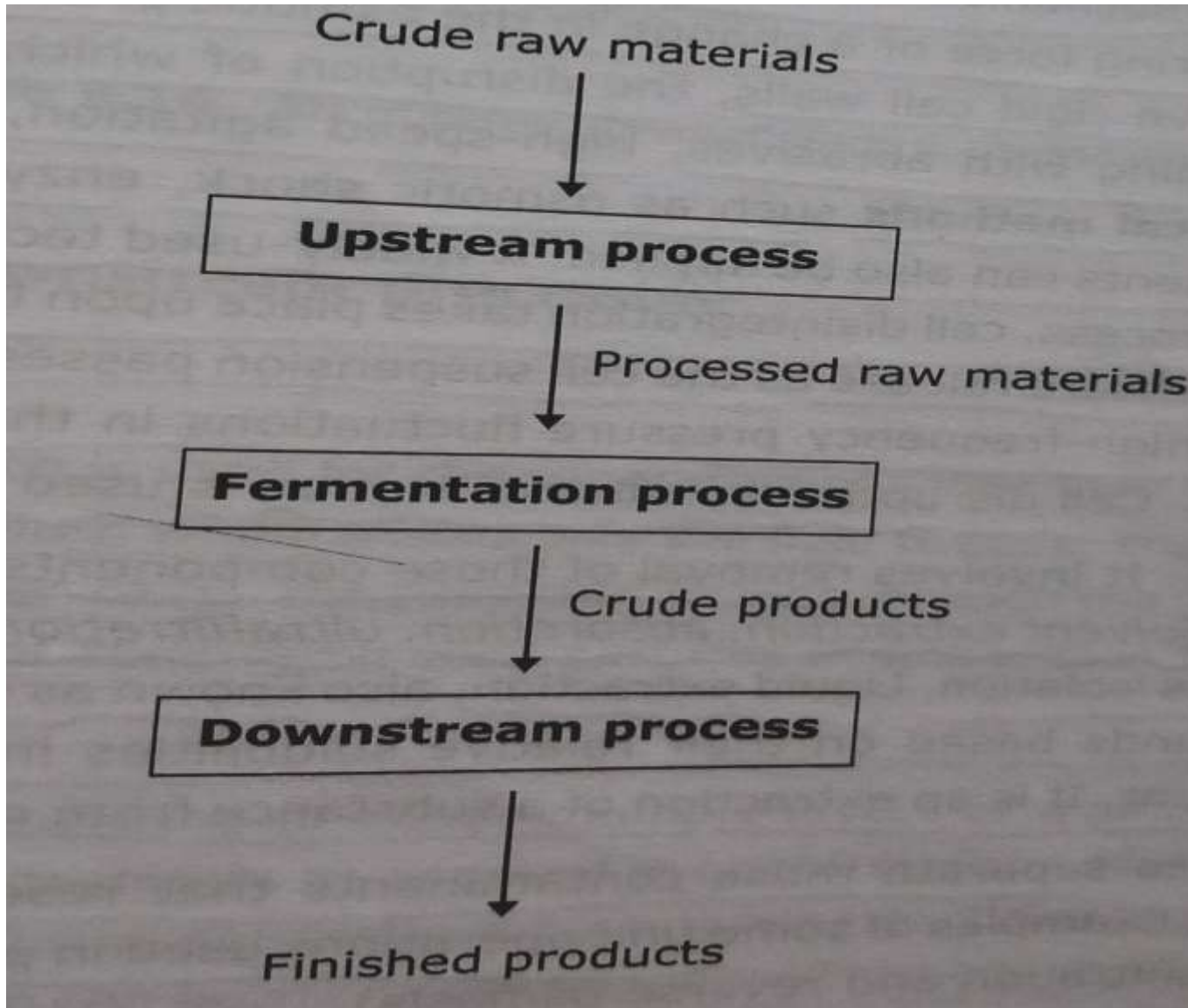


Industrial Microbiology (CC 12)

Downstream Processing (Unit 4)

