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 finally, 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

   the specific activity of the pure target molecule = 40.0 U/mg $_{prot}$

   Initial specific activity: 2.2 / 0.36 U/mg $_{prot}$. $purity = \frac{\frac{2.2}{0.36}}{40} * 100\% = 15.28\%$

   Final specific activity: 43.2 / 1.11 U/mg $_{prot}$. $purity = \frac{\frac{43.2}{1.11}}{40} * 100\% = 97.30\%$

2. The initial and final specific activity [U/mg prot]

   Initial specific activity: 2.2 / 0.36 = 6.11 U/mg $_{prot}$

   Final specific activity: 43.2 / 1.11 = 38.92 U/mg $_{prot}$

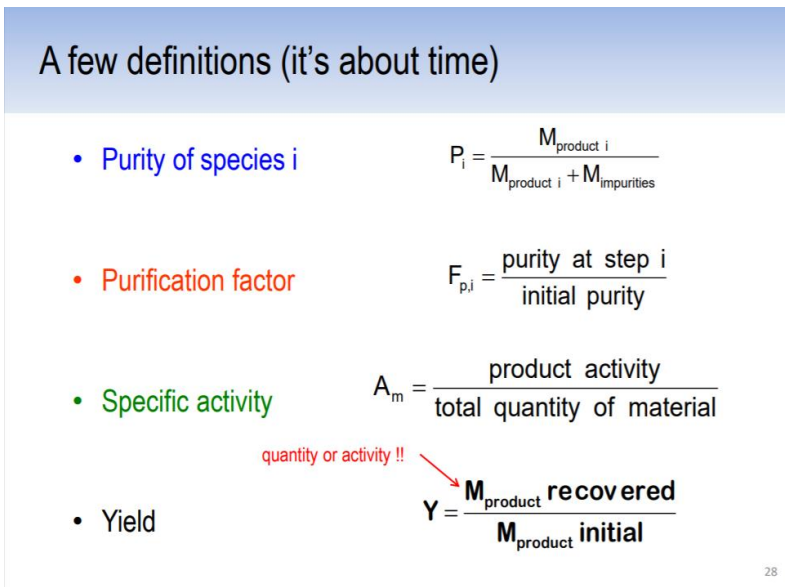
3. The purification yield

   $$purification\ \ yield = \frac{2.0 * 10^3 * 43.2}{100 * 10^3 * 2.2} = 39.3\%$$

4. The purification factor

   $$purification\ \ factor = \frac{97.30\%}{15.28\%} = 6.37$$

formula could be found on the slide below:



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 $_{prot}$.
Calculate the average yield of each purification step

$$purification\ \ yield = \frac{2.35 * 92\% * 10^3 * 48.9}{500 * 10^3 * 0.336} = 62.93\%$$

$$average\ \ yield = \sqrt[6]{62.93\%} = 92.6\%$$

Exercise 1.3: Isolation of an enzyme

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 amount of 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?
   The most likely explanation is that the enzyme, although purified, was denatured by the processing conditions in one or the other step. It therefore lost its biological activity while the mass yield remained unchanged.
2. What would you propose to reduce the loss of specific activity?
   Identify the steps and conditions that are responsible for the greatest losses and look for alternative conditions / methods that better preserve the target molecule.

