-Mycin E, Eryliquid, Eryped, Erythro ES, Erythro-Holz, Erythroped, Esinol, Monomycin, Paediathrocin, Pediamycin, Refkas, Sigapedil, Wyamycin E.* Prepn: Brit. pat. **830,846;** R. K. Clark, U.S. pat. **2,967,129** (1960, 1961 both to Abbott). Hydrated crystals from acetone + water, mp 109–110°. $[\alpha]_D$ − 42.5°.

THERAP CAT: Antibacterial.

THERAP CAT (VET): Antibacterial.

Exercise 1.8 Insulin production and producers

The role of insulin and its therapeutic potential were identified around 1920 by Frederick Banting and his student Charles Best, two Canadian researchers. They were also the first to successfully isolate the molecule.

In 2023, the world insulin market amounted to ca. USD 28 billion, and it is expected to grow up to USD 45 billion by 2035. Make a brief documentary search and answer the following questions:

1. How was insulin produced in the early days?
2. Which companies are the largerst producers of insulin today ?
3. What are the main steps in the production process?
4. Do they differ significantly from one producer to the other?
5. How pure does insulin have to be?
6. How is insulin typically administered to the patients?
7. Why isn't there any oral formulation?
8. What are the alternatives?

Exercise 1.9 Specific analytics

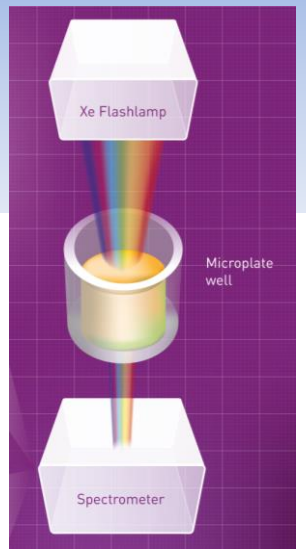
In order to properly monitor the purification of biomolecules you need a minimum of two analytical techniques: a generic method for the determination of global protein concentration (Bradford or Lowry, for example), and a specific assay that enables the quantitative determination of your target molecule.

Propose simple analytical methods that would enable you to specifically quantify the target molecules listed below:

1. GFP
2. Cytochrome C
3. Ethanol
4. IgG
5. ß-galactosidase
6. Penicillin

Exercise 1.10

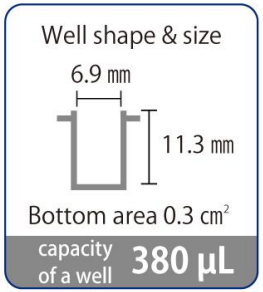
96 well microplates



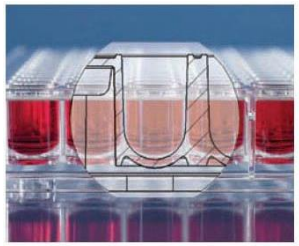
The picture shows a microplate well with the incident light beam coming from above, travelling through the liquid layer of depth h and the bottom of the plate before being measured

96 well microplates have become a universally acknowledged, versatile tool for the fast handling of numerous numbers (high throughput screening). They enable a quick and easy acquisition of spectroscopic measurements (OD_{600}, UV-VIS, fluorescence).
According to the Beer-Lambert law, the interpretation of the measurements requires values for the path length h (cm) the incident beam has to go through.
The internal diameter of each well is d_w = 0.69 cm. The bottom of the well can be flat or hemispheric, for a maximum capacity of ca. 380 µL.

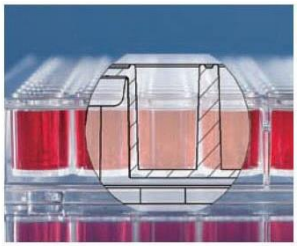
1. Calculate the depth of liquid (path length) h (cm) as a function of the deposited volume V (µL) of liquid for both types of bottom geometries.
2. Give your results some thought … how could you check the accuracy of your assumptions and calculations?



Source: www.shutterstock.com



Round bottom (U)



Flat bottom (F)