Chisti, Y., in *Bioseparation and Bioprocessing: A Handbook*, vol. 2, (Subramanian, G., editor), Wiley-VCH, New York, 1998, pp. 3–30. Strategies in downstream processing.

1 Strategies in Downstream Processing

Yusuf Chisti

1.1 Introduction

Biological products come from many sources: human and animal tissue (e.g., blood, pancreas, pituitary) and body fluids (e.g., milk of transgenics), plant material (e.g., Taxol® from the bark of *Taxus* species, oils), microbial fermentations, cultures of higher eukaryotes, and raw broths from enzyme bioreactors. Irrespective of the source, crude extracts, fluids and broths invariably undergo separation and purification to recover the product in the desired form, concentration, and purity. Processing beyond the bioreaction step is termed downstream processing. Here the 'bioreaction step' includes producing plants and animals.

A recovery process consists of physicochemical operations such as those listed in Table 1-1. The steps of a properly engineered downstream process are integrated with each other and with the bioreaction stage to yield an optimal recovery scheme [19]. This discussion is limited to factors which must be considered in developing any economically viable product purification and concentration scheme based on a

Table 1-1. Bioseparation operations.

Solid-liquid separations [1–3]
Centrifugation, filtration, flocculation, flotation, sedimentation [4,5].
Membrane separations [1–3,6]
Diafiltration and dialysis, microfiltration, pervaporation [7], reverse osmosis, ultrafiltration.
Extractions
Aqueous liquid–liquid extraction [1,8,9], extraction and leaching of solids [10], reversed miceller extraction, liquid membrane extraction [11,12], solvent extraction [1,13], supercritical extraction.
Chromatographic methods [1–3,14]
Affinity, gel permeation, hydrophobic interaction, ion exchange. See also Volume 1.
Thermal operations
Distillation, drying [1], evaporation, freeze drying or lyophilization [15].
Miscellaneous
Adsorption, cell disruption [16–18], crystallization, electrophoresis and other electrokinetic methods, precipitation [1].

small selection of the many available processing operations. Individual operations are detailed in other sources [1–3] including some chapters of this series.

1.2 Overview of Process Considerations

Anyone faced with designing a bioseparation scheme can take comfort in the variety of available separation processes (Table 1-1); however, the same variety can be a source of much distress. The number N_f of possible recovery flowsheets that can be theoretically devised to completely separate a mixture of C components by using S number of separation operations is given by

$$N_f = \frac{[2(C-1]!S^{(C-1)}}{C!(C-1)!} \tag{1}$$

Thus, complete separation of a five-component mixture using two separation methods would generate 224 possible flowsheets! Although only one component, the product, is usually wanted, several 'components' need separating. Examples of such components are cells, water, cell debris, nucleic acid polymers, added salts, and the remainder of the proteins. Often, a process must include additional nonseparating steps such as cell disruption, heating, and mixing. Not all possible flowsheets can be exhaustively evaluated; instead, experience and thorough knowledge of individual bioseparations and relevant fermentation must be relied upon to narrow the choices to a few practicable options for detailed evaluations and experimental testing.

Factors that must be considered in designing a downstream processing scheme include the nature, concentration and stability of the product, the desired purity and end use. Because of contamination and supply considerations, there is a distinct trend to move away from direct extraction of human and animal sources to recombinant cells. Thus, for example, microbially produced recombinant human insulin and growth hormone are now available. For vaccines, too, attempts are underway to engineer safer organisms to produce the antigenic material that would otherwise be obtained from pathogens.

The end use of the product may vary – research, *in vitro* diagnostic, food, animal feeds, soil inoculants, pesticides, medicinals, medical device, cosmetics, etc. The specific form of the product may include live human cells for medical purposes; live microorganisms, viruses (e.g., for vaccines), spores (e.g., for biotransformations, insecticides, and solid state culture), and higher organisms (e.g., nematodes); bioactive polymers, proteins and enzymes; inactive polymers (e.g., food protein, xanthan, PHB); smaller organics (e.g., streptomycin, amino acids, citric acid, ethanol, Taxol®); polypeptides (e.g., cyclosporine); and cellular organelles (e.g., nuclei, mitochondria, chloroplasts).

Location of the product, whether extracellular, intracellular, or periplasmic, affects how it is recovered. Physical and chemical properties of the product and contaminants need addressing, and biosafety issues must be given attention (see Chapter 13).

A further consideration is price relative to existing sources and other competing products. When no competing products or alternative sources can be identified, the estimated production costs would need to be compared with what the market can reasonably be expected to pay.

As far as possible, the requisite purification and concentration should be achieved with the fewest processing steps; generally, no more than six to seven steps are used, a situation quite different from that in chemistry and biochemistry laboratories, where the number of individual steps is often not a major consideration, and purity of the product is usually more important than overall yield or costs [19]. The overall yield of an n-step process with step yield of x percent is $(x/100)^n$. Therefore, n must be minimized for a high overall yield. For example, a train of only five steps, each with 90% step yield, would reduce the overall recovery to less than 60% [20]. To minimize reduction of the overall yield, high-resolution separations such as chromatography should be utilized as early as possible in the purification scheme in keeping with the processing constraints that these steps require (e.g., clean process streams free of debris, particulates, lipids, etc.).

Separation schemes incorporating unit operations which utilize different physical–chemical interactions as the bases of separation are likely to achieve the greatest performance for a given number of steps. Combining two separation stages based on the same separation principle may not be an effective approach. As an example, when two chromatographic steps in series are selected, e.g., gel filtration which separates based on molecular size, and ion exchange chromatography which separates based on difference in charge on the molecules, may be a suitable combination.

Speed of processing is another factor that significantly affects the design of a recovery scheme. The size of the bioreaction step and the frequency of harvest usually determine the turnaround time for the downstream process train. Sometimes during processing, exposure of material to relatively severe environmental conditions is unavoidable. Very many factors affect stability, including temperature, pH, proteases and other degrading enzymes, mechanical forces, microbial contamination, oxidants, and other denaturing chemicals. In severe environments, the duration of exposure must be minimized and especial precautions (e.g., low temperature; addition of chemicals to reduce oxidation, etc.) are necessary to reduce the impact of exposure. The need for speedy processing constrains equipment choice and capacity. For example, the low pH necessary during extraction of penicillins affects stability, hence rapid extraction is essential, thus mixer–settler type extraction is contraindicated.

Typically, a separation process must operate within the physiological ranges of pH and temperature (pH $\sim$ 7.0; temperature $\leq$ 37 °C), but differences from the norms are not unusual. For example, enzymes such as lysozyme, ribonuclease, and acid proteases are quite stable at low pH values [19]. Some biologically active molecules, particularly proteins, may be sensitive to excessive agitation; however, enzymes, with the exception of multienzyme complexes and membrane-associated enzymes, are not damaged by shear in the absence of gas–liquid interfaces [1,21].

Except for the final few finishing operations, downstream processing is usually conducted under non-sterile, but bioburden-controlled conditions; however, prevention of unwanted contamination and cleaning and sanitization considerations require

that the processing machinery be designed to the same high standards as have been described for sterile bioreactors [22,23]. Containment and hygienic processing requirements may severely affect equipment choice (see also Chapters 13 and 14).

A commercial recovery scheme must be reliable and consistent. Process robustness is essential to economic production, process validation, and product quality. Automation assures consistency and rapid turnaround of the process equipment. Operations such as in-place cleaning are often automated [24].

Additional considerations include biosafety and containment. Bioproducts may be potentially allergenic, and they may produce activity associated reactions in process personnel [10]. In addition, process material may be pathogenic, cytotoxic, oncogenic, or otherwise hazardous. Processing of such material requires attention to containment and biosafety both during design and in operation of the bioseparation scheme [10,25]. Certain processing operations are difficult to contain, and may pose peculiar operational problems. For example, gasket failures during high-pressure homogenization could create high-pressure sprays [16] and, unless designed with containment features, operations such as centrifugation may generate aerosols (see also Chapter 13).

Small quantities of multiple products are sometimes produced in the same plant: a series of runs or campaigns of one product is followed by another. The risk of cross-contamination is high and adequate safeguards are essential. Experience suggests that cross-contamination with penicillins and penicillin-containing substances cannot be reasonably prevented in a multi-product facility. Because penicillins may produce adverse reactions in some patients, Good Manufacturing Practices (GMP) regulations demand dedicated penicillin processing facilities that are segregated from non-penicillin products. Separate air handling systems are necessary if a building processes penicillins as well as non-penicillin products.

GMP regulations including the validation requirements [26], affect all aspects of downstream processing. Requirements depend on the kind of product (e.g., food, bulk pharmaceutical, final dosage form, etc.) and the jurisdiction. Willig and Stoker [27] should be consulted for specific guidance.

The final few downstream processing steps include formulation which is highly product specific. How a product is formulated may critically affect its stability, efficacy, and bioavailability. Formulation may involve addition of fillers (e.g., starch, cellulose, sugar, flour), diluants, preservatives, sunlight protectants (e.g., carbon black, dyes, titanium oxide), dispersal aids, emulsifiers, buffers, moisture retainers, adjuvants (e.g., mineral oils and aluminum hydroxide added to improve antigenicity of certain vaccines), flavors, colors, and fragrances. Additional finishing operations may include sterile filtration, vialing, granulation, agglomeration, size reduction, coating, encapsulation, tableting, labeling, and packaging.

