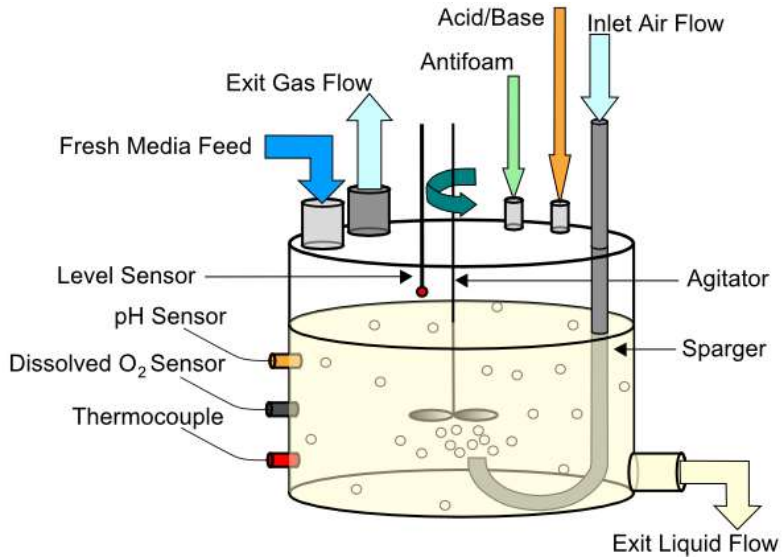
Gojenje GSO v večjem merilu

Molekularni biologi: izolacija, karakterizacija, mutageneza, izražanje genov v (industrijskih) mikroorganizmih
Biokemijski inženirji: rast GS mikroorganizmov v velikem merilu pri optimalnih pogojih

Povečevanje volumnov kultur (scale-up) ne pomeni enostavnega linearnega povečevanja pogojev iz laboratorijskega merila. Potrebna so posebna znanja, povezana predvsem z delovanjem bioreaktorjev. Za doseganje optimalnih pogojev rasti je potrebno uravnavati T, pH, tip in hitrost mešanja ter konc. kisika. Splošno pravilo: za vsako 10-kratno povečanje volumna je potrebno na novo določiti optimalne pogoje.

Koncept bioreaktorja: posoda za kontrolirano rast celic v sterilnih pogojih. Hkrati predstavlja mehanizem biološke varnosti. Preko v bioreaktor vstavljenih sond je mogoče stalno spremljanje parametrov rasti in njihovo uravnavanje.



http://www.ecs.umass.edu/che/henson_group/research/bioreactor.html

Stopnje proizvodnega procesa

Proizvodni postopki, ki vključujejo rast mikroorganizmov, imajo več stopenj:

- priprava gojišča in sterilizacija gojišča in fermentorja;
- založno kulturo gojimo v epruveti, nato v erlenmajericah, predfermentorju in končno proizvodnem fermentorju (sterilen prenos materiala!);
- ločevanje celic od tekočine (centrifugiranje ali filtracija);
- za znotrajcelične proteine celice razbijemo in odstranimo debris;
- za zunajcelične proteine izvedemo izolacijo iz gojišča.

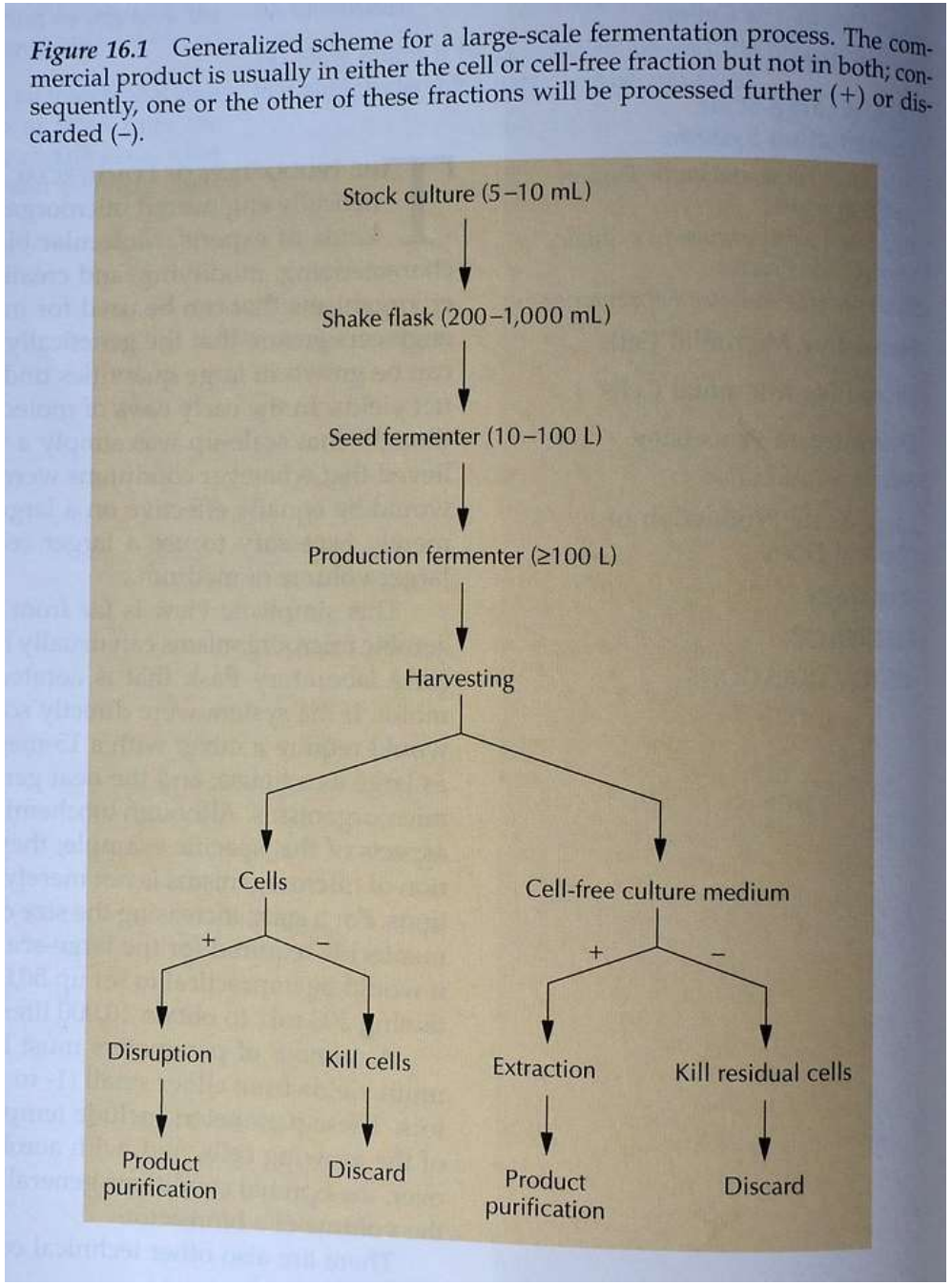


Figure 16.1 Generalized scheme for a large-scale fermentation process. The commercial product is usually in either the cell or cell-free fraction but not in both; consequently, one or the other of these fractions will be processed further (+) or discarded (–).

