4 DOWN STREAM PROCESSING

4.1 INTRODUCTION

Industrial fermentations comprise both upstream (USP) and downstream processing (DSP) stages (Fig. 4.1). USP involves all factors and processes leading to and including the fermentation and consists of three main areas: the producer organism, the medium and the fermentation process. DSP encompasses all processes following the fermentation. In most cases this means recovery of a product from a dilute aqueous solution. The complexity of DSP is determined by the required purity of the product which is in turn determined by its application. The products of biotechnology include whole cells, organic acids, amino acids, solvents, antibiotics, industrial enzymes, therapeutic proteins, vaccines, gums etc. The primary objective in industrial fermentation processes is to recover the product efficiently, reproducibly and safely to its required specification, while achieving maximum product yield at minimum recovery costs. Fermentation factors affecting DSP include the properties of the microorganisms (particularly morphology, flocculation characteristics, size & cell wall rigidity). These factors impact filterability, sedimentation and homogenization efficiency. The presence of fermentation by-products, media impurities & additives like antifoam may interfere with DSP steps. Therefore, a holistic approach is required when developing a new industrial purification strategy. The whole process, both upstream & downstream factors need to be considered, e.g., a cheap carbon and energy source containing many impurities may provide initial cost savings but may necessitate increased DSP costs. Hence overall cost savings may be achieved with a more expensive but purer substrate.

As the products vary greatly in size and nature, different separation techniques are required for their recovery & purification. The lability or sensitivity of many of the bioproducts, particularly proteins, to the environmental conditions, places further demands on the characteristics of the separation processes used for their production. The concentration of the product in the starting material is a major factor in the overall cost of production. The relationship between concentration and selling price for a broad range of products illustrates this (Fig. 4.2). The influence of step yield and the number of steps on overall yield (Fig. 4.3) demonstrates that apart from the significant operating cost associated with each purification step, the cumulative yield loss observed in multi-stage purification can be substantial – even when average step yields are 80-90%. Thus for optimum economic viability, the number of steps required to attain the desired product specifications should be minimised. In addition, some biochemical purification processes require substantial analytical back-up support and some biological materials require complicated and time consuming assay procedures. Such analytically intensive steps should be avoided if possible as the analytical cost component may result in the purification step being cost-ineffective.

Many of the steps in DSP are traditional unit processes used extensively in the chemical industry. The DSP scheme (Figs 4.4 & 4.5) normally employed for isolation and purification of biomolecules can be divided into the following stages: (i) solid-liquid separation or clarification; (ii) concentration and (iii) purification.