Exercise 1.4: Selection of a proper DSP sequence

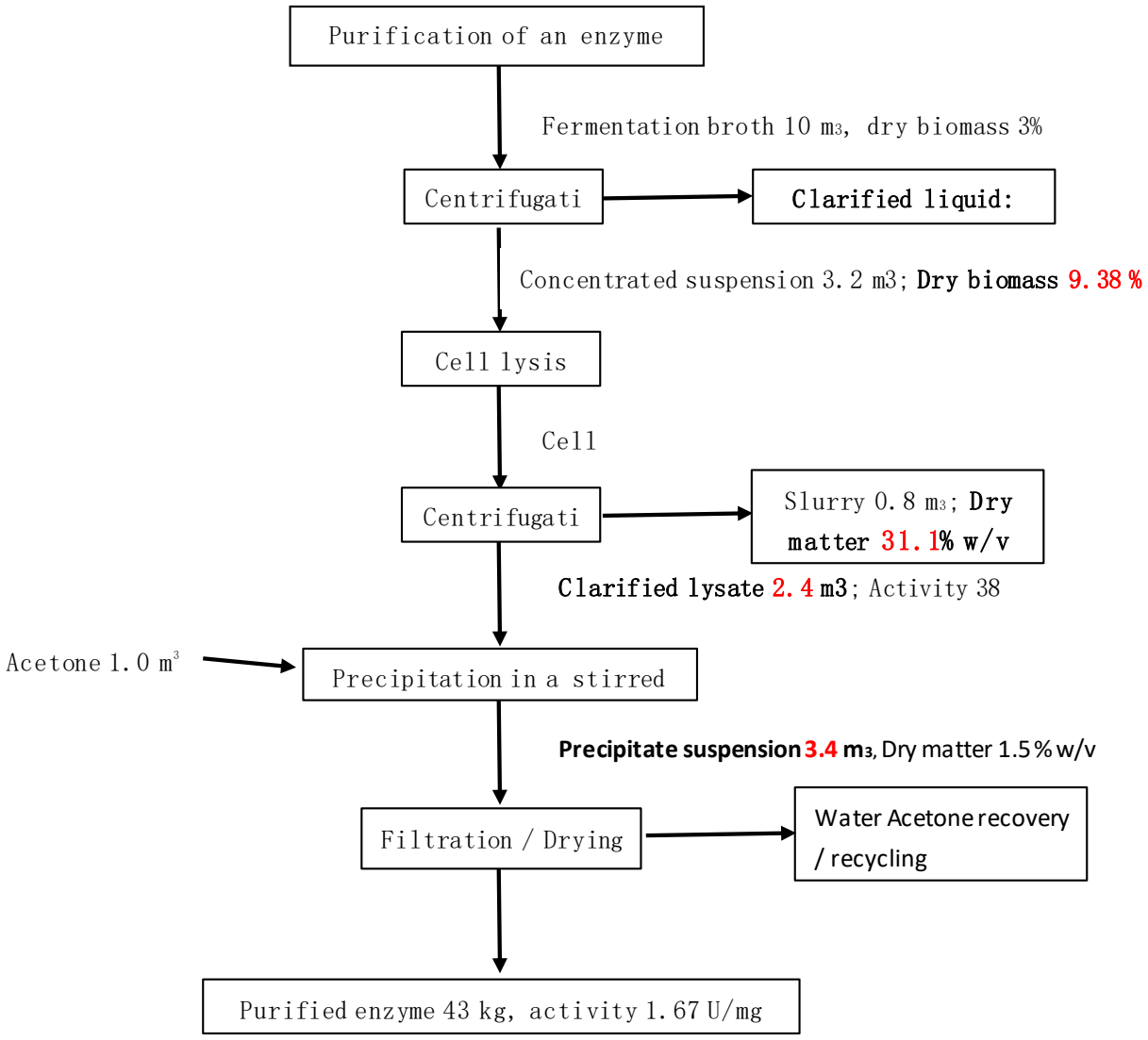
As a development engineer you are asked to purify a recombinant enzyme from a culture of Escherichia coli at industrial scale.
Choosing from the list of techniques below, please select a logical sequence of 6 purification steps that will take you from the content of the bioreactor to a reasonably pure form of your enzyme (many possible answers). Draw the corresponding flow sheet.
NB: it may be 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.

There are a multitude of valid answers to this question, however the steps must respect a logic

associated with their characteristics of resolution, capacity, efficiency and speed. In addition, since E. coli does not secrete the product it is therefore intracellular and an additional cell lysis step and a liquid / solid separation step should be included in the process.
A possible answer could be:
1. Liquid / solid separation (centrifugation) and recovery of the solid fraction
2. Cell lysis (high pressure homogenizer)
3. Solid liquid separation (centrifugation) and recovery of the liquid phase
4. Concentration and fractionation by ultrafiltration
5. Ion exchange chromatography
6. Crystallization

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.



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?

*$0.8 \cdot 10^6$ ml * 38 U /ml = 30.4 units*

3. Which is the global recovery yield of enzymatic activity?

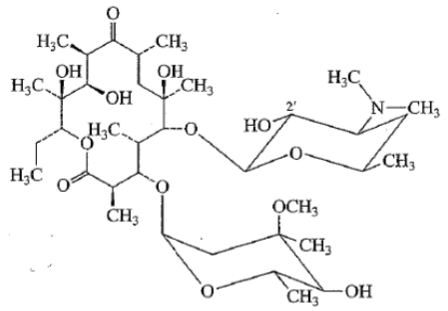
*The ratio of the total enzyme activity recovered in the dried solid and the total enzyme activity in the clarified lysate gives ($43 \cdot 10^6$ mg * 1.67 U /mg) / ($2.4 \cdot 10^6$ ml * 38 U /ml) = 0.79 = 79 %.*

Exercise 1.6: Purification of erythromycin

You have been given the assignment to purify the antibiotic erythromycin. You have at your disposal the Merck Index, which has the information on erythromycin shown on the next page.