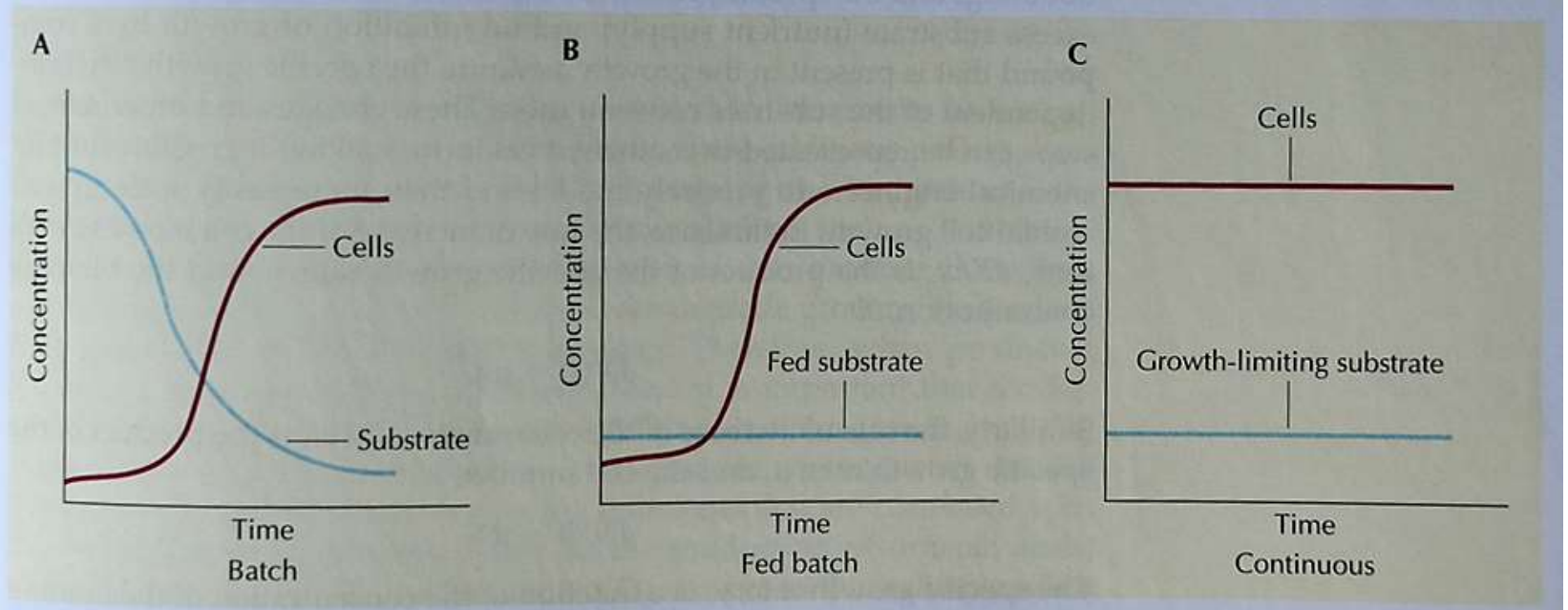
Tipi fermentacijskih postopkov

Šaržna fermentacija: Gojišče pripravimo vnaprej in vanj vcepimo predkulturo.

Šaržna fermentacija z dohranjevanjem: Sestavine gojišča dodajamo postopno, med potekom procesa.

Kontinuirna fermentacija: Hranila dodajamo postopno, ves čas procesa pa tudi odstranjujemo biomaso (z izrabljenim gojiščem).

Figure 16.2 Schematic representation of the time course (progress curves) of cell concentration (mass) and substrate concentration in batch **(A)**, fed-batch **(B)**, and continuous **(C)** fermentations.



Šaržna fermentacija

Razmere v reaktorju se ves čas spreminjajo (konc. hranil, biomase, produkta). Kultura ima značilne faze rasti (lag = faza prilagajanja, faza pospeševanja, log = logaritemska = eksponentna, faza pojemanja, stacionarna, faza odmiranja).