1.3 Product Quality and Purity Specifications

The specifications on product purity and concentration should be carefully considered in developing a purification protocol. Concentration or purification to levels beyond those dictated by needs is wasteful. The acceptable level of contamination in a particular bioproduct depends on the dosage, the frequency of use, and the method of application (e.g., food, drug, oral, parenteral), as well as on the nature and toxicity (or perceived risk) associated with the contaminants [19]. Products such as vaccines, which are used only a few times in a lifetime, may be acceptable with relatively high levels of other than the desired biomolecule. In some cases, contaminating protein levels of about 100 ppm may be acceptable. *In vitro* diagnostic proteins (enzymes, monoclonal antibodies) may tolerate greater levels of contaminants so long as the contaminants do not interfere with the analytical performance of the product. With certain diagnostic proteins, such as the blood typing monoclonal antibodies, cross-contamination causing misdiagnosis is an extreme concern because of possibly fatal consequences of mis-typing. Such concerns influence the design and operation of the downstream process, particularly for multi-product plants.

Parenteral therapeutics usually must be purer than 99.99%. A variety of approaches are used to assure quality. Methods typically used with protein therapeutics are summarized in Table 1-2; Anicetti et al. [29] provide additional details. Requirements relating to some specific contaminants are discussed below.

Table 1-2. Methods for quality assurance of protein therapeutics [28].

Impurity or contaminant	Analytical technique
Protein contaminants (e.g., host cell proteins)	SDS–PAGE electrophoresis, HPLC, immunoassays (ELISA, etc.)
Endotoxin	Rabbit pyrogen test, LAL[a]
DNA	DNA dot–blot hybridization
Proteolytic degradation products	IEF[b], SDS–PAGE, HPLC, N- and C-terminus analysis
Presence of mutants and other residues	Tryptic mapping, amino acid analysis
Deamidated forms	IEF
Microbial contamination	Sterility testing
Virus	Viral susceptibility tests
Mycoplasma	21 CFR[c] method
General safety	As per 21 CFR 610.11

[a] Limulus amoebocyte lysate; [b] Isoelectric focusing; [c] Code of Federal Regulations.

1.3.1 Endotoxins

Products derived from bacteria such as *Escherichia coli* will invariably be contaminated with bacterial cell wall endotoxins which can cause adverse reactions (headaches, vomiting, diarrhea, fevers, etc.) in patients unless reduced to very low levels (e.g., less than 5×10^{-13} kg per kg body weight). Endotoxins are extremely heat-stable lipopolysaccharides that are not easily removed from solutions of macromolecules. Ultrafiltration and reverse osmosis are effective for depyrogenation of water and small solutes. Other pyrogen removal methods are adsorption on activated carbon and barium sulfate, hydrophobic interaction chromatography, and affinity chromatography. Endotoxins bind to polymixin B affinity columns, but this method must be combined with detergent treatment for effectively removing protein-bound endotoxins [28]. Chromatography using LAL affinity matrix also removes endotoxins.

As a guiding principle, processing must aim to minimize endotoxin contamination by controls on process water and other additives. In addition, aseptic and bioburden controlled operation, and frequent cleaning of equipment help to reduce contamination. The equipment cleaning protocol must include procedures proven for depyrogenation. Standard alkali-based cleaning procedures [24] are quite effective in depyrogenation of stainless steel equipment, but other methods are necessary for cleaning chromatographic columns and membrane filters. The depyrogenation step employed during cleaning of membrane filters usually involves a 30-minute, 30–50 °C treatment with sodium hydroxide (0.1 M), hydrochloric acid (0.1 M), phosphoric acid, or hypochlorite (300 ppm free chlorine). Thorough rinsing with pyrogen-free water follows. Similar procedures are used for chromatographic columns.

An endotoxin-free product should be validated using the LAL test. This test is based on endotoxin-induced coagulation of amoebocyte lysate of horseshoe crab (*Limulus polyphemus*) at 37 °C, pH 7.0. Less than 0.3 ng mL^{-1} endotoxin levels are easily detected. Scrupulously clean glassware and water are necessary to prevent false positives. Some known interferences are EDTA, sodium dodecyl sulfate, urea, heparin, and benzyl penicillin.

1.3.2 Residual DNA

Residual DNA from producing cells can potentially contaminate the product. DNA fragments from established animal cells were once believed to be potentially oncogenic, which prompted the U.S. Food and Drug Administration to recommend a contamination level of no more than 10 pg DNA per dose [30]. Less restrictive limits are now accepted because no oncogenic events were observed following injections of large doses of DNA into animals. Nonetheless, DNA is a contaminant and demonstration of its satisfactory clearance is essential to quality assurance of the product [30]. Residual DNA is removed usually by adsorption on strong anion-exchange resins at pH $\geq$ 4. Hydrophobic interaction chromatography is also effective and so is affinity chromatography under conditions that bind the desired protein but not the DNA.

1.3.3 Microorganisms and Viruses

Parenteral products, other than certain vaccines, must be free of microorganisms and viruses [19]. Products derived from potentially contaminated sources such as human donors, animals, and some cell lines, can be especially problematic. For such products, the purification scheme must demonstrate viral inactivation or removal unless the product is terminally sterilized by validated means (see also Volume 1, Chapter 16). Usually, in-series processing with at least two steps, each capable of six log virus removal or deactivation, would be necessary. Viruses can be removed by ultrafiltration, or deactivated by methods such as heating, treatment with chemicals (e.g., β-propiolactone), solvents and detergents, and ultraviolet or gamma irradiation. In one study with plasma derived human serum albumin, heat treatment at 60 °C for 10 h in the final container produced more than five log reduction of vaccinia, polio-1, vesicular stomatitis, Sindbis and HIV-1 within 10 minutes [31]. In another case, freeze-dried coagulation factors were treated at 80 °C for 72 h in the final vial. For Factor VIII, inactivation of HIV-1 occurred within 24 h, without significant deterioration of the product [31]. For a Factor IX preparation, treatment with solvent/detergent combination of tri-(*n*-butyl) phosphate and Tween-80 for 5 h inactivated a range of typical enveloped viruses within an hour [31]. Up to six log reduction of some typical enveloped viruses such as herpes simplex-1 and Sindbis could be achieved in spiked samples using protein G column chromatography with acid elution; however, only three log reduction was observed for acid tolerant non-enveloped polio virus [31].

1.3.4 Other Contaminants

For many biological products, particularly pharmaceuticals, seemingly minor alterations in downstream processing can have important implications on the performance of the product. For example, penicillins may be recovered by liquid–liquid extraction of either the whole fermentation broth or solids-free broth. The latter scheme requires an additional solid–liquid separation step than the whole broth process. However, the whole-broth extracted product has been known to cause more frequent cases of allergenic reactions in comparison with the other processing alternative. In fact, some pharmaceutical companies now demand of contract suppliers that, in addition to meeting product specifications in terms of measurable contamination, the product they supply must conform to a certain production method, in this case extraction after removal of fungal solids. When raw penicillin is for bulk conversion to semi-synthetic penicillins, whole-broth extraction may be acceptable in view of the security afforded by the additional steps involved in making and purifying 6-aminopenicillanic acid from raw penicillin [19].

1.4 Impact of Fermentation on Recovery

Downstream processing should not be considered in isolation with the bioreaction step. Development of biocatalyst by natural selection, mutation, and recombinant DNA technology is a powerful means of influencing downstream processing [32]. Similarly, modification of fermentation feeding strategies, culture media and conditions profoundly affect the downstream process [32].

1.4.1 Characteristics of Broth and Microorganism

Composition of the fermentation medium affects downstream recovery. Relatively poorly defined complex media components are often acceptable for producing commodity chemicals and bulk antibiotics, but usually not for parentral proteins. Low-serum and protein-free media are commonly employed in animal cell culture to greatly simplify recovery of sparing amounts of proteins produced. Similarly, the type of antifoam and its concentration must accommodate the recovery constraints.

For some processes, alternative microorganisms may be a viable option. Preference should be given to faster growing, easy to process organisms. Selection of a producer must consider the overall productivity of the process, not just that of the fermentation step. Production of recombinant proteins in *Saccharomyces cerevisiae* may have important advantages relative to production in genetically modified bacteria such as *Escherichia coli* [33]. *S. cerevisiae* is generally recognized as safe for food and pharmaceutical use. In addition, unlike bacteria, the yeast does not produce endotoxins, and its broths are much easier to process than those of mycelial fungi and filamentous bacteria [33]. Unlike the DNA-laden homogenates of bacteria such as *E. coli*, yeast lysates are not excessively viscous. In yet other cases, it may be possible to naturally select autoflocculating strains, as has been done with certain brewing yeasts and bacteria. Cells may also be genetically modified into flocculating ones.

Genetic engineering of producing organisms and products provides new opportunities for influencing downstream bioseparations. For example, recombinant fusion proteins with added polypeptide 'affinity tags' have been produced to facilitate purification [34,35]. Affinity tags have been developed for ion exchange, hydrophobic interaction, affinity, immunoaffinity and immobilized metal ion chromatography. Specific cleavage sites between the tag and the protein allow removal of the tag after purification [34]. Some of the available affinity tags and the chromatographic methods applied with those tags are listed in Table 1-3. Reagents and enzymes that have been used to cleave the tags, and the specific cleavage sites, are noted in Table 1-4.