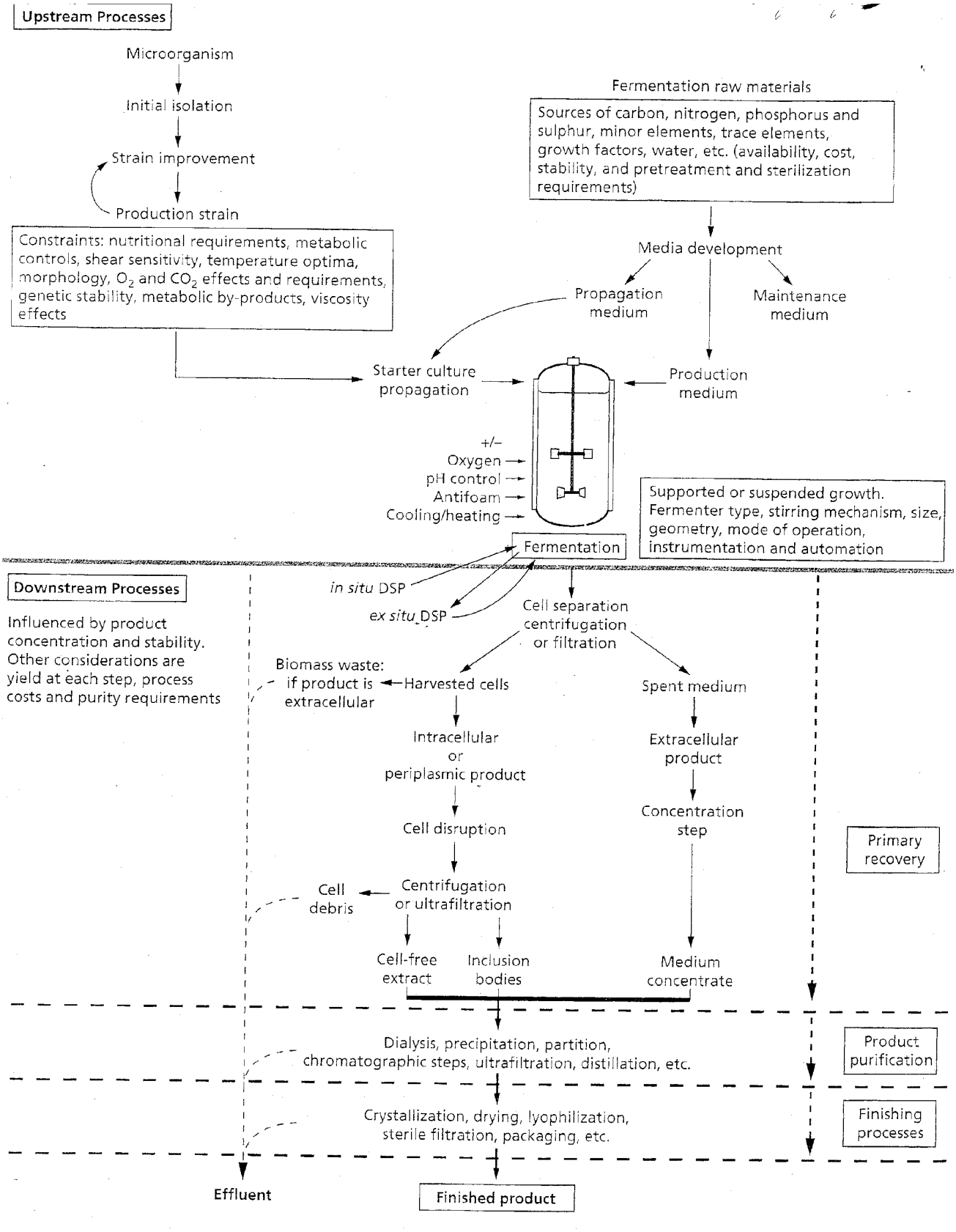


Fig. 4.1: An outline of upstream and downstream processing application.

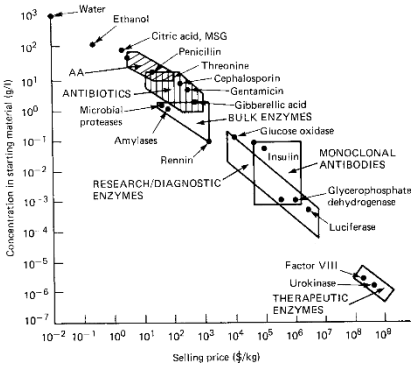


Fig. 4.2: Relationship between product concentration in starting material and its selling price (Ward, 1992).

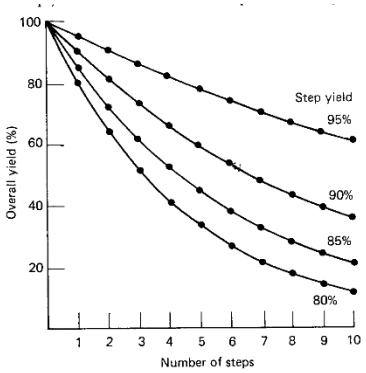


Fig. 4.3: Effect of the number of purification steps and step yield on overall process yield (Ward, 1992).

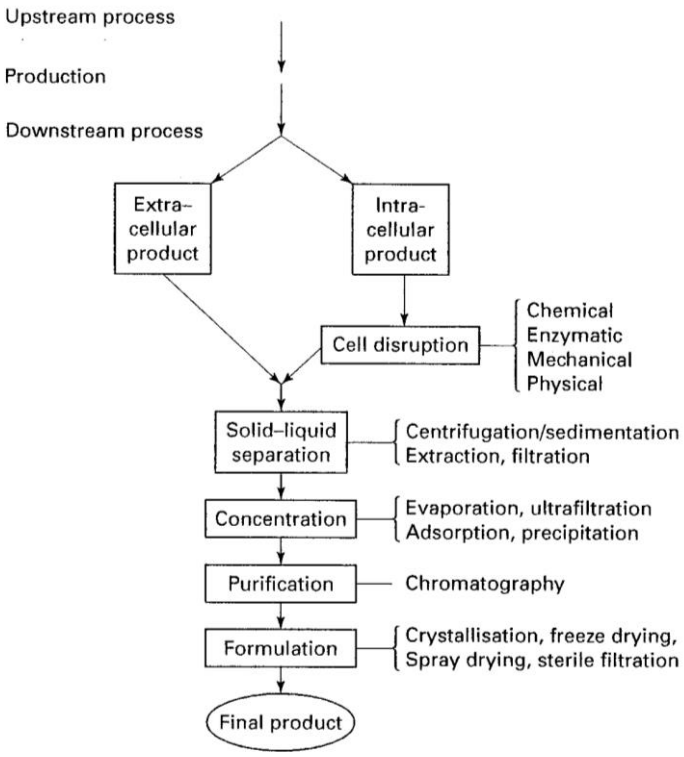


Fig. 4.4: Downstream processing. Different stages in the isolation and purification of the products of biotechnology (Ratledge and Kristiansen, 2004).