What do you think are the most likely unit operations that should could be used for the isolation and purification of this antibiotic?

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.

There is quite a lot of information on the page of the Merck Index dedicated to erythromycin. However, the most relevant appears near the end of the section: it is said there that erythromycin has a rather low water solubility of 2 g/L, whereas it is readily soluble in organic solvents such as ethanol, acetone or acetonitrile. However, these solvents are water-miscible in about all proportions and would not be much help in terms of purification.

In this respect, chloroform or – even better since it is less toxic - ethyl acetate appear to be very

interesting candidates for a liquid-liquid extraction step performed directly from the fermentation broth. Depending on the partition coefficient of the antibiotic in the organic phase, this could lead to a significant concentration of the product, together with the removal of many contaminants that would remain in the aqueous phase.

Exercise 1.7: Production of lactic acid

In 2017, around 370'000 metric tons of lactic acid were produced worldwide. Make a brief documentary search and answer the following questions:

1. Which companies are the largest producers of lactic acid

   It is difficult to find recent, accurate figures, but the largest companies most often cited include Archer Daniels Midland Company (USA), NatureWorks LLC (USA), Purac (The Netherlands), Galactic S.A. (Belgium) and several Chinese companies, among them are the CCA (Changzhou) Biochemical Co. Ltd., Henan Jindan Lactic Acid Co. Ltd., and Musashino Chemical Co. Ltd.

2. What are the principal uses and applications of lactic acid?

   In the cosmetic industry, lactic acid is used in the manufacture of hygiene and esthetic products, owing to its moisturizing, antimicrobial and rejuvenating effects on the skin, as well as of oral hygiene products. Lactic acid derivatives such as lactate esters are widely used because of their hygroscopic and emulsifying properties (Gao, Ma, & Xu, 2011). In the pharmaceutical industry it is used as a supplement in the synthesis of dermatologic drugs and against osteoporosis

   As a food additive it is approved for use in the EU, USA and Australia and New Zealand. It is listed by its INS number 270 or as E number E270. Lactic acid is used as a food preservative, curing agent, and flavoring agent. It is an ingredient in processed foods and is used as a decontaminant during meat processing.

   Lactic acid is also employed in pharmaceutical technology to produce water-soluble lactates from otherwise-insoluble active ingredients. It finds further use in topical preparations and cosmetics to adjust acidity and for its disinfectant and keratolytic properties.

   Lactic acid is also the monomer for the production of biodegradable polymers (PLA and PLGA).

   For the anecdote, lactic acid has historically been used to assist with the erasure of inks from official papers to be modified during forgery.

3. In which ways is it commonly produced and purified

   Lactic acid is produced industrially by bacterial fermentation of carbohydrates, or by chemical synthesis from acetaldehyde that comes from coal or crude oil. In 2009, lactic acid was produced predominantly (70–90%) by fermentation.

   Fermentative production

   Fermented milk products are obtained industrially by fermentation of milk or whey by Lactobacillius species bacteria: Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus delbrueckii subsp. bulgaricus (Lactobacillus bulgaricus), Lactobacillus helveticus, Lactococcus lactis, and Streptococcus salivarius subsp. thermophilus

(Streptococcus thermophilus).

As a starting material for industrial production of lactic acid, almost any carbohydrate source containing C5 and C6 sugars can be used. Pure sucrose, glucose from starch, raw sugar, and beet juice are frequently used. Lactic acid producing bacteria can be divided in two classes: homofermentative bacteria like Lactobacillus casei and Lactococcus lactis, producing two moles of lactate from one mole of glucose, and heterofermentative species producing one mole of lactate from one mole of glucose as well as carbon dioxide and acetic acid/ethanol.

Chemical production

For lactic acid chemical synthesis, acetaldehyde is let to react in liquid phase and under high pressure with hydrogen cyanide in the presence of a base to produce lactonitrile.

After its recovery and purification by distillation, hydrochloric acid or sulfuric acid is added to hydrolyze lactonitrile to lactic acid, which is then esterified with methanol to produce methyl lactate, and this is recovered and purified by distillation. The purified methyl lactate is finally hydrolyzed in acidic aqueous solution to lactic acid and methanol, the latter being recycled in the same processSynthesis of both racemic and enantiopure lactic acids is also possible from other starting materials (vinyl acetate, glycerol, etc.) by application of catalytic procedures.