Another strategy for simplifying downstream recovery is genetic manipulation to enable extracellular secretion of the recombinant protein. Failing outright secretion, it may be possible to achieve secretion into the periplasm of microorganisms such as *E. coli*. Relatively mild disruption or extraction conditions can then be used for

Table 1-3. Affinity tags and corresponding chromatographic separations [34].

Affinity tag	Chromatography scheme
Polyarginine	Ion exchange
Polyphenylalanine	Hydrophobic interaction
β-Galactosidase	Affinity
Protein A	Affinity
Antigenic peptides	Immunoaffinity
Polyhistidine	Metal ion chelate

recovery in comparison with products produced in the cytoplasm. Periplasmic secretion has additional advantages: periplasm of *E. coli* contains only seven of the 25 cellular proteases [36], hence, the likelihood of proteolysis is reduced. Moreover, periplasm contains only 100–200 proteins [36], therefore, selective extraction of periplasm yields a less complex, easier to purify mixture. In addition, the oxidative environment of periplasm is more favorable to formation of disulfide bonds than the environment of cytoplasm. Disulfide linkages determine the correct folding of the polypeptide chain and, therefore, its biological activity. Chemicals such as chloroform, Triton X-100, and combinations of lysozyme and EDTA [36] facilitate release of periplasmic proteins. Extraction chemicals should be tested for possible effects on protein stability. In one study, Garrido et al. [33] observed loss of β-galactosidase activity even at 4 °C when the enzyme was extracted with a mixture of chloroform and sparing amounts of sodium dodecyl sulfate (SDS). In larger quantities, SDS is a well-known protein denaturant [37].

Table 1-4. Chemicals and enzymes for specific cleavage of fusion proteins [34].

Cleavage reagent	Cleavage site
Cyanogen bromide	Met ↓
Formic acid	Asp ↓ Pro
Hydroxylamine	Asn ↓ Gly
Collagenase	Pro-Val ↓ Gly-Pro
Factor Xa	Ile-Glu-Gly-Arg ↓
Enterokinase	Asp-Asp-Asp-Lys ↓
Rennin	His-Pro-Phe-His-Leu-Leu ↓
Carboxypeptidase A	C-terminal aromatic amino acids
Carboxypeptidase B	C-terminal basic amino acids

Secretion or extracellular leakage of an otherwise intracellular product is sometimes achieved simply by modifying the fermentation conditions. For example, addition of penicillin during growth in certain amino acid fermentations produces cells that leak the amino acid which is recovered by isoelectric precipitation from the extracellular fluid.

1.4.2 Product Concentration

Concentration of the product in the source material affects the cost of recovery. Concentrations are usually quite low; some values typically seen in culture broths are noted in Table 1-5. In addition to the product, the broth contains many contaminants – proteins, lipids, surfactants, carbohydrates, nucleic acid polymers, salts, components of the culture medium, pigments, organic acids, alcohols, aldehydes, esters, amino acids, and other metabolic products – some of which may be quite similar to the desired product. Some of the contaminants may be toxic or otherwise hazardous (e.g., endotoxins, mycotoxins).

Downstream processing typically represents 60–80 % of the cost of production of fermentation products. Thus, superficially it may appear that process improvement should focus on downstream. This is not so. Even small improvements in the yield or purity of the product in the bioreaction step can have a significant effect on downstream recovery costs. As a rough guide, the selling price P (US\$ kg^{-1}) of a product (i.e., a reflection of cost of production) depends on its concentration C_i in the broth or the starting material. This dependence can be described by the equation

$$P = 528 \cdot C_i^{-1}, \quad (2)$$

which is based on data compiled by Dwyer [38]. The potential for yield improvement at the bioreaction stage is usually high. Major yield enhancements have been fairly commonly achieved by strain selection, medium development, optimization of feeding strategies, and environmental controls.

Table 1-5. Typical concentration of various products in raw fermentation broth.

Product	Final concentration ($kg\ m^{-3}$)
Vitamin B_{12}	0.06
Monoclonal antibodies	0.1–0.5
Riboflavin	0.1–7
Antibiotics	0.2–35
Gibberelic acid	1–2
Amino acids	2–100
Yeast	30–60

1.4.3 Combined Fermentation–Recovery Schemes

In keeping with a global approach to process improvement or intensification, schemes that combine the bioreaction stage and parts of downstream processing are potentially attractive [32]. Such schemes include extractive fermentations, fermentationa–distillation, perfusion culture using membranes, inclined settlers or 'spinfilters' to retain the cells in the bioreactor, fermentation–adsorption using chromatographic media, as well as other methods. Combining fermentation and recovery not only reduces the number of individual processing steps, but the productivity of the fermentation may also be substantially enhanced by eliminating or reducing the inhibitory effects of certain products.

A novel scheme for retaining particles, particularly animal cells, in perfusion bioreactors relies on standing sound waves applied perpendicular to a vertically aligned harvest flow channel [39–41]. The sound waves concentrate the suspended cells in bands aligned with the flow [42,43]. Gravity sediments such aggregated particles against the flow once the sound is switched off; hence, a clarified liquor leaves the flow channel whereas the solids are concentrated in the feed vessel. This type of separation in ultrasonic flow fields provides an effective means of retaining cells in continuous flow bioreactors. This technique allows easy maintenance of sterility as no mechanical items penetrate the sedimentation chamber. Moreover, there is nothing to clog, foul, or breakdown. Process-scale implementation of this method is being developed.

1.5 Initial Separations and Concentration

The first few processing operations in a purification train are aimed at volume reduction to minimize processing costs by reducing the size of the downstream machinery. Removal of suspended material and substances which might interfere with further downstream operations are additional requirements of some of the early separation steps. Further, because viscous broths are difficult to handle, viscosity reduction should be achieved as early as possible to simplify pumping, mixing, filtration, sedimentation, etc. Removal of suspended solids, digestion of carbohydrates, or removal of nucleic acids are some of the operations that may be needed to improve broth handling.

Typically, solid–liquid separation would be among the first processing steps for extracellular as well as intracellular products. For the latter, solid–liquid separations are usually a means of concentration of the biomass, or removal of the suspending culture fluid prior to disruption or other downstream treatment. Cell or other solid product washing operations often employ solid–liquid separation steps. The commonly used methods of solid–liquid separation are filtration and centrifugation. Centrifuges are used also to separate difficult to break emulsions and other liquid–liquid systems. Some examples are recovery of cream from milk, recovery of oil drops, fats (e.g. in rendering and meat processing plants) and waxes, and liquid–liquid extraction.

Table 1-6. Types and applications of centrifuges [1,19].

Tubular bowl. Tubular bowl machines are capable of high *g*-forces, usually up to 20 000 *g* in industrial devices. Solids accumulate in the bowl and must be removed manually at the end of operation. Bowl capacity limits solids-holding capability. To ensure sufficient interval between bowl cleaning, the solids concentration in the feed should usually be ≤ 1 % volume/volume; higher concentrations can be processed with smaller batches, for example, in production of certain vaccines. Good dewatering of solids is obtained.

Multichamber bowl. Similar to tubular bowl machines. Division of bowl into multiple chambers increases solids-holding capacity. Solids must be discharged manually; hence, economic operation is feasible only with feeds with low concentration of solids. Good for polishing of otherwise clarified liquors. Capable of high *g*-forces. Gradation of *g*-forces from inner to outer chamber. Smallest particles sediment in the outermost chamber. Good dewatering of solids.

Disc-stack. Lower *g*-forces than tubular bowl machines. Solids may be retained, or discharged intermittently or continuously by various mechanisms (e.g., periodic ejection of solids by hydraulic separation of upper and lower parts of the bowl; nozzle discharge under pressure; valves; etc.). Not all discharge methods are suitable for all solids. Solids must flow. Poor dewatering. Not suited for mycelial solids; good for slurries of yeasts and certain bacteria. Depending on the mechanism of solids discharge, may handle feeds with up to 30 % (v/v) solids.

Scroll discharge. Scroll discharge decanter centrifuges are suitable for slurries with high concentration of relatively large, dense solids. Feed solids concentrations of 5–80 % (v/v) can be handled. Solids are discharged continuously. The *g*-forces are low. Suitable for fungal broths and dewatering of sewage sludge.

Perforated bowl or basket centrifuges. Also known as filtering centrifuges. Useful for low-*g* recovery of relatively large, mostly crystalline solids. The perforated bowl is lined with filter cloth to retain solids, whereas the liquid passes through. Sedimented cake may be washed and recovered as fairly dry material. Not effective for particles below 5 µm, and loadings < 5 % (v/v) [44].