4.2 SOLID-LIQUID SEPARATION

Solid-liquid separation is a primary recovery operation for the separation of whole cells from culture broth, removal of cell debris, collection of protein precipitate, collection of inclusion bodies, etc. The unit operations commonly used are centrifugation and filtration.

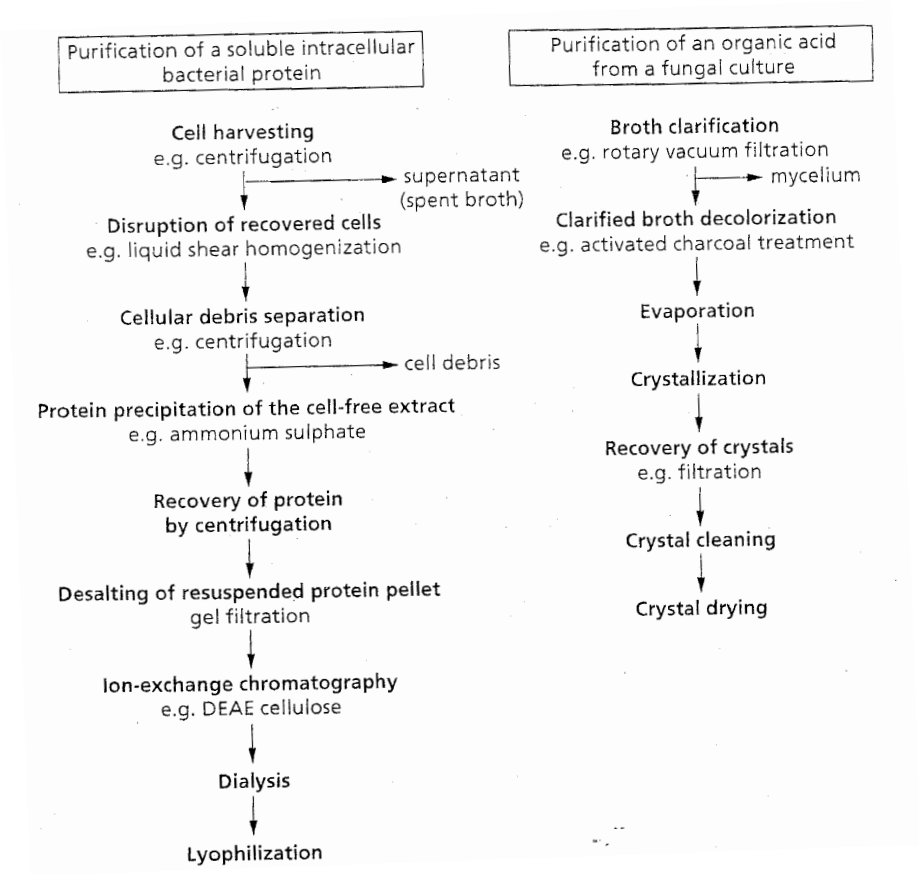


Fig. 4.5: Schema for the downstream processing for two microorganisms.

4.2.1 FILTRATION

A filter medium constitutes the separating agent and retains the particles according to size while allowing the passage of the liquid through the filter. In cake filtration, the particles are retained as a cake on the filter medium. The flow through the filter layers is dependent on area of the filter & flow resistance provided by the filter medium & the cake. If the particles do not penetrate the filter medium, then its flow resistance will remain unchanged. However, the cake layer, as it grows thicker will provide increasing resistance. The cake layers, especially in biomass separation, are compressible & the changing effective pressure difference will influence the flow through the filter, e.g., of filter media are perforated sintered metal, cloth, synthetic fibres, cellulose, glass wool, ceramics & synthetic membranes. Many types of filtration equipment are available.

4.2.1.1 Plate and frame filters

These are cheap and versatile - the surface area can be adjusted by varying the number of plates (Fig. 4.6). Not suitable for the removal of large quantities of solids from broths as the plates have to be dismantled for solids recovery. They are used as polishing devices to filter out low residual solids.

1.2.1.2 Filter Press

A filter press is built of a sequence of perforated plates alternating with hollow frames. The plates are covered with a suitable filter medium (cloths) that create a series of chambers through which the slurry can be forced. Solids are retained in the chambers and the filtrate discharges into the hollows on the plate surface and drain out.

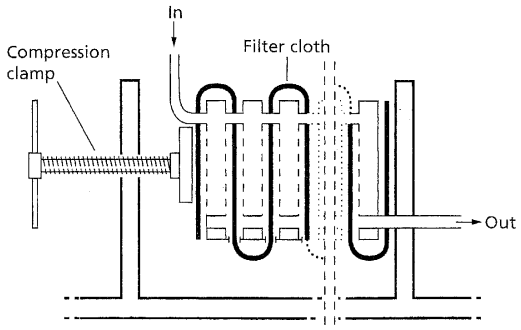


Fig. 4.6: A plate and frame filter (Brown *et al.*, 1987).