- Fermentation : In industrial microbiology, fermentation describes any anaerobic as well as aerobic process for the production of the product by the mass culture of a microorganism
- Industrial fermentations comprise both upstream and downstream processes
- Upstream processing (USP) involves all factors and processes leading to, and including, fermentation, such as, sterilization of the fermenter, preparation & sterilization of culture medium and the preparation and growth of a suitable inoculum of microbial strain.
- USP includes –
 1. The formulation of the medium to be used in culturing the process organism during the development of the inoculum and in the production fermenter



2. The sterilization of the medium, fermenters and ancillary equipments
3. The production of an active, pure culture in sufficient quantity to inoculate the production vessel
4. The growth of the organism in the production fermenter under optimum conditions for product formation
 - Downstream processing (DSP) encompasses all processes following the fermentation. It includes –
 1. The extraction of the product and its purification
 2. The disposal of effluents produced by the process
 - It has the primary aim of efficiently, reproducibly & safely recovering the target product, while maximizing recovery yield and minimizing costs

- Fermentation broths are complex, aqueous mixtures of cells, comprising the soluble extracellular, intracellular products and any unconverted substrates
- The fermentation broth has to be processed and passed through several stages of separation and purification
- Any treatment of the culture broth after fermentation to concentrate and purify the product is known as downstream processing
- The target product may be recovered by processing the cells (intracellular product) or the spent medium (extracellular product)
- The level of purity is usually determined by the specific use of the product

- Individual operations or steps that alter the physical properties of the materials are called unit operations
- Unit operations involve centrifugation, chromatography, crystallization, membrane processes (dialysis, ultrafiltration, microfiltration, reverse osmosis), distillation, drying, evaporation, mixing, precipitation, solvent extraction etc.
- The physical & chemical properties of the product along with its concentration and location, are key factors in determining the initial separation steps and overall purification strategy
- Stability of the product also influences the requirement for any pretreatment necessary to prevent product inactivation &/or degradation

Fermentation Factors affecting DSP

1. Properties of microorganisms (morphology, flocculation characteristics, size & cell wall rigidity)
2. Media impurities
3. Fermentation additives present (eg. antifoams)
4. Stability of the biological materials
5. Nature of the end product & its concentration
6. Byproducts present
7. Necessary degree of purification

- The selection of specific unit steps is influenced by the economics of the process, the required purity of the product, the yield at each step and safety aspects
- The number of unit operation steps should be kept to a minimum to prevent the overall losses due to multistage purification