Solid–liquid separations can be implemented in a variety of ways that are best suited to particular applications. Thus, as detailed in Table 1-6, many different designs of centrifuges are available [1,44]. Similarly, filtration may be performed in conventional filter presses, horizontal and vertical leaf-type pressure filters, rotary drum pressure or vacuum filters with or without filter aid (or body feed or admix) and using different means of solids discharge. Production scale rotary drum filters tend to be quite large: 0.9–4.3 m drum diameter and up to 6 m drum width. Sterile operation is usually not feasible, and containment is difficult. Alternatively, solids may be recovered by membrane filtration either in dead end (e.g., in many filter sterilizations) or cross-flow modes; the latter may be implemented in flat plate, hollow fiber or spiral wound static membrane cartridges, as well as in dynamic modes [1]. While the variety of available options helps to ensure that specific needs are met, careful consideration of the problem at hand is required for selection of the optimal processing method. Alternatives should be considered whenever possible. For example, rotary drum filters with string discharge usually perform well in separating mycelial solids from penicillin broths, but this discharge mechanism, without filter aids, causes problems with broths of *Streptomycetes* and other bacteria [19]. Precoat drum filtration may be used with bacterial broths when biomass is not the desired

product. A knife blade (or doctor blade) discharge mechanism is used to continuously remove the deposited solids along with a thin layer of the precoat. Knife discharge without precoat or filter aids is suitable for recovering yeast from the filter cloth on drum filters; however, knife blades are not suited to cleanly cutting away a layer of deposited mycelial fungi because of the stringy nature of solids. Similarly, because of the concentration and the morphology of the solids, the disc stack centrifuge is not suitable for fungal fermentation broths, but properly selected scroll discharge machines are effective. Leaf filters are generally batch devices that are inexpensive to install, but labor-intensive to operate. Leaf filters are suitable for broths with little solids, e.g., in polishing of beer [19]. Gravity sedimentation may be employed as a volume reduction step prior to removal of solids by other means, but sedimentation by itself is not common for biomass removal in processing of high value products. Gravity sedimentation in thickeners and clarifiers [4,5] is encountered widely in sludge recovery in biological wastewater treatment. Certain solids may be recovered using hydrocyclones, but this method is little used in bioprocessing.

When more than one processing option its technically feasible, evaluations of the economics of use in terms of capital expenditure on equipment and its operating costs (processing time, yields, labor, cleaning, maintenance, analytical support) is necessary for optimal process selection. Economic evaluations should be performed over the expected lifetime of the equipment [19]. For example, for separation of solids from fermentation broth, centrifugation and microfiltration may be two competing alternatives [1]. In still other applications, for example when very fragile cells are to be separated from suspending liquid, centrifugation may not be an option.

Some other concentration steps, applicable to products in solution, are precipitation [1,45], adsorption, chromatography [14], evaporation, pervaporation [7] and ultrafiltration [1]. Some of these operations are equally capable purification steps (e.g., chromatographic separations). Certain steps (e.g., some chromatographic separations; membrane separations) may require a relatively clean process stream, free of debris, lipids or micelles which may cause fouling of the equipment. Such steps are often used downstream of steps which can handle cruder material [19].

Sometimes the characteristics of fermentation broth or process liquor may be modified by pretreatment to enable processing by a certain method. Major changes in processing characteristics may be achieved by pH and/or temperature treatment, use of additives such as polyelectrolytes, other flocculants and enzymes, and changes in ionic strength [19]. Flocculants (e.g., alum, calcium and iron salts, tannic acid, quaternary ammonium salts, polyacrylamide) can enhance sedimentation rates by thousands of fold relative to unflocculated suspension. Aging of protein precipitates and crystals can substantially improve filtration and sedimentation. Addition of salts is sometimes helpful in dewatering difficult to dewater solids such as protein precipitates. Water is drawn out of the pores of the solid into the salt containing liquid film on the outside. Osmosis or chemical potential difference drives the flow. Among other factors, time of harvest can beneficially alter processing behavior of the broth as well as the stability of the labile product. Culture conditions and methodology influence microbial morphology, product formation and downstream recovery. For example, cells grown in defined media are generally easier to disrupt

than ones cultured in complex media [16]. Also, high specific growth rates produce less robust cells.

1.6 Intracellular Products

In general, a biological product is either secreted into the extracellular environment, or it is retained intracellularly. In comparison with the total amount of biochemicals produced by the cell, very little material is usually secreted to the outside; however, this selective secretion is itself a purification step which simplifies the task of the biochemical engineer. Extracellular products, being in a less complex mixture, are relatively easy to recover. On the other hand, because a greater quantity and variety of biochemicals are retained within cells, intracellular substances are bound to eventually become a major source of bioproducts [16]. Among some of the newer intracellular products are recombinant proteins produced as dense inclusion bodies in bacteria and yeasts. Recovery of intracellular products is more expensive as it requires such additional processing as cell disruption [16–18], lysis [16], permeabilization [46], or extraction. Intracellular polymers such as poly-β-hydroxybutyrate (PHB) may be recovered either by cell disruption [17,37] or solvent extraction. In principle, selective release of the desired intracellular products is possible, but in practice it is neither easily achieved nor sufficiently selective. Hence, the desired product must be purified from a relatively complex mixture, complicating processing and adding to the cost [19]. Nevertheless, an increasing number of intracellular products are in production. Economics of production may be improved by recovering several products (intracellular and extracellular) from the same fermentation batch [21].

As for other separations, many options exist for the disruption of cells (Table 1-7). Of these, high-pressure homogenization is apparently the most suitable for bacterial broths, whereas bead mills are more widely used for fungal cultures [1,16]. For dissolved products, cell disruption conditions (e.g., pressure, number of passes) must be selected to prevent excessive micronization of debris because micronization complicates solid–liquid separation further downstream [16]. However, when the product is an intracellular solid that is undamaged by homogenization, micronization of debris actually favors product recovery. This strategy is useful with protein inclusion bodies, certain cellular organelles, and sometimes with granules of bioplastics such as polyhydroxyalkanoates. Nonetheless, overzealous disruption conditions should be avoided in view of the recently published evidence that suggests loss of intracellular solids by micronization [37].

Disruption of bacterial cells releases large amounts of nucleic acids which increase the viscosity of the broth, often producing viscoelastic behavior. To ease further purification, the nucleic acids are usually removed by precipitation (e.g., with manganous sulfate, streptomycin or polyethyleneimine) [1]; alternatively, viscosity may be reduced by enzymatic digestion of nucleic acids or high-shear processing in high-pressure homogenizers [19]. Another alternative for eliminating nucleic acid polymers is heat shock treatment prior to disrupting the cells. Heat shock treatment

Table 1-7. Cell disruption options [16–18].

High-pressure homogenization. Frequently used for large-scale disruption of yeasts and non-filamentous bacteria. Generally not suitable for mycelial broths. Broth must be free of large suspended solids, tight cell clumps and flocs. Maximum acceptable particle size is about 20 μm, but a lower size is preferred. Slurry viscosity should not normally exceed 1 Pa s [1,16]. Optimal viscosity and solids concentration ranges are narrower than for bead mills.
Bead milling. Bead mills come in vertical and horizontal configurations with different mechanisms for retention of grinding media, and different types of agitators. Agitators that reduce back-mixing are preferred. Vertical mills are susceptible to fluidization and accompanying loss in performance. Typically three to six passes should achieve complete disruption. Useful for yeasts, mycelial fungi, algae; less efficient with bacteria. Grinding bead size affects disruption. Smaller the microbial cell, smaller the optimal bead size [16,33].
Autolysis. Under suitable conditions certain cultures would autolyse in the stationary phase upon completion of fermentation. Baker's yeast can autolyse.
Osmotic shock. Useful for animal cells and in specific cases for bacteria. Large dilutions may be necessary.
Thermolysis. Sufficiently heat-stable products may be released by heat shocking the cells. Microbial susceptibility to heat shock treatment varies widely. Monvalent metal ions such as Na^+ and K^+ may aid thermolysis. Suited to specific cases.
Enzymes and chemicals. Detergents, EDTA, solvents (e.g., toluene), antibiotics, and lytic enzymes may be used. Sometimes enzymes and chemical additives are used in combination with homogenization or bead milling to reduce the severity of mechanical treatment. Treatment with acids and alkalis may be useful in specific cases. Especially useful for extraction from periplasm.
Others. Ultrasonication, desiccation, freeze–thaw, extrusion of frozen paste. Applicable only to laboratory scale.

would typically require rapid heating to at least 64 °C and a holding time of 20–30 minutes. This treatment should digest almost all DNA/RNA. Shorter holding times may be satisfactory if complete degradation is not necessary for processability. Rapid temperature rise preferentially destroys proteases relative to RNA-hydrolyzing enzymes. Thermal treatment may be feasible for heat stable products [37] as well as for those produced as denatured inclusion bodies.

Processing considerations relevant to some specific bioseparations are discussed in the following section.

1.7 Some Specific Bioseparations

1.7.1 Precipitation

Proteins are easily concentrated by precipitation with organic solvents (e.g., ethanol, acetone), polymers (e.g., poly(ethylene glycol), poly(propylene glycol), dextran),

and salts. Fractional precipitation allows for a degree of separation [1]. Fractionation with ammonium sulfate is commonly used. Organic solvents produce a denaturing environment making low temperature processing necessary [1]. Alcohol precipitation is frequently used in recovering biologically inactive dissolved polymers such as polysaccharides. Examples include precipitation of xanthan and gellan with isopropanol. Precipitation methods can handle large amounts of crude material, are easily scaled up, and can be implemented in continuous processing modes [1,47]. However, precipitation is generally not useful for recovery from very dilute animal cell culture fluids. Ammonium sulfate precipitation for recovery of recombinant β-galactosidase from *S. cerevisiae* has been detailed by Zhang et al. [47].