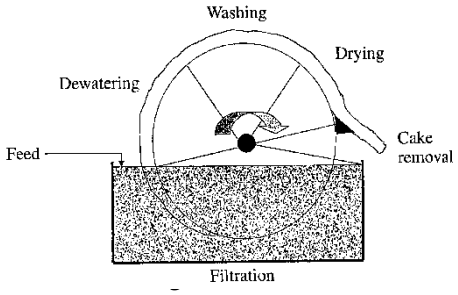
1.2.1.3 Membrane Filter Press

The cake chambers are covered with a rubber membrane that is inflated using air or water, allowing *in situ* compacting of the cake. These allow higher yield and drier cake but involve a higher capital investment. Filtration using microporous membranes operated under pressure is a viable alternative to centrifugation.

1.2.1.4 Vacuum filters

Vacuum filters are used for clarification of fermentation broths (containing 10-40% solids by volume with particle sizes ranging between 0.5-10 μm) due to simplicity of operation and low cost.

The best known vacuum filters are the rotary drum vacuum filters (Fig. 4.7) which are used for filtration of filamentous fungi and yeast cells. They are used to clarify large volumes of liquid with automatic solids discharge. Tangential flow (cross flow filtration) is an effective method to separate cells from liquid where high value product is involved. The parallel motion of fluid to the membrane helps reduce the thickness of the cell layer on the filter surface.



ıg drum, maintained
tially immersed in a
ł of 0.25-5 rpm, the
te is drawn and the
Continuous rotation
tions of dewatering,
n the filter cake prior
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Fig. 4.7: A rotary drum vacuum filter.

1.2.2 CENTRIFUGATION

In centrifugation the removal of solids relies on the density difference between the particles to be separated and the surrounding medium. Choice of centrifugation equipment depends on the organism size and the required throughput. Separation efficiency in many cases has to be balanced against throughput. For large scale fermentations, solid recovery has to be continuous and these centrifuges need to have a solids discharge mechanism. Nozzle discharge types are suitable for recovery of yeast and bacteria but tend to get clogged by fungal mycelium or large particulate matter. Solids ejecting centrifuges (Fig. 4.8) have continuous or intermittent mechanisms to discharge solids and may be used for the recovery of mycelia or bacterial biomass. The screw decanter centrifuge which is suitable for dewatering of coarse solid materials at high solid concentrations has been used for the recovery of yeasts and fungi.

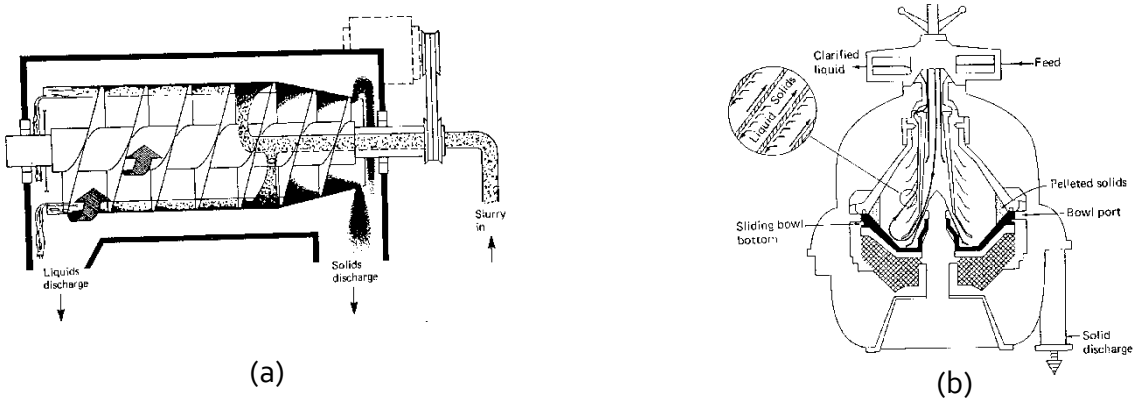

Fig. 4.8: Centrifuges for large scale fermentation solids recovery. (a) Solids discharge centrifuge, (b) Screw decanter centrifuge (Ward, 1992).

4.2.3 PRETREATMENT OF BROTH TO FACILITATE CLARIFICATION

Pre-treatment/conditioning of the broth by changing the biomass particle size, fermentation liquor viscosity and the interactions between biomass particles is sometimes required to ensure efficient solid-liquid separation. This is achieved by using a filter aid (body-feed) in the broth and/or pre-coating the filter medium. Filter aids are incompressible, discrete particles of high permeability ranging between 2-20µm and should be inert to the broth being treated – the most common are Diatomite (skeletal remains of aquatic plants), Perlite (processed volcanic rock) and inactive carbon.

Agglomeration of individual cells or cell particles into large flocs which are easily separated at low centrifugal force is achieved by adding flocculating agents, e.g., polycations – either cellulosic or based on synthetic polymers, inorganic salts or mineral hydrocolloids. Cationic filter aids also reduce the load of pyrogen, nucleic acid and acidic protein which normally foul chromatography columns.