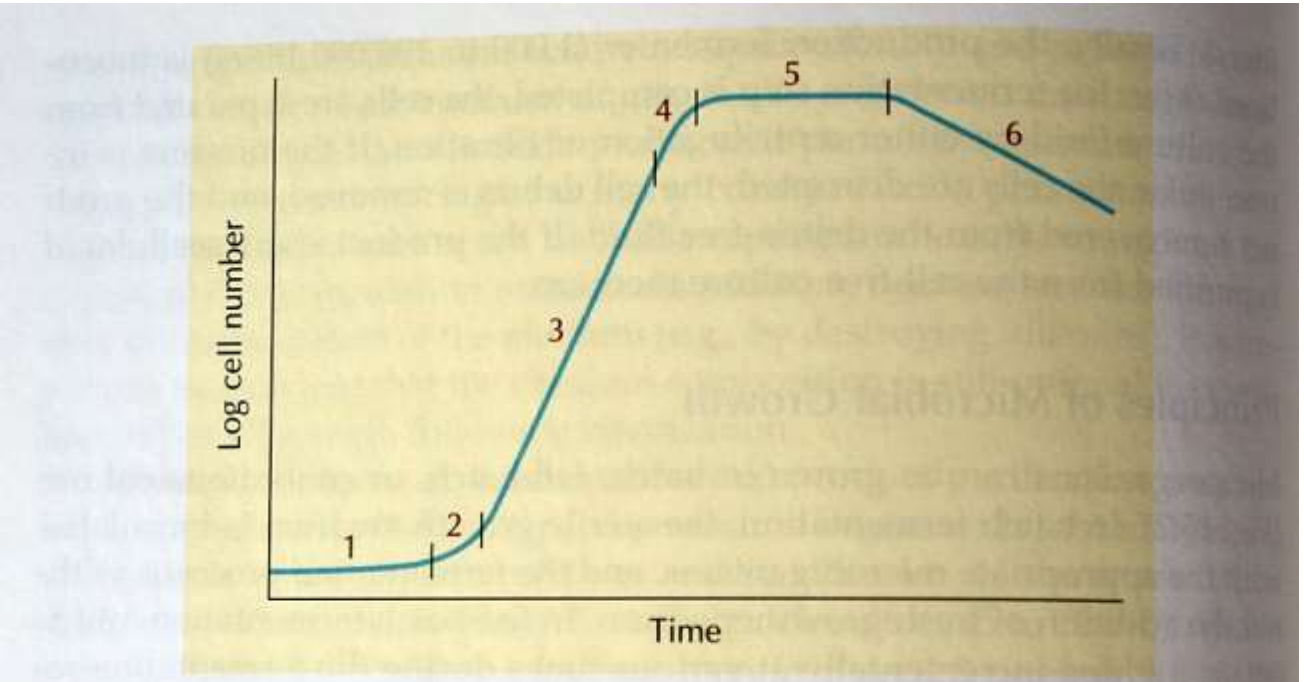


Figure 16.3 Pattern of microbial cell growth in a batch fermenter. The six phases of the growth cycle are (1) lag, (2) acceleration, (3) log (exponential), (4) deceleration, (5) stationary, and (6) death.

Šaržna fermentacija z dohranjevanjem

Ker sestavine gojišča dodajamo med potekom procesa, se logaritemska in stacionarna faza podaljšata, s tem pa se poveča koncentracija biomase. Produktov, ki nastajajo v stacionarni fazi (npr. antibiotiki), je več kot pri šaržnih postopkih. V stacionarni fazi se poveča tudi proteoliza, zato za proizvodnjo rekombinantnih proteinov postopek ustavimo pred to fazo.
Kdaj je treba dodati sveže gojišče, določimo posredno, preko sprememb v pH ali konc. CO_2. Spremljanje procesa mora torej biti bolj natančno kot pri šaržnem postopku. Po drugi strani pa so izpleni lahko tudi več kot 10-krat večji.
Ta način gojenja je za delo z insektnimi in sesalskimi celicami v kulturi edino uporaben.

Kontinuirna fermentacija

Vzdržujemo konstantno gostoto celic ob konstantnem volumnu kulture. S stališča stroškov je ta tip gojenja najugodnejši, čeprav ga sorazmerno redko uporabljajo za mikrobne celice. Reaktorji so manjši od šaržnih (za enak izplen), zaključni procesi potekajo neprestano (ni ozkih grl in prekinitev) in v manjšem merilu, celice so fiziološko ves čas enako aktivne in hitrost proizvodnje je enakomerna. Po drugi strani pa zaradi dolgih fermentacij opažajo izgubljanje vektorjev, lažje pride do težav s sterilnostjo, zaradi morebitnih novih šarž gojišča med potekom kontinurine fermentacije pa lahko pride do zastoja v rasti in proizvodnji (prilagajanje na nove pogoje).

Povečevanje učinkovitosti fermentacije

Med fermentacijo spremljamo osnovne parametre gojenja, to so koncentracija raztopljenega kisika, T, pH, raven mešanja. Ti parametri najmočneje vplivajo na rast, proizvodnjo in stabilnost produkta.

Koncentracijo kisika uravnavamo z reguliranim dotokom sterilnega zraka. Spreminjamo lahko tudi velikosti mehurčkov (bolje, če so manjši). Če so potrebe po kisiku zelo velike, lahko fermentor deluje pri povišanem tlaku, ali pa v kulturo namesto zraka uvajamo kisik.

Spremembe pH so posledica izločanja metabolitov. Preko senzorjev spremljamo spremembe in s črpalkama, ki (ob mešanju) uvajata kislino ali bazo, uravnamo pH na vrednost, ki je za izbrani mikroorganizem optimalna.

Proizvodnja naj bi potekala tako, da bi v čim krajšem času v danem volumnu dobili čim več produkta. Za visoko proizvodnjo je ključna visoka gostota celic. Za *E. coli* so visoke vrednosti >50 g (dw)/l kulture v šaržnih postopkih. Suha teža (dw) predstavlja ~20 % mokre teže (ww). Tako visoke vrednosti biomase dobimo samo z optimalno sestavo gojišča in pogojev gojenja. Nekatera hranila in nekateri metaboliti lahko v previsokih koncentracijah zavirajo rast celic (glukoza > 50 g/l, fosfor > 10 g/l, amonijak 3 g/l). Acetat, ki nastaja pri anaerobni rasti in v prisotnosti prekomerne konc. glukoze, deluje na rast zaviralno, zato glukozo lahko nadomestimo z glicerolom, ali pa znižamo T.