1.7.2 Foam Fractionation

Foam fractionation, microflotation or froth flotation is potentially useful for concentrating particles (cells, organelles, other small solids such as granules of PHB) and proteins into a foam phase for further recovery. The technique involves gentle bubbling of air (or other inert gas) at the base of a column of broth or solution. Hydrophobic solids and surface active molecules accumulate at the gas–liquid interface and rise with the bubbles. Collector surfactants and other promotors are often added to improve attachment. Additives such frothing agents and stabilizers may be necessary. Enrichment in the foam depends on physical collection efficiency of bubbles (i.e., on bubble size, hydrodynamics, bubbling rate, concentration of particles) and adsorption chemistry. Empirical investigation is essential for selecting suitable additives, concentrations, hydrodynamic regimes, and for assessing performance, including recovery from the foam phase. Culture conditions may be used to influence adsorption behavior. Froth flotation is encountered only occasionally in bioprocessing. Potentially, fermenters used in batch cultivation could subsequently be employed for froth flotation. Airlift bioreactors with gas–liquid separators [48] and added means of skimming the gas-floated biomass are used in activated sludge treatment of wastewater. Part of the harvested sludge is returned to the reactor as inoculum.

1.7.3 Solvent Extraction

Rapid solvent extraction can be carried out in centrifugal extractors such as the Podbielniak and the Alfa Laval machines that are commonly used in antibiotics processing [1,13]. These devices were originally designed to handle solids-free liquids, but have been adapted to media containing limited amounts of small particles. Other more conventional extractors are banks of mixer–settlers, York–Scheibel column (suitable for solids-free liquids), and the reciprocating plate Karr column (suitable for whole broths). Supercritical extraction of solids and liquids with carbon dioxide or other solvents (e.g., pentane) may be useful for small organic solutes. In these

cases a concentrated solute is obtained easily by boiling off the solvent. Recently, serum albumin has been extracted into aqueous reverse micelles formed in carbon dioxide using a perfluoropolyether surfactant [49]. This opens up new opportunities for purification of proteins and other large molecules.

1.7.4 Aqueous Liquid–Liquid Extraction and its Variants

Conventional liquid–liquid extraction based on partitioning between an aqueous phase and a water-immiscible organic solvent is not suitable for proteins and protein-based cellular organelles because of low protein stability in organic solvents. A suitable alternative is partitioning between two immiscible aqueous phases [1,8,9]. Such phases are obtained by adding two incompatible polymers – for example, poly(ethylene glycol) and dextran – to water, or by mixing a relatively hydrophobic polymer solution with salts. Examples of such systems are aqueous mixtures of PEG-PVA, PPG-dextran, PPG-potassium phosphate, PEG-ammonium sulfate, as well as others. Partitioning of solutes is brought about by differences in net charge and hydrophobicity. Higher-polarity molecules solubilize preferentially in the salt-rich phase, whereas the relatively hydrophobic molecules concentrate in the polymer-rich phase. Polymers with attached affinity ligands – hydrophobic and ionizable functional groups – can improve partitioning behavior. Partitioning is strongly affected by pH, composition and type of phases (e.g., molecular weight of polymer, ionic strength, salt, polymer). In addition, the volume ratio of the phase mixture to that of the protein solution should be such that neither phase approaches saturation with protein. Aqueous two-phase systems have been successfully employed for enrichment of proteins, cells, organelles, and small molecules. Proteins that extract into the polymer phase are back extracted into the salt phase for recovery. Phase separation can be slow because of high viscosity and small density differences. Gravity separation is generally satisfactory for PEG-salt systems, but centrifugal separation may be necessary for PEG-dextran. Aqueous two-phase extraction is commercially employed, but it is relatively uncommon.

Among relatively new developments in liquid–liquid extraction is reversed miceller extraction [12] also known as liquid membrane emulsion extraction. Reversed micelles are surfactant stabilized microdroplets of an aqueous phase suspended in a water-immiscible solvent. Contacting the reversed micelle-laden organic phase with an aqueous mixture of proteins or other solutes results in preferential transfer of one or more species from the aqueous phase to the organic phase, and from there to the aqueous core of the reversed micelles. The intervening organic phase constitutes a liquid 'membrane.' Extraction is influenced by pH and ionic strength of the bulk aqueous phase, and the nature of the reversed miceller core. Usually, a protein solubilizes in the reverse miceller phase at pH values below its isoelectric pH when the ionic strength is low. Once a component has been extracted, reversed micelles can be back-extracted with buffers to yield a solution rich in the desired substance. Back-extraction is favored by altering the pH and ionic strength. Factors such as hydrophobicity of the protein also contribute to partitioning behavior.

A variation of the liquid membrane emulsion extraction is the supported liquid membrane extraction [11; see also Volume 1, Chapter 11]. No stabilizing surfactant is necessary in this case; instead, the liquid membrane-forming organic phase is supported in the pores of a porous solid that separates the two aqueous phases. Additives may be employed to enhance mass transfer through the organic phase [11]. Reversed micelles and liquid membranes are not widely used at present.

1.7.5 Membrane Separations

Cross-flow membrane filtration flux typically ranges over 10–120 $L \cdot m^{-2} \cdot h^{-1}$; the exact value depends on the membrane pore size and the viscosity of the suspending fluid. Microfiltration of animal cells and microbial homogenates is done best at transmembrane pressures less than 1.38×10^4 Pa. Higher pressures, typically 6.9–34.5 $\times 10^4$ Pa, are used in recovering microbial cells. Because of the small pore size, ultrafiltration membranes invariably require high transmembrane pressures (13.8–27.6 $\times 10^4$ Pa) for reasonable flux.

Polymer membranes predominate in bioprocessing, but ceramic and sintered metal membranes are used occasionally. Hydrophilic membranes are preferred for liquids. Hydrophobic polymer membranes are easily fouled by silicone antifoams which may cause as much as 50 % decline in flux. Low-molecular weight poly(propylene glycol) or poly(ethylene glycol) based antifoams are usually better. Mechanical foam control [24,50] during fermentation is sometimes helpful in eliminating or reducing antifoam consumption.

Even without antifoams, membrane performance deteriorates over time, making periodic replacement necessary. Prior experience or experimentation are the only reliable predictors of membrane life [6]. Membranes are not easily cleaned; detectable residues of bioactive material may remain after any reasonable cleaning. Such situations require product-dedicated filters to prevent cross-contamination. Furthermore, polymer-based membrane filters cannot usually be heat sterilized; chemical sanitization and atmospheric steaming are the only options. Chemical cleaning, sanitization, and steaming lower membrane life; hence the choice of chemicals and cleaning conditions need to be carefully assessed.

The major costs associated with ultrafiltration and microfiltration are the initial capital expense and the cost of membrane replacement; energy is not a major expense. The frequency of membrane replacement determines feasibility of membrane separations. In contrast, in reverse osmosis where the high transmembrane pressure is unavoidable, pumping expense and membrane replacement costs are major contributors to operating costs. As with centrifuges, membrane filter selection requires experimental evaluations [1,6].

Even in cross-flow operation, membrane filters experience performance loss due to concentration polarization or accumulation of a solute layer at the surface of the membrane. Small amounts of relatively large, dense inert solids such as cellulose fibers or polymer beads added to the feed are known to reduce concentration polarization by disturbing the fluid boundary layer on the membrane surface. Cross-flow

channels are sometimes also inserted with static turbulence enhancers such as wire screens, but such filter modules are not suitable for mycelial or filamentous biomass especially at high concentration of solids. Mechanical methods of increasing turbulence are employed in dynamic filters, but few such devices have gained any commercial acceptance. One dynamic configuration utilized two porous concentric cylinders with microfiltration membranes supported on the surfaces of the annulus. The inner cylinder rotated at high speed; differences in angular velocities of the fluid elements along the width of the annular gap produced Taylor vorticés that substantially enhanced filtrate flux relative to static cross-flow operation [51]. Nonetheless, limited scale-up potential prevented further development. A variation on the concentric cylinder theme has recently been introduced by Pall Filters. This design consists of a stack of supported circular microfilter membranes with mechanically agitated circular steel discs mounted inbetween. Rotation of discs dramatically enhances filtrate flux [52]. The stack supports up to 1.5 m^2 membrane surface, but this may be substantially increased in future designs simply by increasing the overall height of the stack. The device is suited to recovering yeasts and non-filamentous bacteria from relatively less viscous broths.

Membrane filters are used also in the diafiltration mode for buffer exchange, washing of solids, desalting, and removing other small molecules from solution of macromolecules.

Pervaporation is another membrane separation that is particularly useful for low energy recovery of relatively volatile liquids (e.g., ethanol) from fermentation broths [7]. Permselective membranes separating the broth from a vapor phase allow only selective permeation of the desired solvent to the other side, where hot air or heat supplied to the membrane continuously evaporates the solvent, hence maintaining a mass transfer driving force. Membrane chemistry determines permselectivity.