4.2.4 FLOTATION

Particles are adsorbed on gas bubbles, get trapped in a foam layer and can be collected. The gas may either be sparged into the particulate feed or very fine bubbles can be generated from

dissolved gases by releasing the overpressure or by electrolysis. Foam is stabilized by the addition of "collector substances" such as long chain fatty acids or amines.

4.3 RELEASE OF INTRACELLUAR COMPONENTS

Some target products are intracellular, including many enzymes and recombinant proteins, several of which form inclusion bodies. The breaching of the cell wall/envelope and cytoplasmic membrane can pose problems, especially when cells possess strong cell walls, e.g., a pressure of 650 bar is needed to disrupt yeast cells. General problems associated with cell disruption include the liberation of DNA which can increase the viscosity of the suspension which may affect further processing. Addition of DNAse can help prevent this problem. If mechanical disruption is used then heat is invariably generated and cooling measures need to be implemented. Also products released by eucaryotes are often subject to degradation by hydrolytric enzymes (proteases, lipases, etc.). This damage can be reduced by the addition of enzyme inhibitors, cooling the cell extract and rapid processing.

Cell disruption can be achieved by mechanical and non-mechanical means. The disruption process is quantified by monitoring changes in absorbance, particle size, total protein concentration or the activity of a specific enzyme.

4.3.1 MECHANICAL CELL DISRUPTION METHODS

Several mechanical methods are available for cell disruption. Those based on solid shear involve extrusion of frozen cell preparations through a narrow orifice at high pressure. This is useful at a laboratory scale but not for large scale operations. Methods employing liquid shear are generally more effective. The French press (pressure cell) is often used in the laboratory and high pressure homogenisers, such as the Manton and Gaulin homogeniser (APV type mill) are employed for pilot and production scale cell disruption. They may be used for bacterial and yeast cells and fungal mycelium. In these devices the cell suspension is drawn through a check valve into a pump cylinder where it is forced under pressure (up to 1 500 bar) through a very narrow annulus or discharge valve after which the pressure drops to atmosphere. Cell disruption is achieved by high liquid shear in the orifice and the sudden pressure drop upon discharge causes explosion of the cells.

The rate of protein release is independent of the cell concentrations but is a function of the pressure exerted, the number of cycles through the homogeniser, and the temperature. Disruption of yeast cells requires three cycles at 650 bar whereas *E. coli* requires 1 100-1 500 bar. During processing the temperature rises about 20°C over one pass at 800 bar. Therefore pre-cooling of the cell suspension is essential. A problem with this method of cell disruption is that all intracellular materials are released. Thus, the product of interest must be separated from a complex mixture of proteins, nucleic acid and cell wall fragments. Some fragments of cell debris are not readily separated, making the solution difficult to clarify. Also, proteins may be denatured if the equipment is not sufficiently cooled and filamentous microorganisms may block the discharge valve. With certain categories of microorganisms, the homogenisers have to be contained to prevent escape of aerosols.

On a laboratory scale, manual grinding of cells with abrasives, usually alumina, glass beads, kieselguhr or silica can be an effective means of disruption but results may not be reproducible. In industry, high speed bead mills equipped with cooling jackets are often used to agitate a cell suspension with small beads (0.5-0.9 µm diameter) of glass, zirconium oxide or titanium carbide. Cell breakage results from shear forces grinding between beads and collisions with beads. The efficiency of cell breakage is a function of agitation speed, concentration of beads, bead density and diameter, broth density, flow rate and temperature. Cell concentration is also a major factor (optimum 30-60% dry weight) which is an important difference from the liquid shear homogenisers described above.

Ultrasonic disruption of cells involves cavitation, microscopic bubbles or cavities generated by pressure waves. It is performed by ultrasonic vibrators that produce a high frequency sound with a wave density of approx. 20kHz. A transducer converts the wave into mechanical oscillations *via* a titanium probe immersed in the concentrated cells. However, this technique also generates heat, which can denature thermolabile proteins. Rod shaped bacteria are easier to break than cocci and Gram-negative organisms are more easily disrupted than Gram-positive cells. Sonication is effective on a small scale and is not routinely used in large scale operations due to problems with the power transmission and heat dissipation. Cells can be made permeable with an osmotic shock. Other permeabilisation techniques include the use of basic proteins such as protamine; the cationic polysaccharide chitosan is effective for yeast cells; and streptolysin permeabilises mammalian cells.

4.3.2 NON-MECHANICAL CELL DISRUPTION METHODS

An alternative to mechanical cell disruption is to cause their permeabilization. This can be achieved in several ways: autolysis, osmotic shock, rupture with crystals (freezing/thawing) or heat shock.