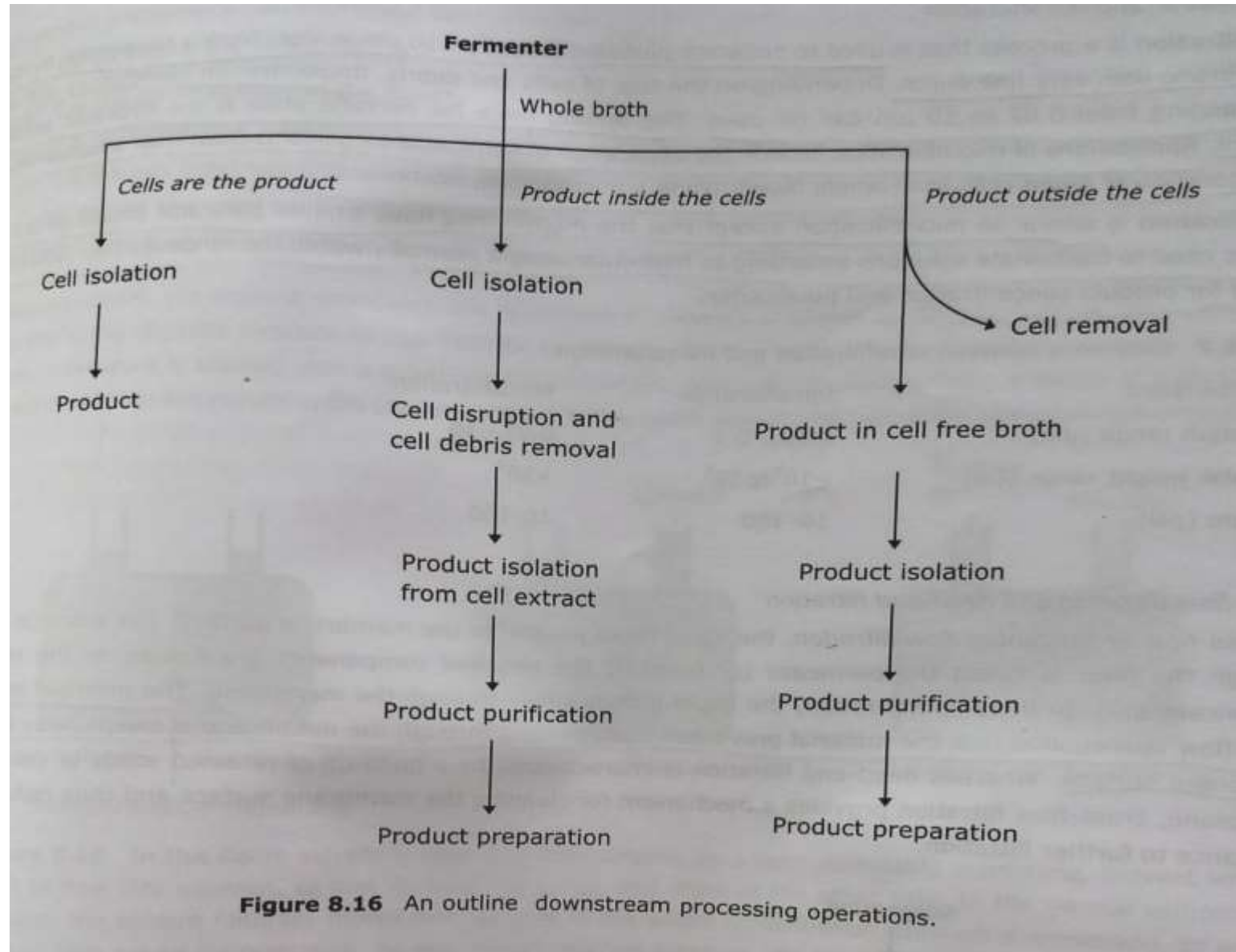


Figure 8.16 An outline downstream processing operations.

Different strategies of DSP

1. Cell separation (harvesting):
 - 1st step in DSP of suspended cultures
 - Basically it is a solid – liquid separation for the removal of cells from the spent medium
 - each fraction can be further processed, depending on whether the product is intracellularly located or has been secreted into the periplasmic space or the medium
 - Selection of method is influenced by the size & morphology of the microorganism (single cells, aggregates or mycelia), the specific gravity, viscosity & rheology of the spent fermentation medium
 - This step involves unit operations such as filtration, centrifugation & flocculation

Flocculation

- Process by which the suspended particles are clumped together to form larger-size clusters
- The clusters may then float on the top of the liquid or settle down to the bottom of the liquid
- Prior to flocculation, colloids are suspended in a liquid & not actually dissolved in a solution (precipitation)
- Flocculants/ flocculating agents/ flocking agents are chemicals that promote flocculation by causing colloids & other suspended particles in liquids to aggregate, forming a floc. Example – polyelectrolytes act by charge neutralization & hydrophobic interactions to link cells to each other
- Flocculants are widely used in the effluent-treatment industries for the removal of microbial cells and suspended colloidal matters

Filtration

- Conventional filtration involves **Depth filters** composed of porous media (cloth, glass wool, cellulose) that retain the solids & allow the clarified liquid (the filtrate) to pass through
- Here, the filtering unit has depth & captures contaminants within its depth, as oppose to on the surface
- These techniques are generally useful for harvesting filamentous fungi, but are less effective for collecting bacteria
- Two commonly used conventional filters in the industry are – **plate & frame filters & rotary vacuum filters**