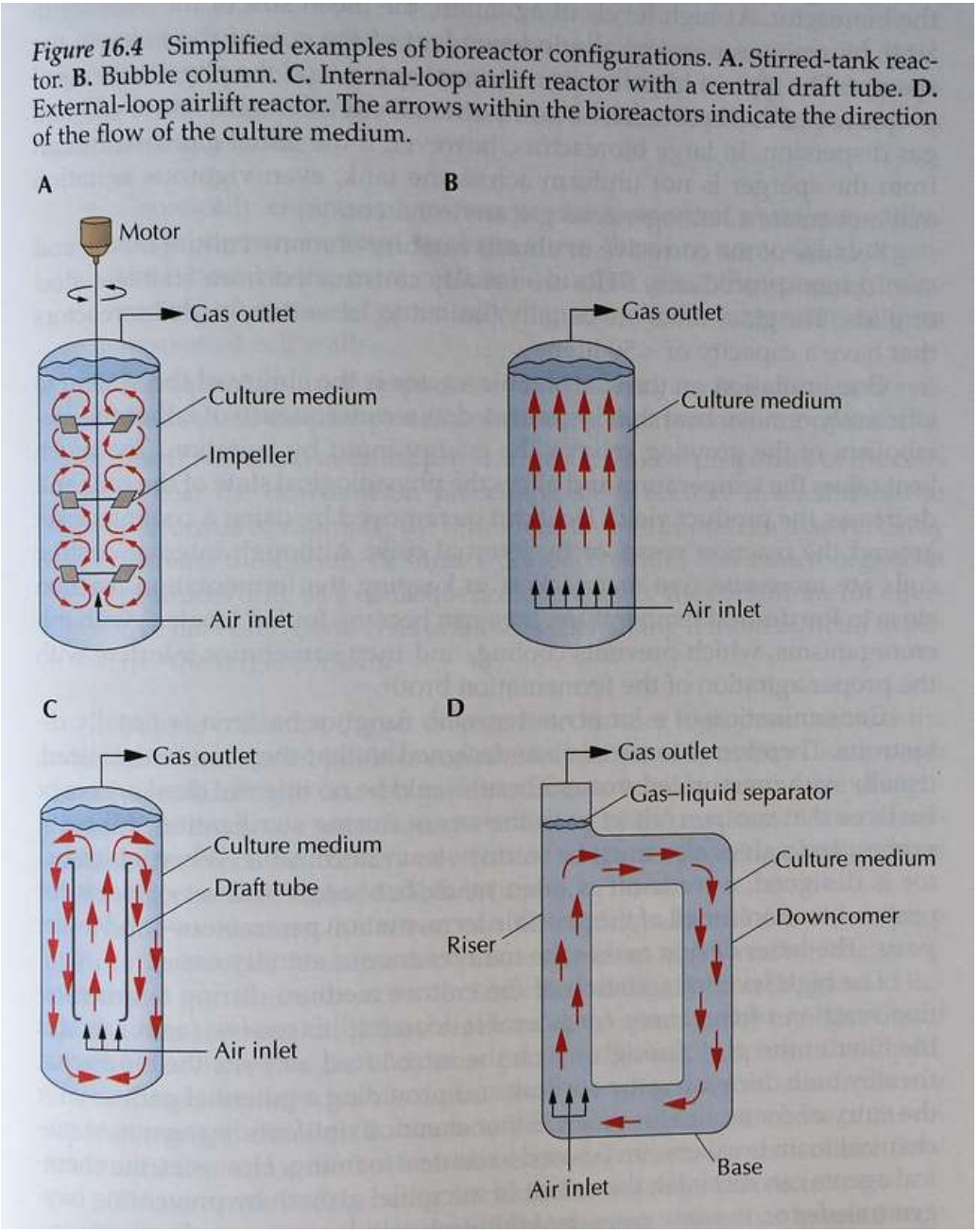
Visoke celične gostote lažje dosežemo v šaržnem postopku z dohranjevanjem, pri čemer uporabljajo različne protokole dovajanja svežih hranil (linearno, stopenjsko, eksponentno).

Konfiguracije bioreaktorjev

V grobem tipe bioreaktorjev razdelimo glede na način mešanja kulture: mešalni tank (STR = stirred-tank reactor), kolonski reaktor (z mehurčki; bubble column), reaktor z dvigovanjem zraka (airlift reactor) z notranjo ali zunanjo zanko.

Obstajajo tudi posebne izvedbe, npr. predalni bioreaktor (trdna gojišča), fotobioreaktor (potreben dostop svetlobe, minimalna globina).

Najpogosteje uporabljamo STR.



Dvostopenjske izvedbe procesov

Gojenje lahko ločimo v več faz, npr. takrat, ko je potrebna sprememba temperature med gojenjem (indukcija izražanja).