Autolysis has been used in the production of yeast extract and other yeast products. Its biggest advantage is the low cost and the microbes own enzymes catalyses the process so there are no foreign substances introduced into the process.
Osmotic shock can be achieved by equilibrating cells in 20% (w/v) buffered sucrose, rapidly harvesting and resuspending in water at 4°C. It is useful for the release of products from the periplasmic space.

Several other techniques (various chemicals and enzymes) have been developed for small scale applications but may lead to problems with subsequent purification steps. Organic solvents (butanol, chloroform and methanol) have been used to release enzymes and other substances from microorganisms by creating channels through the cell membrane. Treatment with detergents or alkali can also be effective.

Cell wall degrading enzymes such as lysozyme, which degrade β-1,4 glycosidic linkages is useful in Gram-positive organisms. Addition of EDTA to chelate metal ions improves the effectiveness of lysozyme. EDTA sequesters the divalent cations that stabilize outer membrane proteins. Yeast cell walls can be degraded by snail gut enzymes (containing a mixture of glucanases).

Antibiotics such as penicillin can be used on actively growing bacterial cells (in combination with osmotic shock).

4.4 PRODUCT RECOVERY

Recovery of extracellular proteins is from the clarified medium, whereas disrupted cell preparations are used both for intracellular proteins and those held within the periplasmic space. Some recombinant proteins expressed at high levels sometimes form inclusion bodies that are released by cell breakage. Following cell disruption, soluble proteins are separated from the cell debris by centrifugation. The resultant supernatant containing the proteins is then processed in a similar way to growth medium containing excreted proteins. Several methods are available for this process, viz., microfiltration and ultrafiltration.

4.4.1 MEMBRANE FILTRATION

Membrane filtration involves the use of membrane technology for the separation of biomolecules and particles and the concentration of process fluids. During separation a semi-permeable membrane acts as a selective barrier retaining the molecules/particles bigger than the pore size while allowing the smaller molecules to permeate through the pores. Membrane filtration processes can be distinguished according to the type of force driving the transport through the membrane and are named by the size of the pores in the filter.

4.4.1.1 Microfiltration

Microfiltration is used for the separation of particles 0.02-10 μm in diameter, primarily during the primary recovery stages of DSP. Microfilters are available in materials such as ceramic and steel that can be aggressively cleaned and sterilised.

4.4.1.2 Ultrafiltration

Ultrafiltration separates solutes in the 0.001-0.02 μm in range; used during the primary recovery stages of DSP and membranes are made of ceramic and steel. The membranes have an anisotropic structure and lie on top of a thick, highly porous structure. Flat membranes are available but for larger scale operations, hollow fibres systems (Fig. 4.9) are usually preferred. Several ultrafiltration units can be linked together to produce a sophisticated purification system. These methods are extensively applied for the purification of proteins and for separating and concentrating materials. Ultrafiltration is also effective in removing pyrogens (bacterial cell wall lipopolysaccharides), cell debris and virus from media and for whey processing. Another variation of the ultrafiltration is diafiltration, where water or other liquids are filtered to remove unwanted low molecular weight contaminants. This can be used as an alternative to gel filtration or dialysis for removing ammonium sulphate from a protein preparation precipitated by this salt (de-salting), for changing a buffer or in water purification.

4.3.1 SALTING/PRECIPITATION

An older but effective method used at this stage is salting out of proteins, followed by the recovery of precipitated proteins by centrifugation. Precipitation is achieved by the addition of inorganic salts at high ionic strength and usually in the form of solid or saturated solutions of ammonium sulphate. Ammonium sulphate is popular due to its high solubility, low toxicity and

low cost but it can liberate ammonia at high pH and is corrosive to metal surfaces, e.g., centrifuges. The solubility of the salt varies with temperature, so strict temperature control is required. Addition of organic solvents, such as acetone, ethanol and isopropanol at low temperature reduces protein solubility and the dielectric constant of the medium resulting in precipitation of the proteins.

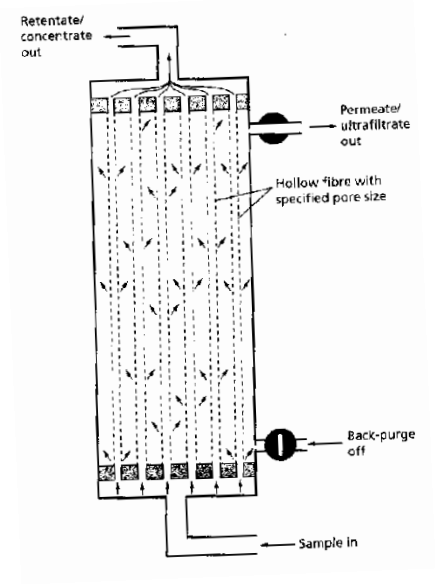

Fig. 4.9: Diagrammatic representation of a hollow fibre ultrafiltration unit.