A. Plate & frame filters/ filter presses:

- They are in the form of an alternating horizontal stack of porous plates and hollow frames. The stack is mounted in a support structure where it is held together with a hydraulic or screw ram. Filter cloths are held in place between the plates that contain flow channels for the feed and permeate streams
- This forms a series of cloth-lined chambers into which the cell suspension is forced under pressure
- After completion of filtration, the apparatus must be dismantled to remove the collected filter cake
- These batch filtration systems are used for harvesting microorganisms from fermentations, including the preparations of blocks of baker's yeast, the recovery of protein precipitates & the dewatering of sewage sludge

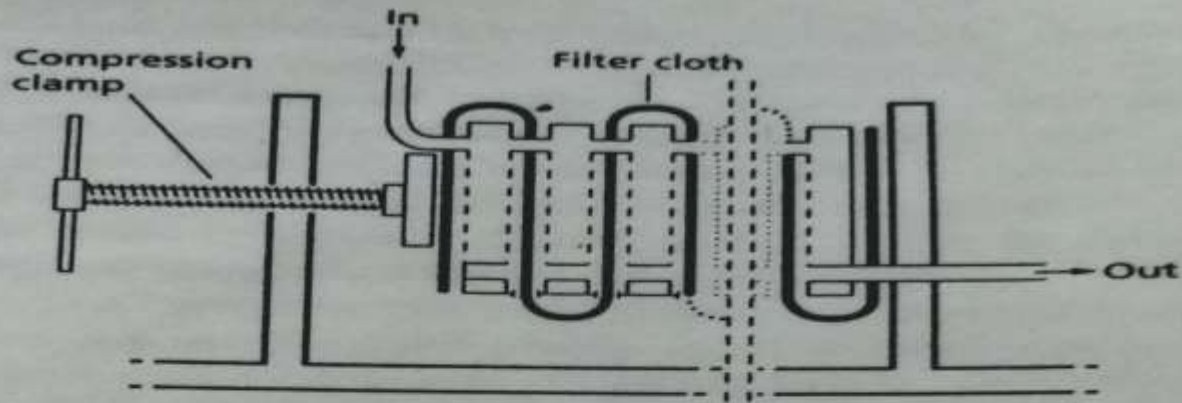


Fig. 7.8 A plate and frame filter (from Brown *et al.* (1987)).

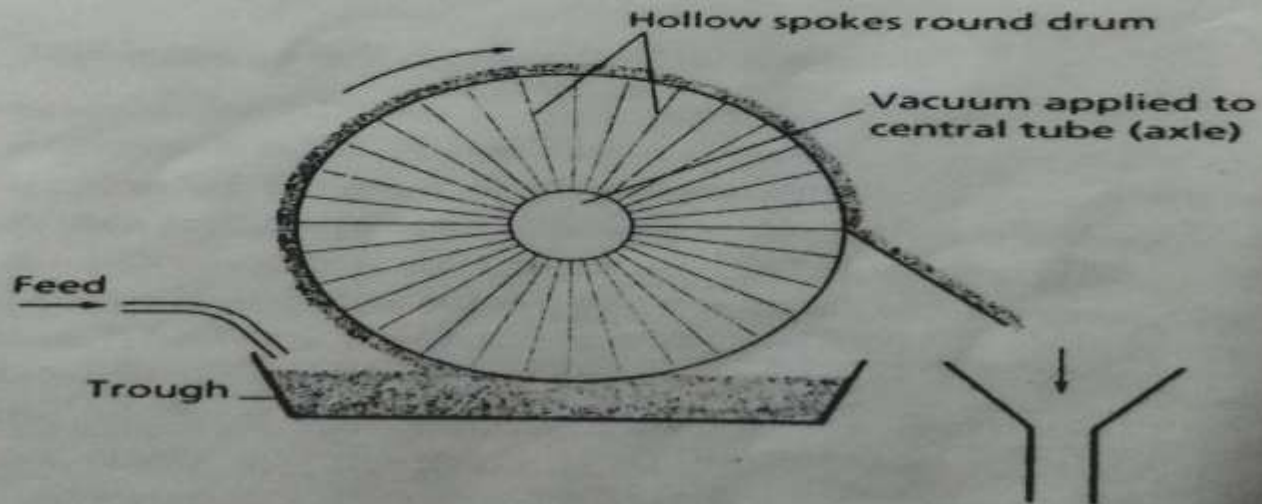


Fig. 7.9 A rotary vacuum filter showing the removal of filter cake using a knife.