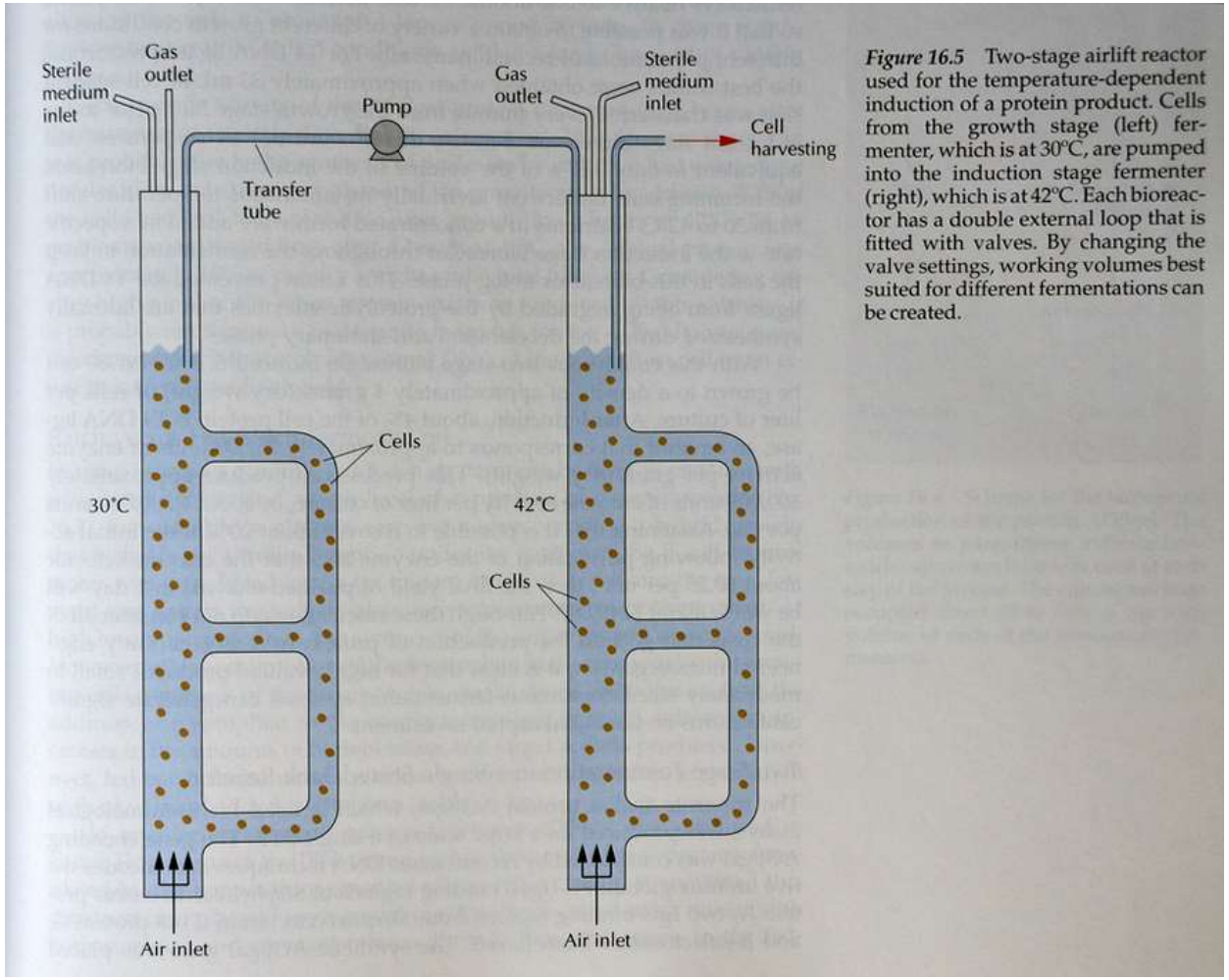


Figure 16.5 Two-stage airlift reactor used for the temperature-dependent induction of a protein product. Cells from the growth stage (left) fermenter, which is at 30°C, are pumped into the induction stage fermenter (right), which is at 42°C. Each bioreactor has a double external loop that is fitted with valves. By changing the valve settings, working volumes best suited for different fermentations can be created.

Izbor tipa procesa

Proizvodnja rekombinantnega proteina v istem sevu GS celic poteka v različnih tipih fermentacijskih procesov različno. Vsak tip ima svoje prednosti in slabosti.

Table 16.1 Comparison of batch and fed-batch fermentation for the production of a fusion protein including the insulin B peptide

Product	Yield in fermentation system:		
	Batch	Batch + Trp	Fed batch
Biomass (g [DW]/L)	6.7	12	20
Fusion protein ÷ total protein (%)	4.6	7.9	11
Total amount of fusion protein (g/L)	0.17	0.53	1.21
Plasmid-bearing cells (%)	86	62	90

Adapted from Gosset et al., *Appl. Microbiol. Biotechnol.* **39**:541–546, 1993.

Biomass is measured in grams (dry weight) per liter (g [DW]/L). In the "Batch + Trp" fermentation, 0.1 g of tryptophan was added. In the fed-batch fermentation, 0.1 g of tryptophan was added every 2 hours, for a total of five times during the course of the 10-hour fermentation. A higher level of tryptophan added to the batch fermentation did not increase the amount of either biomass or target protein produced.

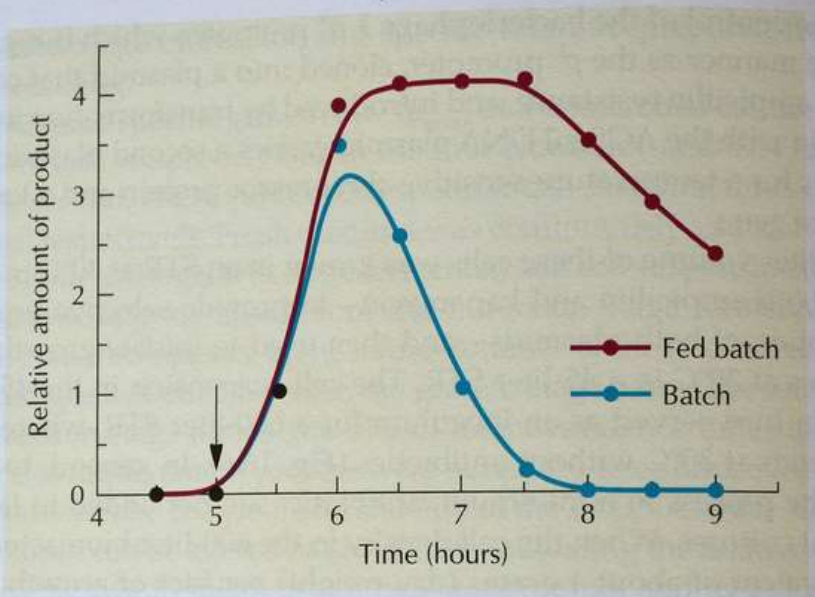


Figure 16.7 Schematic representation of the amount of foreign protein produced as a function of time by recombinant *E. coli* following induction (arrow) in the mid-log phase of growth. Prior to induction, no foreign protein is synthesized. After induction, in batch mode, in the absence of additional nutrients, the cells soon enter stationary phase and synthesize proteases that degrade the foreign protein product. After induction, in fed-batch mode, the added nutrients ensure that the cells remain in log phase for an extended period of time and do not produce any proteases until one and a half to two hours later than the cells in batch mode; therefore, the foreign protein is more stable and is easier to recover. In addition, the provision of nutrients during fed-batch mode makes it less likely that plasmids carrying foreign genes will be lost compared with cells in batch mode. The time represents the number of hours from the start of the fermentation.