4.3.2 AQUEOUS TWO-PHASE SEPARATION

This involves partitioning the protein between the two phases, depending on its molecular weight and charge. Commonly used systems include dextran and polyethylene glycol (PEG) or PEG and potassium phosphate. The two phases can then be separated by centrifugation. This method is cheap, gentle and versatile, and can be scaled up for industrial applications including the purification of enzymes, e.g., RNA polymerase from *E.coli*.

Many alkaloids, antibiotics, steroids and vitamins are recovered by liquid-liquid extraction methods using organic solvents. Antibiotics, e.g., are extracted from culture media into solvents such as amyl acetate. The solvents used should be non toxic, selective, inexpensive, and immiscible with broth and must have a high distribution coefficient for the product. Effective recovery of the solvent for re-use is an essential aspect of overall process economics.

4.4.4 CHROMATOGRAPHY

Chromatographic techniques are usually employed for higher value products. Those methods, normally involving columns of chromatographic media (stationary phase), are used for de-salting, concentration and purification of protein preparations. A number of factors need to be considered when choosing a chromatographic technique. For proteins these include molecular weight, isoelectric point, hydrophobicity and biological affinity. Each of these properties can be exploited by specific chromatographic methods. The following chromatographic parameters need to be considered: capacity, recovery and resolving power (selectivity). Capacity refers to

the sample size that can be applied to the system in terms of protein concentration and volume and the recovery is the yield of product at each stage. Yield values should be as high as possible otherwise the overall process will be uneconomic. Resolving power and selectivity relate to the ability to separate two components, one being the product and the other being the impurities. This is particularly important at the final purification stage.

1.4.4.1 Adsorption chromatography

Adsorption chromatography separates according to the affinity of the protein or other material for the surfaces of the solid matrix. Alumina (Al_2O_3), hydroxyapatite ($Ca_{10}(Po_4)_6(OH)_2$) or silica (SiO_2) are used for purifying non-polar molecules. Polystyrene based resins are effective matrices for polar molecules. This technique involves hydrogen bonding and/or van der Waal's forces. The adsorbed proteins are eluted by increasing the ionic strength, often by using a gradient of increasing phosphate ion concentration.

1.4.4.2 Affinity chromatography

This is a powerful and highly selective purification technique that often results in a several thousand-fold purification in a single step. However, this method is expensive on an industrial scale. The technique involves specific chemical interactions between solute molecules such as proteins and an immobilised ligand (functional molecule). Ligands are covalently linked to the matrix material, e.g., agarose *via* a spacer arm to avoid steric hindrance. Some ligands interact with a group of proteins, e.g., nicotinamide adenine dinucleotides, adenosine monophosphate and Procion and Cibracon dyes; other ligands are highly specific especially substrates, substrate analogues and antibodies. Since monoclonal antibodies have become more readily available, immunoaffinity chromatography methods have been developed for the purification of various antigens. The loading capacity of affinity chromatography columns is large, as the volume of the sample is unimportant and the high resolution and elution is achieved using specific cofactors or substrates; alternately non-specific elution may be performed with salts or pH change.

1.4.4.3 Gel filtration chromatography

This involves separation on the basis of molecular size (molecular sieving), although molecular shape can also influence separation performance, consequently it is particularly useful for de-salting protein preparations. The stationary phase consists of porous beads composed of acrylic polymers, agarose, cellulose, cross-linked dextran, etc., which have a defined pore size. These support materials should be sterilisable, chemically inert, stable, highly porous and hydrophilic, containing some ionic groups. Mechanical rigidity is important to maintain good flow rates. The initial choice of stationary phase material is also key as some may interact with the target product, e.g., carbohydrate based matrices interact with glycoproteins. Solute molecules below the exclusion size of the support material pass in and out of the beads. Molecules above the exclusion size, pass only around the outside of the beads through the interstitial spaces and the apparent volume of the column is smaller for these larger excluded molecules. As a result they flow faster down the column separating from smaller molecules and eluting first. Smaller molecules able to enter the pores are then eluted in decreasing order of size (Fig. 4.10).

1.4.4.4 Ion exchange chromatography

This involves the selective adsorption of ions or electrically charged compounds onto ion exchange resin particles by electrostatic forces. The matrix material is based on cellulose substituted with various charged groups either cations or anions, e.g., anion exchange resin diethylaminoethyl (DEAE) cellulose. Proteins possess positive, negative or no charge depending on their isolectric point (pI) and the pH of the surrounding buffer. If the pH of the buffer is below the pI, the protein has an overall positive charge; a buffer at the pI results in a protein with no charge. A protein with pI 4.2 will be uncharged at pH 4.2 and will not bind to either positively or negatively charged resins. When the pH is raised to pH 7.0, the protein is negatively charged and will bind to positively charged resins. A protein in an anionic state will be able to adsorb to DEAE cellulose and any contaminants will pass through the column. The product can then be desorbed as a purified fraction by altering the ionic strength of the buffer.