B. Rotary vacuum filters:

- Continuous filtration systems, used in several industrial processes. Particularly for harvesting fungal mycelium during antibiotic production, for baker's yeast production and in dewatering sludge during waste-water treatment
- This device comprises a hollow perforated drum that supports the filter medium. This drum slowly rotates in a continuously agitated tank containing the suspension to be filtered
- Solids accumulate on the filter medium as liquid filtrate is drawn, under vacuum, through the filter medium into the hollow drum to a receiving vessel
- As the drum rotates, collected solids held on the filter medium are removed by a knife that cuts them off into a collection vessel

- Filter media may be precoated with a filter aid – diatomaceous earth – which can be continuously replenished
- The rate of filtration (flow of filtrate) is determined by the resistance of the cake & the filtration medium (for a constant pressure/ vacuum and incompressible cake)
- In terms of biosafety, these two filtration systems are unsuitable for processing toxic products, pathogens or certain recombinant DNA microorganisms and their products

Membrane Filtration

- Modern methods of filtration involve absolute filters rather than depth filters
- These consist of supported membranes with specified pore sizes that perform separations by retaining particles larger than its pore size on the surface of the membrane. Particles smaller than the pore size may pass through the membrane or may be captured within the membrane by other mechanisms
- It can be divided into three main categories – microfiltration, ultrafiltration and reverse osmosis membranes (in decreasing order of pore size)
- The rate of filtration per unit area of membrane depends on a range of pore & membrane characteristics, such as the pore size distribution, pore density, surface porosity & membrane porosity