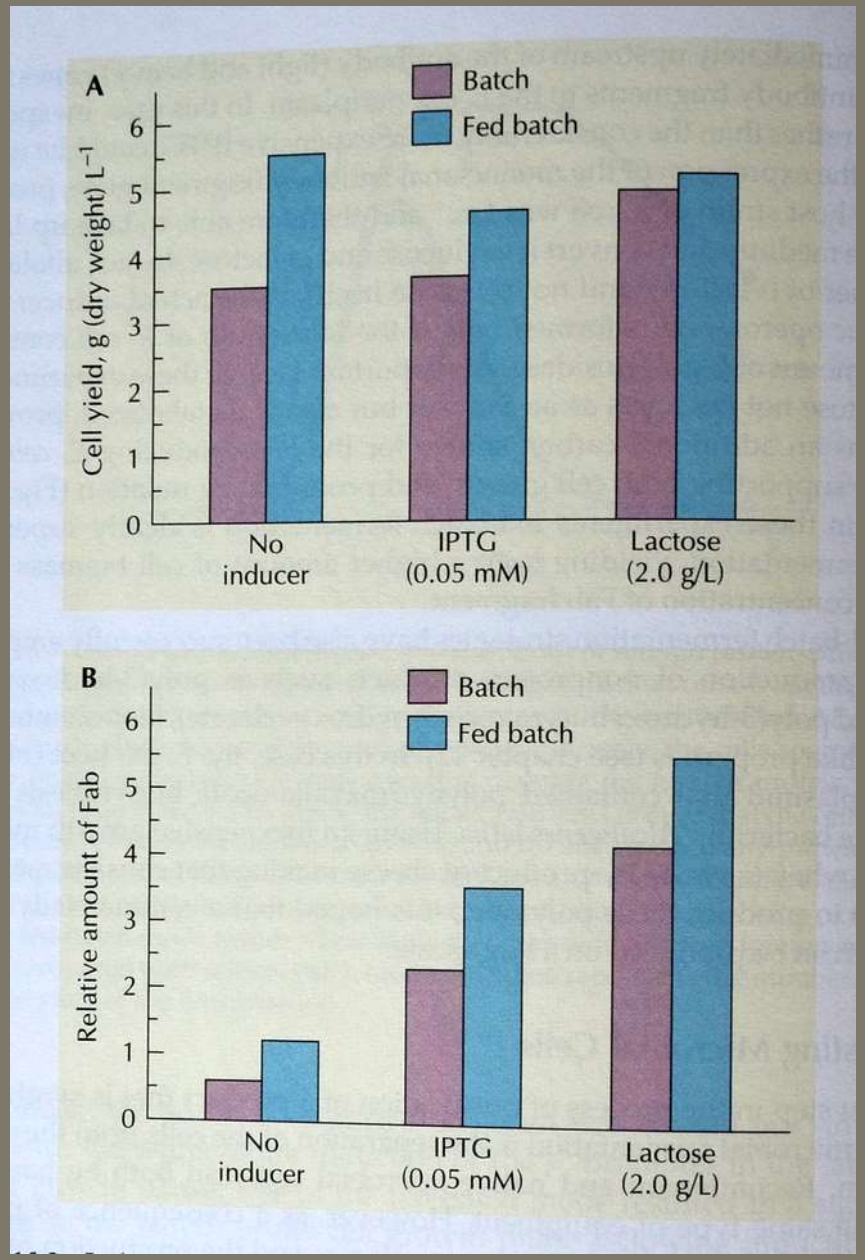


Figure 16.8 Induced recombinant protein production with batch and fed-batch *E. coli* cells grown to late logarithmic phase. Cells were either not induced, induced with 0.05 mM IPTG, or induced with 2.0 grams of lactose per liter. **A.** In batch culture, the cells that were induced with lactose, which acts as both an inducer and a growth substrate, grew to a significantly greater extent than either the noninduced or the IPTG-induced cells. With fed-batch cells, the lesser extent of growth of induced versus noninduced cells probably reflects the resources that the induced cells direct to the synthesis of the Fab antibody fragment. **B.** With both batch and fed-batch conditions, more of the Fab antibody fragment was produced when the cells were induced with lactose than with IPTG. Fab antibody fragment synthesis in the absence of inducer represents incomplete repression of the *lac* promoter.

Zaključni procesi: filtracija kulture

Za zbiranje celic iz kulture uporabljamo centrifuge ali membranske filtracijske sisteme.

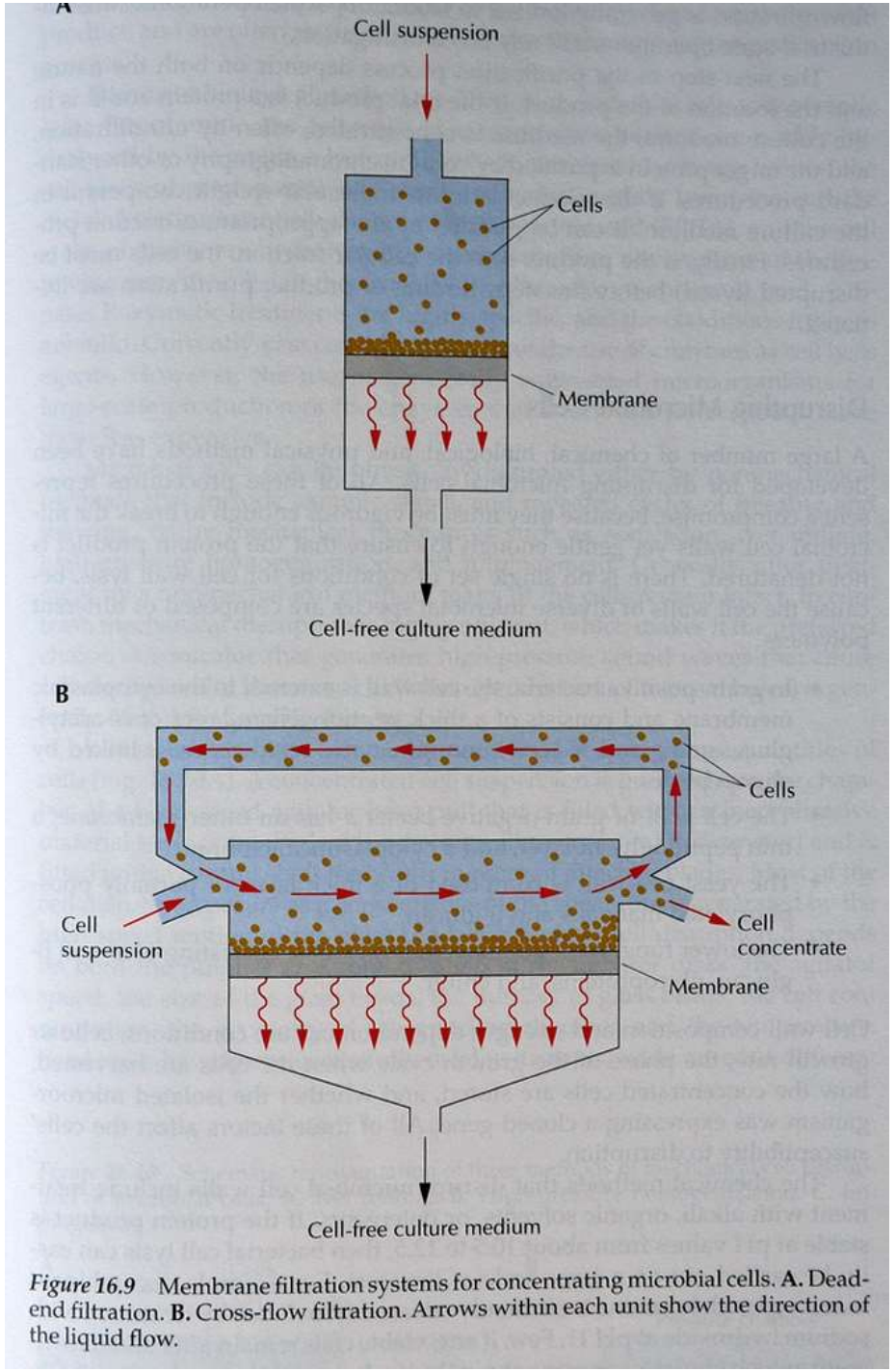


Figure 16.9 Membrane filtration systems for concentrating microbial cells. **A.** Dead-end filtration. **B.** Cross-flow filtration. Arrows within each unit show the direction of the liquid flow.