4.4.4.5 High performance liquid chromatography (HPLC)

HPLC was originally developed for the separation of organic molecules in non aqueous solvents but is now used for proteins in aqueous solution. Columns are densely packed - containing very small rigid particles, 5-50 µm diameter of silica or a cross-linked polymer making high pressures a requirement. The method is fast and gives high resolution of solute molecules.

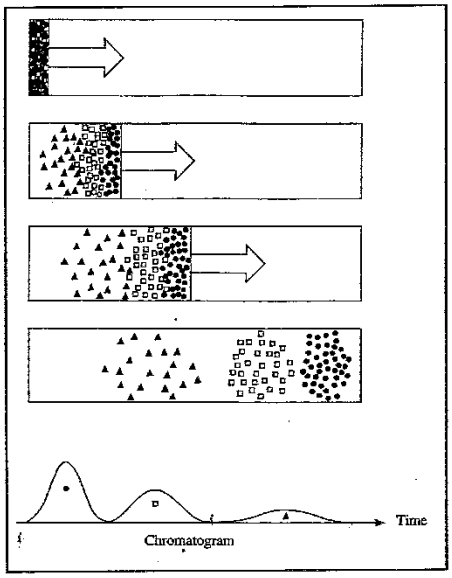

Fig. 4.10: Chromatographic separation of components in a mixture. Three different solutes are shown schematically as circles, squares and triangles (Belter *et al.*, 1988).

4.4.4.6 Dialysis and electrodialysis

These membrane separation techniques are used for the removal of low molecular weight solutes and inorganic ions from a solution. The membranes are size selective with specific molecular weight cut-offs. Low molecular weight solutes move across the membrane by osmosis from a region of high concentration to a region of low concentration. Electrodialysis methods separate charged molecules from a solution by the application of a direct electrical

current carried by mobile counter-ions. Membranes used contain ion-exchange groups and have fixed charge, e.g., positively charged membranes allow the passage of anions and repel cations. These membranes are essentially ion-exchange resins in sheet form and have been used for desalination of water.

4.4.4.7 Distillation

Distillation is used to recover fuel alcohol, acetone and other solvents from fermentation media and for the production of potable spirits. Batch distillation in pot stills continues to be used for the production of some whiskies but for most other purposes continuous distillation is the method of choice. With ethanol, the continuous process produces a product with maximum ethanol concentration of 96.5% (v/v). This azeotophic mixture is the highest concentration that can be achieved from aqueous ethanol, unless a dehydration step is introduced using a water entrainer such as benzene or cyclohexane.

Some continuous stills may be in the form of four or five separate columns but the Coffey type still comprises two columns – the rectifier and analyser, each containing a stack of 30-32 perforated plates. Incoming fermentation broth is heated as it passes down a coiled pipe within the rectifier column by the ascending hot vapour produced by the analyser column. The now hot broth is released into a trough at the top of the analyser column and as it falls down the column it is heated by steam. Hot vapours generated are then conveyed from the top of the analyser column to the bottom of the rectifier column. As it passes upwards it is condensed on the coils carrying incoming broth. There is a temperature gradient in the rectifier column and each volatile compound condenses at its appropriate level, from where the fraction is collected.

4.5 FINISHING STEPS

1.5.1 CRYSTALLIZATION

Product crystallization may be achieved by evaporation, low temperature treatment, or the addition of a chemical reactive with the solute. The product's solubility cannot be reduced by adding solvents, salts, polymers, e.g., non-ionic PEG and polyelectrolytes or by altering the pH.

1.5.2 DRYING

Drying involves the transfer of heat to the wet material and removal of the moisture as water vapour. Usually this must be performed in such a way as to retain the biological activity of the product. Parameters affecting drying are the physical properties of the solid-liquid system, intrinsic properties of the solute, conditions of the drying environment and heat transfer parameters. Heat transfer may be by direct contact, convection or radiation. Rotary drum driers remove water by heat conduction. A thin film of solution is applied to the steam heated surface of the drum which is scraped with a knife to recover the dried product. In vacuum tray driers the material to be dried is placed on heated shelves within a chamber to which a vacuum is applied. This lowers the temperatures to be used due to the lower boiling point of water at reduced pressures. The method is suitable for small batches of expensive materials, such as some pharmaceuticals. Spray drying involves atomisation and spraying of product solution into a heated chamber and resultant dried particles are separated from gases using cyclones. Pneumatic conveyor driers use hot air that suspends and transports particles.

Freeze drying (lyophilisation) is often used where the final products are live cells, as in starter culture preparation or for thermolabile products. This is especially useful for some enzymes, vaccines and other pharmaceuticals where retention of biological activity is critical. In this method, frozen solutions of antibiotics, enzymes or microbial cell suspensions are prepared and the water is removed by sublimation under vacuum, directly from solid to vapour state. This method eliminates thermal and osmotic damage.