A. Microfiltration:

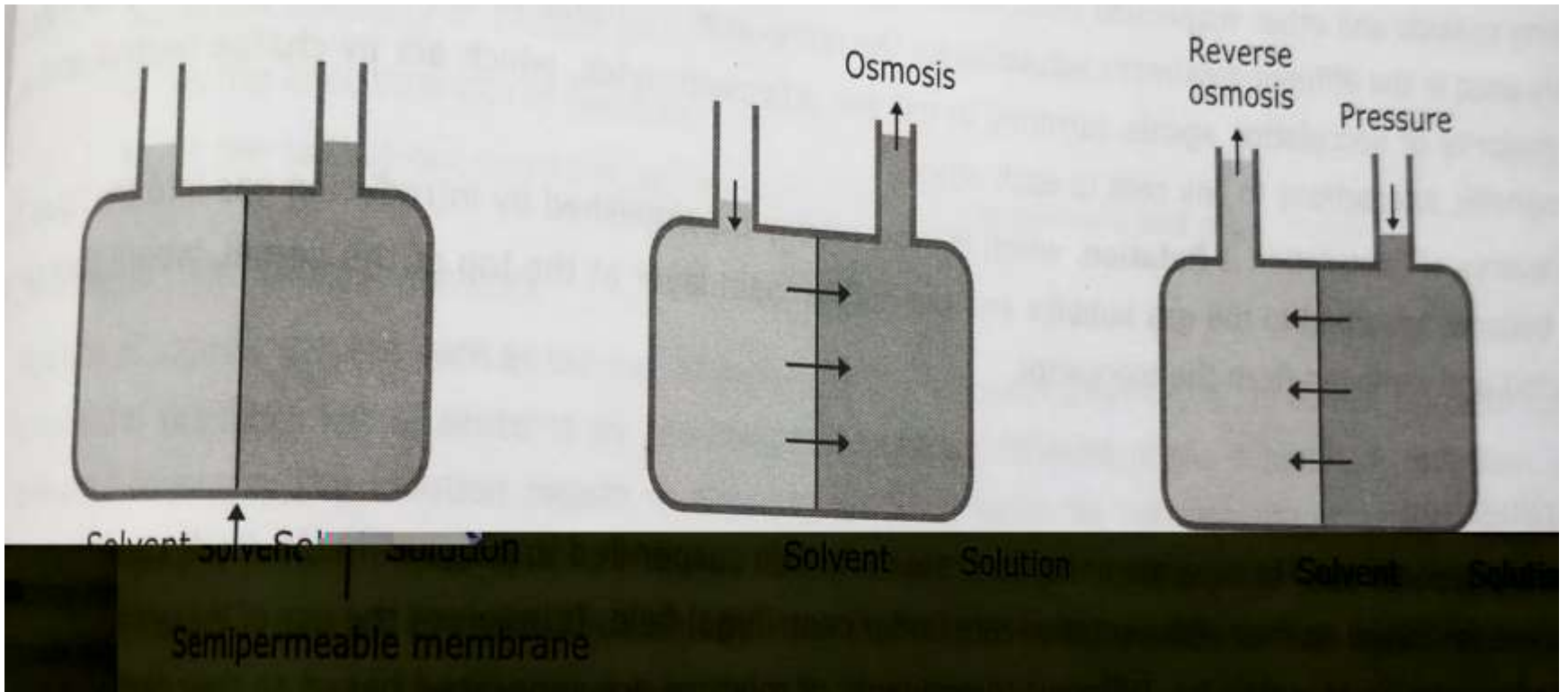
- Used to separate particles of 10^{-2} μm to 10 μm , including removal of microbial cells from the fermentation medium and the separation of blood cells from whole blood
- The driving force for microfiltration is the hydraulic pressure gradient
- It is relatively expensive due to high cost of membranes, but include quiet operation, lower energy requirement, good temperature control, easy washing of the product, no bioaerosol production
- It is also suitable for handling pathogens and recombinant microorganisms

B. Ultrafiltration:

- Similar to microfiltration except that the membranes have smaller pore sizes (0.001 – 0.1 μm) and are used to fractionate solutions according to molecular weight, normally within the range 2 kDa – 500 kDa
- The membranes have anisotropic structure, composed of a thin membrane with pores of specified diameter providing selectivity, lying on top of a thick, highly porous, support structure
- Used for product concentration & purification; also effective in removing pyrogens (bacterial cell wall LPS), cell debris and viruses from media and for whey processing

C. Reverse osmosis:

- Membranes have pore size of 10^{-2} - 10^{-4} μm diameter
- Used for dewatering or concentration steps
- Employed to desalinate sea water for drinking
- Here, the solvent molecules are forced by an applied pressure to flow through a semi-permeable membrane in the opposite direction to that dictated by osmotic forces, hence the term reverse osmosis
- A pressure is applied onto a solute-containing phase, which drives solvent from a region of high to a low solute concentration and results in the concentration of solute molecules on one side of the semipermeable membrane
- A pressure of 30-40 bar is needed to dewater a 0.6 mmol/L salt solution



Sedimentation

- A process where molecules or particles sediment downward due to gravitational force
- Separates insoluble particles from liquids
- Rate of sedimentation is a function of both size and density. Hence, larger & denser particles sediment faster
- This low-cost technology is relatively slow & is suitable only for large flocs ($>100 \mu\text{m}$ diameter)
- Extensively used for primary yeast separation in the production of alcoholic beverages and in waste water treatment

Centrifugation

- A method to separate molecules based on their sedimentation rate & centrifugal field
- Used to separate or concentrate materials suspended in a liquid medium
- Can separate particles as small as 0.1 μm diameter and is also suitable for some liquid-liquid separations
- Its effectiveness depends on particle size, density difference between the cells and the medium & medium viscosity
- The applied force on particles depends on the rotation speed & the radius of centrifuge rotor. Thus, centrifugal force causes denser and larger components to migrate outward from the axis of rotation