Zaključni procesi: razbijanje celic

Razbijanje celic izvedemo v industrijskem merilu z mokrim mletjem, homogenizacijo z visokim tlakom ali s postopkom ‚inpingement‘.

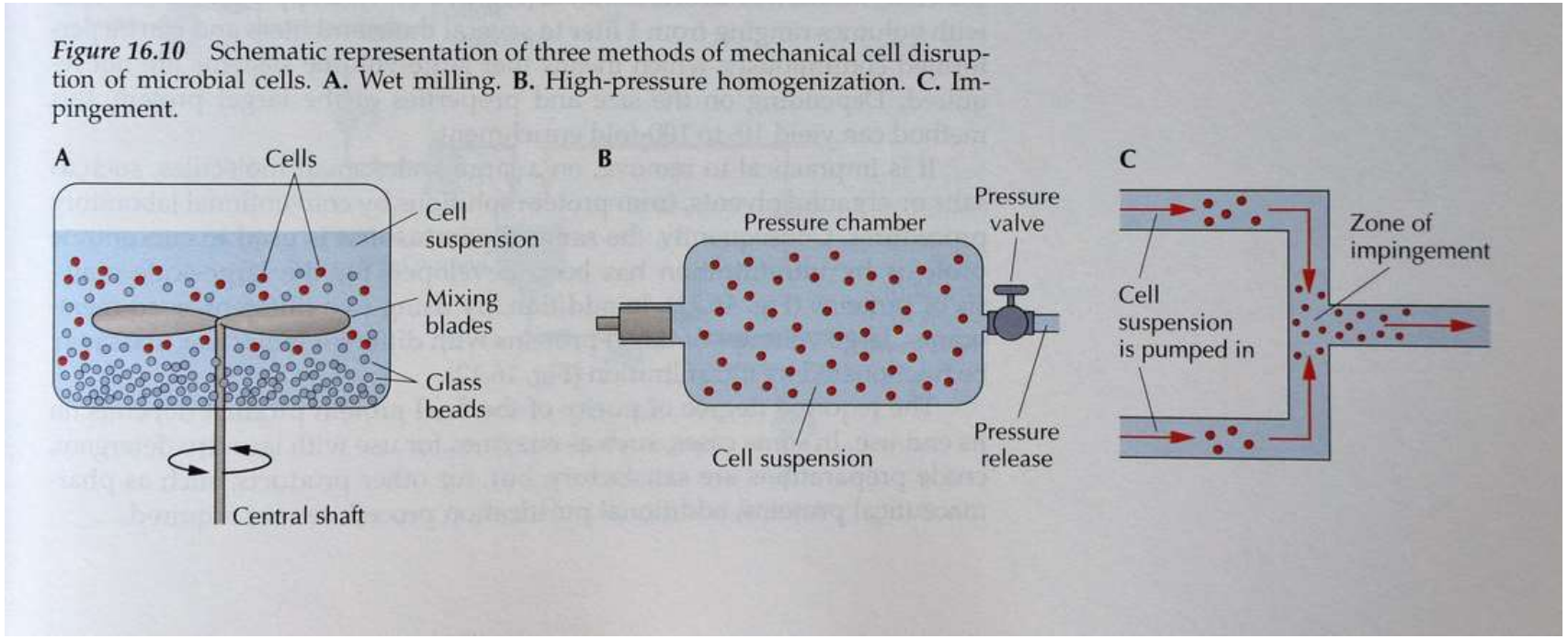


Figure 16.10 Schematic representation of three methods of mechanical cell disruption of microbial cells. **A.** Wet milling. **B.** High-pressure homogenization. **C.** Impingement.

Zaključni procesi: izolacija rekombinantnega proteina

Iz celičnega supernatanta ali lizata izoliramo proteine s podobnimi osnovnimi tehnikami kot v laboratorijskem merilu, a s posebnimi izvedbami, prirejenimi za industrijsko merilo.