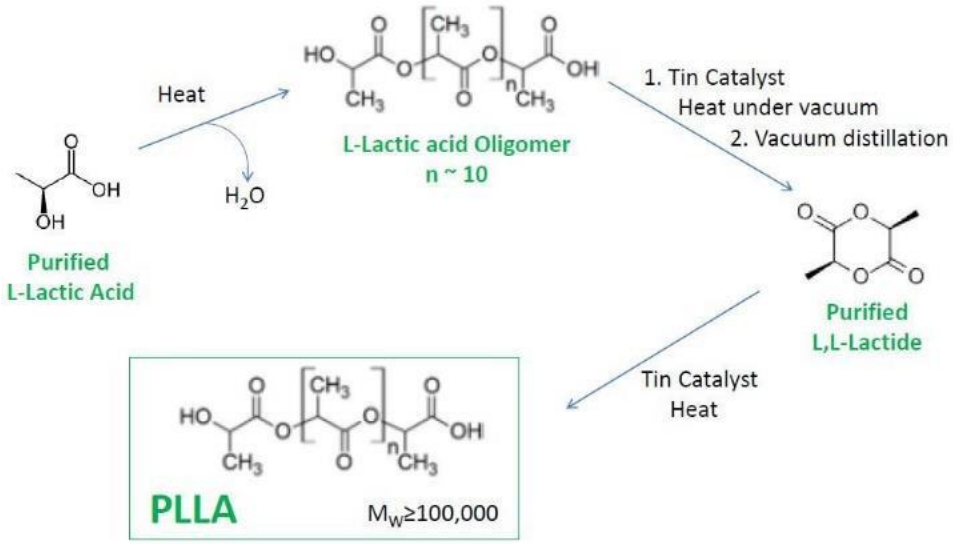
Purification

Lactic acid purification is one of the most costly steps of the production process. Great attention should be paid to the addition of low-cost residues or other nutrients to the medium, because removal of impurities can significantly increase the costs of purification steps. Methods to reduce impurities in the final product include extraction, membrane separation, ion exchange, electrodialysis and distillation with chemical reaction.

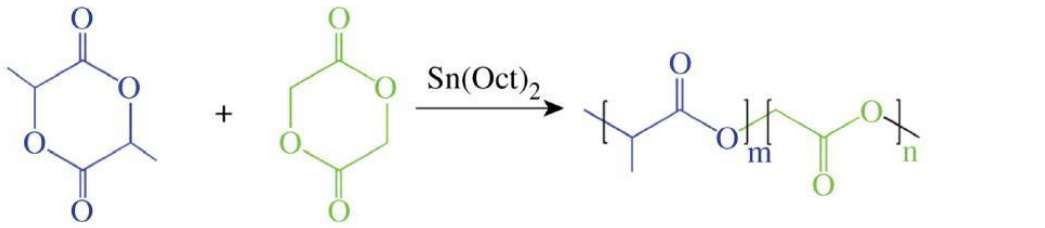
According to some authors, distillation is extremely difficult owing to the low volatility of lactic acid, and electrodialysis cannot separate charged components, especially contaminating amino acids and organic acids. On the other hand, nanofiltration combined with bipolar electrodialysis in downstream purification can replace multiple purification steps with only two steps, while yielding a monomer grade lactic acid from a mixture of unconverted sugars and lactic acid. Ion exchange chromatography is widely used in bioseparations, and several different ion exchangers have been successfully employed in the past few years to recover lactic acid.

4. How are PLLA and PLGA produced?

Lactic acid polymerization can be performed by means of opening the ring of the lactide, composed of two lactic acid molecules that form a ring in the presence of tin salts, heat, and vacuum. ROP of lactones has been widely studied over the past 40 years. Carothers and coworkers explored this technique for lactones, anhydrides, and carbonates.



In the present day, the process for obtaining PLA via ROP employs polycondensation to obtain low molecular weight PLA, depolymerization to form the cyclic dimer (lactide), and ring-opening polymerization to obtain high molecular weight polylactic acid with tin(II) octoate or another organic stannous salt as a catalyst (see picture above). As far as the polycondensation reaction is concerned, the prepolymer formed must have a molecular weight between 500 and 1000 Da, given that molecular weights below 500 do not favor the formation of the cyclic dimer, and molecular weights greater than 1000 lead to transport phenomena problems due to the increased viscosity.
PLGa is synthesized in a similar manner (ROP) and with the same catalysts, starting from a mixture od lactide and glycolide in different proportions to achieve specific properties.



5. What are these compounds used for?

Their polymers are biodegradable, used as materials for packaging and labeling, and biocompatible, being useful for the manufacture of prosthetic devices, sutures and internal drug dosing. Among them, the polylactic acid has several applications in the textile, medical and pharmaceutical industries.