1.7.6 Electrically Enhanced Bioseparations

Electric fields may be used to enhance bioseparations [53,54], but commercial use is limited at present because of the damaging effects of ohmic heating that accompanies current flow. Electrolysis can be another problem. Nevertheless, electrokinetic forces on charged particles have been demonstrated to reduce concentration polarization and membrane fouling during microfiltration and ultrafiltration, thereby enhancing filtration rates [53]. Up to sevenfold enhancement of transmembrane flow has been recorded during microfiltration with direct current (DC) electric field strengths of 100–120 V cm^{-1} [53]. Some of the problems associated with electric fields may be reduced by replacing the steady DC fields with pulsed direct current fields [53]. Electric discharges have been used also to break foams instantaneously during processing.

The separation potential of electric fields is best illustrated by electrophoresis, which is a well-established extremely high resolution method for separation of proteins. Differences in molecular charge and weight are the bases of separation. However, despite attempts to scale-up [55], electrophoresis remains confined mostly to

laboratory use. Except for small volumes, rapid removal of heat generated has proven difficult without convective mixing that would destroy any separation.

1.7.7 Chromatographic Separations

Enhancing speed has been a major preoccupation with chromatographic processing. Except for bed height dependent gel permeation, the speed of most chromatographic processes can be enhanced by replacing the usual high-resistance packed vertical columns with radial flow devices [55]. Adsorption media used in conventional columns can still be utilized, but the medium is packed in the annulus between two porous concentric cylinders. Radial flow columns attain 10- to 50-fold greater flow rates than conventional columns [55]. Industrial-scale simulated moving bed chromatographic systems are now available [56].

Among other improvements, better, more rigid yet porous chromatographic media that are less susceptible to bed compression have been developed [14]. Other novel media have enabled extremely high speed or perfusion chromatography. Unlike conventional media, perfusion media contain throughpores for bulk flow of fluid through the particle. Diffusional pores as in conventional media are also present. Throughpores allow high flow rates – up to 100-fold greater than in diffusive media [55]. Resulting convection within the particles reduces diffusive transport limitations.

Another high-rate chromatographic system is expanded or fluidized bed chromatography. The medium bed is expanded or fluidized during loading by upflow of unclarified fermentation fluid or cell homogenate [55]. There is little pressure drop through the expanded bed. Plug flow of fluid is desired and easily attained. After adsorption, the microbial solids are washed away by upflow of water or buffer. The adsorbed product is recovered as in conventional chromatography by downward elution of settled, packed bed. Because this method handles unclarified fluids, some solid-liquid separation steps are eliminated. Fluid bed chromatography has been demonstrated with numerous fluids including broths *E. coli,* yeast, mammalian cells [55], autolysed yeast, and blood plasma.

A further rapid chromatographic method that may potentially handle solids-laden fluids is membrane chromatography. This technique employs ion exchange groups or other high-specificity adsorption ligands attached to inner surfaces of pores of conventional microfiltration membranes. Rapid flow through pores reduces diffusion limitations, hence speeding adsorption, and, later, desorption. Hollow fiber membrane modules that allow compact packing of large membrane areas have been used for membrane chromatography [57].

Some especially high-resolution chromatographic separations include HPLC and bioaffinity-based methods. Process-scale HPLC continues to be useful for small batches [38], but this method is expensive, slow, and the high-pressure columns appear to have reached an upper limit of about 0.3 m diameter and 2.4–3.0 m height. Bioaffinity chromatography with affinity ligands – receptors, antibodies, enzymes, and other active proteins – immobilized onto the support media has been used for

quite some time, but it remains expensive. Other problems are often poor stability of the affinity matrix, and ligand leakage into the product (see also Volume 1, Chapter 17). With few exceptions (e.g., protein A affinity columns can be cleaned with the strong denaturant guanidine hydrochloride (6 M) which solubilizes adsorbed proteins without affecting the ligand), ligand stability limits the column cleaning regimen. Because of those factors, a trend toward replacing labile bioaffinity ligands with inexpensive and robust alternatives (e.g., dyes, metal ions) is apparent.

Note that some of the speed-enhancing techniques used with chromatography are equally applicable to non-chromatographic adsorptions. Adsorption using columns or slurries of activated carbon is commonly encountered in bioprocessing, particularly for removing pigments.

1.8 Recombinant and other Proteins

Many of the newer recombinant biotechnology products are proteins [30,58]. While the general features of a bioseparation scheme for these products are the same as for other proteins, there are some unique constraints. Genetically modified microorganisms and cells of higher life forms are often more fragile than the corresponding wild strains [59,60]. This has implications for the design of cell–liquid separation stages. Also, recombinant proteins formed in bacteria and yeasts frequently precipitate inside the cell as dense, insoluble, denatured inclusion bodies. In this form proteins which may otherwise be toxic to the cell may be overproduced and remain protected against proteolytic activity within the cell.

Most bacteria and fungi used in producing recombinant proteins also produce a variety of proteases that may degrade some of the desired protein within the cell and during recovery, soluble, non-inclusion body proteins being particularly susceptible to degradation. Degradation by acid proteases with a pH optimum of 2–4 may be minimized by processing at higher pH and low temperatures. Neutral proteases are not particularly thermostable and may be inactivated by heating to 60–70 °C for 10 minutes [19]. Many proteases are metalloproteins and require a divalent metal ion for proteolytic activity; chelating agents such as ethylenediaminetetraacetic acid (EDTA) or citric acid may be used to inactivate such proteases by binding the metal ions. Alkaline proteases of *Bacillus* sp., such as subtilisin, contain serine at the active site and are not affected by EDTA, but are inhibited by diisopropylfluorophosphate. The short-lived reagent phenylmethylsulfonyl fluoride protects against serine proteases. Antioxidants such as vitamin E and ascorbic acid protect against oxidation [19].

Proteins tend to be more stable in concentrated solutions. Addition of poly(ethylene glycols) and other proteins such as albumins may have a stabilizing effect. Glycerol, sucrose, glucose, lactose, and sorbitol are often used as stabilizers in concentrations of 1–30 %. Enzyme substrates usually have an stabilizing effect, as do high concentration of salts such as ammonium sulfate and potassium phosphate. Metalloproteins may be stabilized by addition of metal salts. Divalent metal ions such as Ca^{2+}, Cd^{2+}, Mn^{2+} and Zn^{2+} stabilize various enzymes [19].

Some commonly used sequences of protein purification methods have been outlined by Bonnerjea et al. [45] and by Wheelwright [3]. Chromatographic procedures are indispensable to producing high-purity proteins. Typically, the mean recovery or yield of separation steps such as those listed in Table 1-1 is ~ 60–80 % [45]. Average and high values of purification factors associated with some protein purification operations are shown in Table 1-8 which is based on data compiled by Bonnerjea et al. [45]. Clearly, affinity chromatography far outperforms other methods, but compared with operations such as ion-exchange chromatography, the scope for further improving performance is small because many affinity separations already operate close to theoretical maximum [45].

Changes in processing volume, product yield, and total and specific activities occur during processing as illustrated in Table 1-9 for a relatively simple purification of brain tumor plasminogen activator (PA) from supernatants of cultured, anchorage dependent rat cells [61]. The purification in Table 1-9 was done at 4 °C. The serum-free conditioned medium used for recovery had an initial plasminogen activator activity of only 9 IU mL^{-1} [61]. Zinc chelate-agarose chromatography was used as the first concentration/purification step. The culture fluid (6 L) was applied to the column (5 × 8 cm) at a flow rate of 200 mL h^{-1}. The column was washed with Tris–HCI buffer (0.02 M, pH 7.5, 1 L) that contained 1 M sodium chloride, aprotinin and Tween-80 (0.01 %, vol/vol). Aprotinin, a protease inhibitor, and Tween-80 (poly(oxyethylene sorbitane monooleate)), a surfactant, are generally added at all stages of PA processing to, respectively, suppress proteolysis and overcome the surface adherent tendency of plasminogen activators [30]. After the wash, the column was eluted with a linear gradient of imidazole (0–0.05 M) in the wash buffer (1 L, 120 mL h^{-1}). Pooled PA fractions were further purified on a concanavalin A-agarose affinity chromatography column. Dialysis was used to concentrate the pooled fractions, and a final gel filtration step (Sephadex G-150 superfine) was employed. The overall yield was 39 % [61]. This figure is fairly typical of large-scale protein

Table 1-8. Approximate values of purification factors observed during protein purifications. Based on Bonnerjea et al. [45].

Operation	Purification factor	
	Average	High
Affinity chromatography	100	3000
Dye–ligand affinity	17	–
Inorganic adsorption	12	100
Size-exclusion chromatography	6	100
Hydrophobic interaction chromatography	15	60
Ion-exchange chromatography	8	50
Detergent extraction	4	12
Precipitation	3	12

Table 1-9. Purification of tumor plasminogen activator [61].