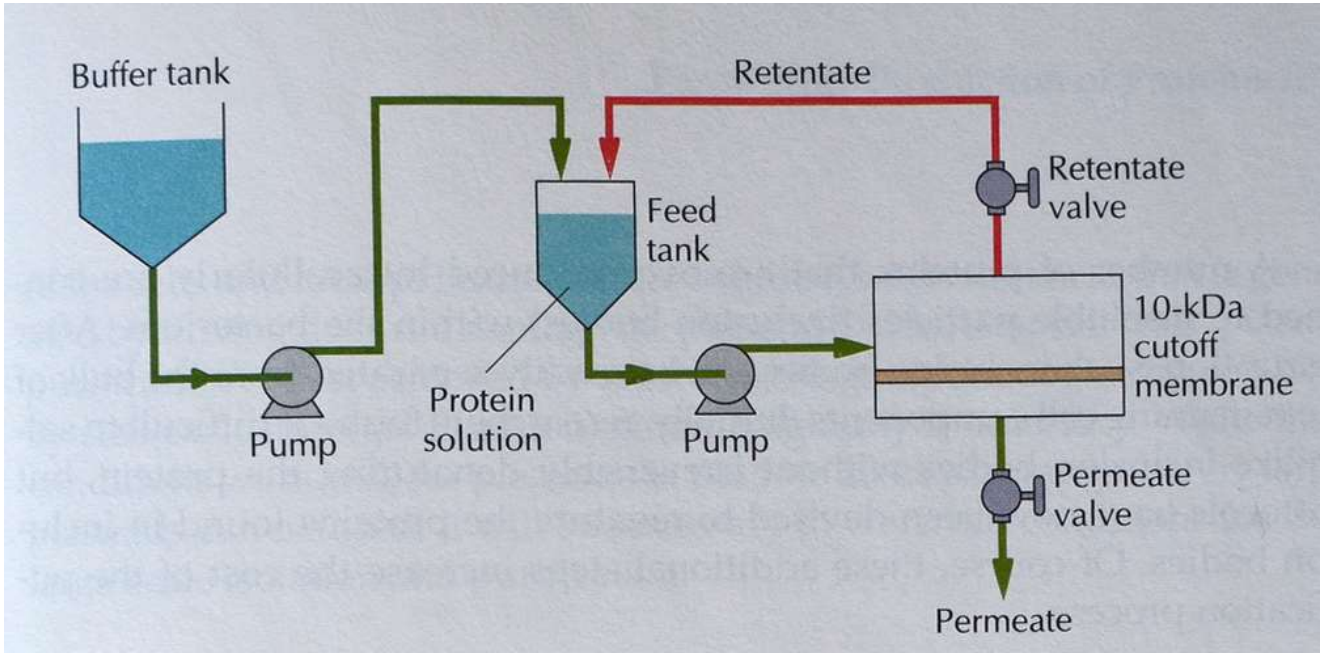


Figure 16.11 Schematic representation of large-scale ultrafiltration dialysis of a protein solution. The solution from the feed tank is pumped across the ultrafiltration membrane, with only a small fraction of the solution actually passing through the membrane and the rest being used to sweep the membrane clean of protein. The volume that passes through the membrane is matched by an equal volume of buffer added to the system. A membrane with a nominal molecular weight cutoff below the size of the target protein is used. For example, a 10-kDa cutoff membrane might be used to retain a 30-kDa protein while removing salt from the solution. Following dialysis, the target protein is found, in a dilute solution, in the feed tank. This solution may be concentrated by ultrafiltration using an identical setup except that no buffer tank is used. The arrows indicate the direction of liquid flow. The relative volume of permeate (liquid that passes through the membrane) compared with the retentate (retained liquid) is controlled by adjusting the permeate and retentate valves; this helps to keep the membrane relatively free of protein that might otherwise clog its pores.

Zaključni procesi: izolacija rekombinantnega proteina /2

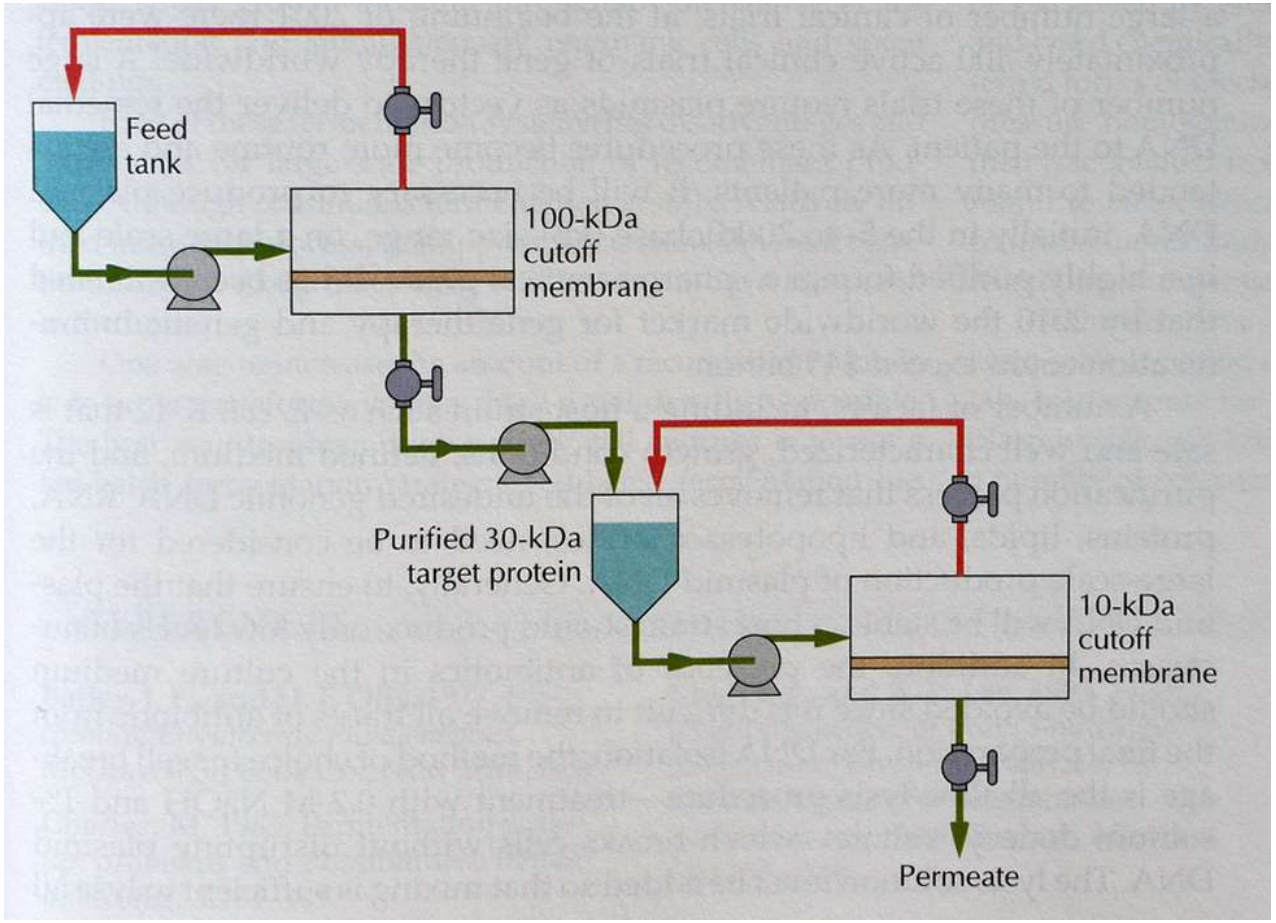


Figure 16.12 Schematic representation of large-scale protein purification using ultrafiltration membranes. In the example shown, two membranes are used sequentially, a 100-kDa cutoff membrane and a 10-kDa cutoff membrane, in order to purify a 30-kDa protein. The solution, which contains a protein mixture, is pumped through the 100-kDa cutoff membrane, with the larger proteins being retained and the smaller proteins, including the 30-kDa target protein, passing through the membrane. This protein solution is next pumped through a 10-kDa cutoff membrane, with the 30-kDa target protein being retained. The arrows indicate the direction of the liquid flow. The symbols are identical to those found in Fig. 16.11.