	Volume (mL)	Total protein (mg)	Total activity (IU)	Volumetric activity ($IU\,mL^{-1}$)	Specific activity ($IU\,mg^{-1}$)	Yield (%)	Purification factor
Clarified medium	6000	270	53 000	8.8	196	100	1
Zinc chelate-agarose	100	138	50 000	500	362	94	1.9
Concanavalin A-agarose	52	2.4	19 400	373	22 750	37	116
Gel filtration	7.5	0.53	20 800	2773	39 000	39	199

recovery. For example, overall recoveries of 23–47 % were noted for a variety of processes (e.g., recombinant BST, recombinant human α-interferon, L-leucine dehydrogenase for use in chiral syntheses) reviewed by Wheelwright [3]. One exception was a somewhat impractical process for tissue-type plasminogen activator (tPA) for which the overall yield was only 6 % [3]. Other methods for large-scale tPA recovery have been presented by Rouf et al. [30].

1.8.1 Inclusion Body Proteins

When possible, production of recombinant proteins as inclusion bodies has important advantages. Some proteins that form inclusion bodies are listed in Table 1-10. Inclusion bodies are easy to isolate, highly concentrated forms of the desired recombinant protein. Typically, inclusion bodies are spheroidal particles, $0.2–2.0 \times 10^{-6}$ m in diameter and 1100–1300 kg m^{-3} density. The sequence of steps in recovery of inclusion body proteins is cell disruption, centrifugal separation of the inclusion body, washing, solubilization of the protein, and renaturation [19]. Cell disruption by homogenization is the preferred technique in large-scale processing. Disruption by high-pressure homogenization has been detailed by Chisti and Moo-Young [16]. Inclusion bodies are not affected by homogenization. Cell homogenates are centrifuged to sediment the dense inclusion body fraction. Centrifugation at 1000–12 000 *g* for 3–5 minutes is sufficient. Sedimentation of cell debris can be minimized by

Table 1-10. Some proteins produced as inclusion bodies [19].

Bovine pancreatic ribonuclease	Human interleukin-2	Lysozyme
Bovine somatotropin (BST)	Human interleukin-4	Porcine phospholipase
Epidermal growth factor	Human macrophage-colony stimulating factor	Prochymosin
Human insulin	Human serum albumin	Pro-urokinase
Human γ-interferon	Immunoglobulins	Tissue-type plasminogen activator

increasing the density and viscosity of the homogenate with additives such as 30 % sucrose or 50 % glycerol. The inclusion body fraction is washed with buffers containing 1 M sucrose, 1–5 % Triton X-100 surfactant [62] and, in some cases, low concentrations of proteolytic enzymes and denaturants. The wash steps remove soluble contaminants, membrane proteins, lipids and nucleic acids. At this stage the remaining solids fraction is > 90 % recombinant protein. The protein solids are solubilized in highly denaturing chaotropic media. Typically, 6–8 M guanidine hydrochloride or 8 M urea are used for solubilization at pH 8–9, 25–37 °C for 1–2 h [62]. Reducing agents are added to the solubilization media to break any inter- and intra-molecular disulfide bonds to fully solubilize the protein. Some reducing agents are 2-mercaptoethanol, dithiothreitol, dithioerythritol, glutathione, and 3-mercaptopropionate. Some typical concentrations are 0.1 M 2-mercaptoethanol, or 10 mM dithiothreitol [62]. The latter has a shorter half-life than 2-mercaptoethanol, but does not have the odor of 2-mercaptoethanol. Stability of thiol compounds in solution is dependent on pH, temperature, and the presence of metal ions such as Cu^{2+}, which lower stability, and of stability enhancers such as EDTA. Good yields of some proteins can be obtained by solubilization without the reducing reagents, but for others reducing agents are essential. Of the denaturants, guanidine hydrochloride is preferable to urea, which may contain cyanate causing carbamylation of the free amino groups on the protein, particularly during long incubation periods in alkaline environments. Note though, that for some proteins, one denaturant may produce significantly higher overall yield than if solubilization with the other is used [19]. Performance has to be empirically evaluated.

For refolding of solubilized protein into active entities, concentration of the denaturant and the reducing agent are reduced by dilution with a refolding buffer. Denaturants can be completely removed by ultrafiltration with addition of renaturing buffer, dialysis, or gel filtration. Renaturation from concentrated protein solutions produces lower yields of the active protein because of intermolecular aggregation in these solutions. Thus, renaturation is done at low protein concentrations, typically $1–20 \times 10^{-3}$ kg m^{-3} protein [62]. Yield of the active protein is enhanced by refolding in the presence of small, non-denaturing amounts (1–2 M) of urea or guanidine hydrochloride [62]. Presence of high-molecular weight polymers such as poly(ethylene glycol) may also improve yield [19].

During refolding, formation of the disulfide bonds is achieved in one of three ways. The air oxidation method uses dissolved oxygen for oxidation of the cystine residues. The refolding buffer containing solubilized protein is aerated or exposed to atmosphere. Oxidation is accelerated by Cu^{2+} ions at approximately 10^{-6} M. Typical reaction conditions are pH 8–9, 4–37 °C for up to 24 h [62]. Traces of 2-mercaptoethanol may enhance yield. Air oxidation is difficult to control [19].

The glutathione reoxidation method typically uses a 10:1 mixture of reduced and oxidized forms of glutathione at a concentration of 10^{-3} M reduced glutathione [62]. Air oxidation is suppressed by using deaerated buffers held under a nitrogen atmosphere. The ratio of the reduced and oxidized forms of glutathione, the ratio of the glutathione and the cystine residues on the protein, the reoxidation temperature (4–37 °C) and time (1–150 h) provide flexibility to this method [62]. Low-molecular weight thiols other than glutathione may also be used. The third method of disulfide

bond formation, the mixed disulfide interchange technique, has been detailed by Fischer [62].

The inclusion body production stage should be optimized to rapidly form relatively pure, large and dense inclusion bodies which are easy to recover and solubilize. Production of proteolytic activity should be suppressed as far as possible. Purification and concentration are greatly simplified because of the already high starting protein concentration and purity in the inclusion bodies which are easy to separate from the bulk of the soluble proteins by centrifugation. The recovery of active protein from inclusion bodies is variable, but can approach 100%. In general smaller polypeptides are easier to refold into active forms [19]. Because of added processing, and the need to refold in dilute solutions, inclusion body-produced proteins tend to be expensive. With certain proteins such as tPA production as an inclusion body in bacteria is technically feasible but is not competitive with animal cell culture-derived product [30], even though the latter is a fairly expensive production method.

1.9 Conclusions

The variety of bioseparations is vast, but usually a small selection of the available methods is sufficient to achieve the requisite purity. The aim always is to employ the fewest possible process steps consistent with the product quality specifications. In-depth knowledge of individual separations must be combined with insight into the bioreaction step to design an efficient, consistent and integrated overall production process. Whereas the scientific understanding of bioseparations continues to improve and several new capable separations have been introduced, downstream processing of biologicals remains an empirical art. Invariably, experimentation must be relied upon to aid process selection, implementation, and scale-up.

Abbreviations and Symbols

BST	bovine somatotropin
C	number of components
C_i	concentration of product in broth or starting material, kg m^{-3}
DC	direct current
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
g	gravitational acceleration, m s^{-2}
GMP	Good Manufacturing Practice
HIV	human immunodeficiency virus
LAL	Limulus amoebocyte lysate
n	number of steps
N_f	number of possible flowsheets
P	selling price, U.S. $\$_{1984}$ kg^{-1}

PA	plasminogen activator
PEG	poly(ethylene glycol)
PHB	poly-β-hydroxybutyrate
PPG	poly(propylene glycol)
PVA	poly(vinyl alcohol)
RNA	ribonucleic acids
S	number of separation operations
SDS	sodium dodecyl sulfate
x	step yield

References

[1] Chisti, Y., Moo-Young, M., in: *Biotechnology: The Science and the Business:* Moses, V., Cape, R.E., (Eds.), New York: Harwood Academic Publishers, 1991, pp. 167–209. Fermentation technology, bioprocessing, scale-up and manufacture

[2] Belter, P.A., Cussler, E.L., Hu, W.-S., *Bioseparations: Downstream Processing for Biotechnology.* New York: John Wiley, 1988.

[3] Wheelwright, S.M., *Protein Purification: Design and Scale up of Downstream Processing.* New York: Hanser Publishers, 1991.

[4] Christian, J.B., *Chem Eng Prog,* 1994, *90*(7), 50–56. Improve clarifier and thickener design and operation.

[5] Tiller, F.M., Tarng, D., *Chem Eng Prog,* 1995, *91* (3), 75–80. Try deep thickeners and clarifiers.

[6] Gyure, D.C., *Chem Eng Prog,* 1992, *88*(11), 60–66. Set realistic goals for cross-flow filtration.

[7] Fleming, H.L., *Chem Eng Prog,* 1992, *88*(7), 46–52. Consider membrane pervaporation.

[8] Abbott, N.L., Hatton, T.A., *Chem Eng Prog,* 1988, *84*(8), 31–41. Liquid–liquid extraction for protein separations.

[9] Raghavarao, K.S.M.S., Rastogi, N.K., Gowthaman, M.K., Karanth, N.G., *Adv Appl Microbiol,* 1995, *41,* 97–171. Aqueous two-phase extraction for downstream processing of enzymes/proteins.

[10] Chisti, Y., in: *Encyclopedia of Bioprocess Technology;* Flickinger, M.C., Drew, S.W., (Eds.), New York: John Wiley, 1999; in press. Solid substrate fermentations, enzyme production, food enrichment.

[11] Patnaik, P.R., *Biotechnol Adv,* 1995, *13,* 175–208. Liquid emulsion membranes: principles, problems and applications in fermentation processes.

[12] Pyle, D.L., *J Chem Technol Biotechnol,* 1994, *59,* 107–108. Protein separation using reverse micelles.

[13] Schügerl, K., *Solvent Extraction in Biotechnology,* New York: Springer-Verlag, 1994.

[14] Chisti, Y., Moo-Young, M., *Biotechnol Adv,* 1990, *8,* 699–708. Large scale protein separations: engineering aspects of chromatography.

[15] Snowman, J.W., *Adv Biotechnol Process,* 1988, *8,* 315–351. Lyophilization techniques, equipment, and practice.

[16] Chisti, Y., Moo-Young, M., *Enzyme Microb Technol,* 1986, *8,* 194–204. Disruption of microbial cells for intracellular products.

[17] Harrison, S.T.L., *Biotechnol Adv,* 1991, *9,* 217–240. Bacterial cell disruption: A key unit operation in the recovery of intracellular products.

[18] Middelberg, A.P.J., *Biotechnol Adv,* 1995, *13,* 491–551. Process-scale disruption of microorganisms.

[19] Chisti, Y., Moo-Young, M., *I Chem E Symp Ser,* 1994, *137,* 135–146. Separation techniques in industrial bioprocessing.

[20] Fish, N.M., Lilly, M.D., *Biotechnology,* 1984, *2,* 623–627. The interactions between fermentation and protein recovery.

[21] Dunnill, P., *Process Brochem,* 1983, *18*(5), 9–13. Trends in downstream processing of proteins and enzymes.

[22] Chisti, Y., *Chem Eng Prog,* 1992, *88*(1), 55–58. Build better industrial bioreactors.

[23] Chisti, Y., *Chem Eng Prog,* 1992, *88*(9), 80–85 Assure bioreactor sterility.

[24] Chisti, Y., Moo-Young, M., *J Ind Microbiol,* 1994, *13,* 201–207. Clean-in-place systems for industrial bioreactors: design, validation and operation.

[25] Flickinger, M.C., Sansone, E.B., *Biotechnol Bioeng,* 1984, *26,* 860–870. Pilot- and production-scale containment of cytotoxic and oncogenic fermentation processes.

[26] Lubiniecki, A.S., Wiebe, M.E., Builder, S.E., in: *Large-Scale Mammalian Cell Culture Technology;* Lubiniecki, A.S., (Ed.) New York: Marcel Dekker, 1990; pp. 515–541 . Process validation for cell culture-derived pharmaceutical proteins.

[27] Willig, S.H., Stoker, J.R., *Good Manufacturing Practices for Pharmaceuticals: A Plan for Total Quality Control,* 3rd edition, New York: Marcel Dekker, 1992.

[28] Garg, V.K., Costello, M.A.C., Czuba, B.A., in: *Purification and Analysis of Recombinant Proteins;* Seetharam, S., Sharma, S.K., (Eds.), New York: Marcel Dekker, 1991; pp. 29–54. Purification and production of therapeutic grade proteins.

[29] Anicetti, V.R., Keyt, B.A., Hancock, W.S., *Trends Biotechnol,* 1989, *7*(12), 342–349. Purity analysis of protein pharmaceuticals produced by recombinant DNA technology.

[30] Rouf, S.A., Moo-Young, M., Chisti, Y., *Biotechnol Adv,* 1996, *14,* 239–266. Tissue-type plasminogen activator: characteristics, applications and production technology.

[31] Roberts, P., *J Chem Technol Biotechnol,* 1994, *59,* 110–111. Virus safety in bioproducts.

[32] Chisti, Y., Moo-Young, M., *Trans I Chem E,* 1996, *74A,* 575–583. Bioprocess intensification through bioreactor engineering.

[33] Garrido, F., Banerjee, U.C., Chisti, Y., Moo-Young, M., *Bioseparation,* 1994, *4,* 319–328. Disruption of a recombinant yeast for the release of β-galactosidase.

[34] Hochuli, E., *Pure Appl Chem,* 1992, *64,* 169–184. Purification techniques for biological products.

[35] Beitle, R.R., Ataai, M.M., *Biotechnol Progress,* 1993, *9,* 64–69. One-step purification of a model periplasmic protein from inclusion bodies by its fusion to an effective metal-binding peptide.

[36] French, C., Ward, J.M., *J Chem Technol Biotechnol,* 1992, *54,* 301. Production and release of recombinant periplasmic enzymes from *Escherichia coli* fermentations.

[37] Tamer, I.M., Moo-Young, M., Chisti, Y., *Ind Eng Chem Res,* 1998, *37,* 1807–1814. Disruption of *Alcaligenes latus* for recovery of poly-β(hydroxybutyric acid): comparison of high-pressure homogenization, bead milling, and chemically induced lysis.

[38] Dwyer, J.L., *Biotechnology,* 1984, *2,* 957–964. Scaling up bioproduct separation with high performance liquid chromatography.

[39] Baker, N.V., *Nature,* 1972, *239,* 398–399. Segregation and sedimentation of red blood cells in ultrasonic standing waves.

[40] Kilburn, D.G., Clarke, D.J., Coakley, W.T., Bardsley, D.W., *Biotechnol Bioeng,* 1989, *34,* 559–562. Enhanced sedimentation of mammalian cells following acoustic aggregation.

[41] Whitworth, G., Grundy, M.A., Coakley, W.T., *Ultrasonics,* 1991, *29,* 439–444. Transport and harvesting of suspended particles using modulated ultrasound.

[42] Mandralis, Z.I., Feke, D.L., *AIChEJ,* 1993, *39,* 197–206. Fractionation of suspensions using synchronized ultrasonic and flow fields.

[43] Weiser, M.A.H., Apfel, R.E., *Acustica,* 1984, *56,* 114–119. Interparticle forces on red cells in a standing wave field.

[44] De Loggio, T., Letki, A., *Chemical Engineering,* 1994, *101*(1), 70–76. New directions in centrifuging.

[45] Bonnerjea, J., Oh, S., Hoare, M., Dunnill, P., *Biotechnology*, 1986, *4*, 954–958. Protein purification: the right step at the right time.
[46] Dörnenburg, H., Knorr, D., *Process Biochem*, 1992, *27*, 161–166. Release of intracellularly stored anthraquinones by enzymatic permeabilization of viable plant cells.
[47] Zhang, Z., Chisti, Y., Moo-Young, M., *Bioseparation*, 1995, *5*, 329–337. Isolation of a recombinant intracellular β-galactosidase by ammonium sulfate fractionation of cell homogenates.
[48] Chisti, Y., Moo-Young, M., *Chem Eng Prog*, 1993, *89*(6), 38–45. Improve the performance of airlift reactors.
[49] Brennecke, J.F., *Chem Ind (Lond.)*, 1995, *21*, 831–834. New applications of supercritical fluids.
[50] Chisti, Y., *Bioproc Eng*, 1993, *9*, 191–196. Animal cell culture in stirred bioreactors: observations on scale-up.
[51] Kroner, K.H., Nissinen, V., Ziegler, H., *Biotechnology*, 1987, *5*, 921–926. Improved dynamic filtration of microbial suspensions.
[52] Lee, S.S., Burt, A., Russotti, G., Buckland, B., *Biotechnol Bioeng*, 1995, *48*, 386–400. Microfiltration of recombinant yeast cells using a rotating disk dynamic filtration system.
[53] Brors, A., Kroner, K.H., in: *Harnessing Biotechnology for the 21st Century:* Ladisch, M.R., Bose, A., (Eds.), Washington, DC: American Chemical Society, 1992; pp. 254–257. Electrically enhanced cross-flow filtration of biosuspensions.
[54] Rudge, S.R., Todd, P., *ACS Symp Ser*, 1990, *427*, 244–270. Applied electric fields for downstream processing.
[55] Shanley, A., Parkinson, G., Fouhy, K., *Chemical Engineering*, 1993, *100*(1), 28–33. Biotech in the scaleup era.
[56] Kim, I., *Chemical Engineering*, 1997, *104*(1), 28–33. Biotech's new mandate: more, cheaper, and faster.
[57] Brandt, S., Goffe, R.A., Kessler, S.B., O'Connor, J.L., Zale, S.E., *Biotechnology*, 1988, *6*, 779–782. Membrane-based affinity technology for commercial scale purifications.
[58] Zhang, Z., Moo-Young, M., Chisti, Y., *Biotechnol Adv*, 1996, *14*, 401–435. Plasmid stability in recombinant *Saccharomyces cerevisiae.*
[59] Dunnill, P., *Chem Eng Res Des*, 1987, *65*, 211–217. Biochemical engineering and biotechnology.
[60] Moo-Young, M., Chisti, Y., *Biotechnology*, 1988, *6*, 1291–1296. Considerations for designing bioreactors for shear-sensitive culture.
[61] Bykowska, K., Rijken, D.C., Collen, D., *Thromb Haemost*, 1981, *46*, 642–644. Purification and characterization of the plasminogen activator secreted by a rat brain tumor cell line in culture.
[62] Fischer, B.E., *Biotechnol Adv*, 1994, *12*, 89–101. Renaturation of recombinant proteins produced as inclusion bodies.