- Higher-speed centrifuges are required for the separation of smaller microorganisms such as bacteria, compared with yeasts
- Slow centrifugation effectively recover residual yeast cells remaining in beer after the bulk has sedimented out. However, 20,000g is required to recover suspended bacterial cells, cell debris and protein precipitates from liquid media
- Industrial centrifuges are of 4 types –
 - 1. Tubular centrifuges:**
 - Produce highest centrifugal force of 13,000 – 17,000g
 - They have hollow tubular rotor bowls providing a long flow path for the suspension, which is pumped in at the bottom & flows up through the rotor

- Particulate material is thrown to the side of the bowl and should be removed periodically. The clarified liquid passes out at the top for continuous collection

2. **Multichamber bowl centrifuges:**

- Consists of a bowl that is divided by vertically mounted interconnecting cylinders. The liquid feed passes from the center through each chamber in turn, and the smaller particles collect in the outer chamber
- Operates at 5000 – 10,000g

3. **Disc stack centrifuges:**

- Operates at 5000 – 13,000g
- Centrifuge bowl contains a stack of conical discs whose close packing aids separation

- As liquid enters the centrifuge, particulate material is thrown outwards, impinging on the underside of the conical discs
- Particles then travel outwards to the bowl wall where they accumulate
- Here, the collected material can be discharged periodically during operation

4. **Screw – decanter centrifuge:**

- Operate continuously at 1500 – 5000g and are suitable for dewatering coarse solid materials at high solid concentrations
- Used in sewage systems for the separation of sludge and for harvesting yeasts and fungal mycelium

- Advantages:

- ❖ Availability of fully continuous systems that can rapidly process large volumes in small volume centrifuges
- ❖ No consumable costs for membranes, chemicals or filter aids
- ❖ Allows aseptic processing

- Disadvantages:

- ❖ High initial capital costs
- ❖ Noise generation during operation
- ❖ Cost of electricity
- ❖ Physical rupture of cells may occur due to high shear
- ❖ Temperature cannot be controlled stringently (affect temperature-sensitive products)
- ❖ Bioaerosol generation (serious concern for certain recombinant DNA organisms or pathogens)

2. Broth conditioning:

- Mostly used in association with sedimentation & centrifugation for the separation of cells from liquid media
- In this process some properties of a microorganism or other suspended material have been altered, such that it flocculates and easily precipitates
- However, in certain cases it may be used to promote floatation
- These techniques are very economic and efficiently separate microbial cells from large volumes of medium
- Coagulating agents – simple electrolytes, acids, bases, salts, multivalent ions, polyelectrolytes

3. Cell disruption:

- Some target products are intracellular (enzymes, recombinant proteins), some may be present in the solution within the cytoplasm, while others may be insoluble and exist as membrane-bound proteins or small insoluble particles (inclusion bodies). Insoluble products must be solubilized before further purification
- **Mechanical methods** include grinding with abrasives, high – speed agitation, high-pressure homogenization & ultrasonication
- **Non-mechanical methods** include autolysis, osmotic shock, enzymic digestion of cell wall, treatment with solvent and detergents
- The cell debris generated during cell disruption is separated from the product by filtration or centrifugation

Mechanical methods

- A. Manual grinding of cells with abrasives (alumina, silica, glass beads) can be an effective means of disruption on a small scale, but results may not be reproducible
- B. High – speed agitation:
 - In industry, high-speed bead mills equipped with cooling jackets are used to agitate a cell suspension with small beads of glass, zirconium oxide or titanium carbide
 - Cell breakage results from shear forces, grinding between beads & collisions with beads
 - The efficiency of cell breakage is a function of agitation speed, concentration of beads, bead density & diameter, broth density, flow rate and temperature

C. High-pressure homogenization:

- Widely used technique for bacterial, yeast cell and fungal mycelium disruption
- In these devices, cell suspension is drawn through a check valve into a pump cylinder. At this point, it is forced under pressure (upto 1500 bar) through a very narrow annulus or discharge valve, over which the pressure drops to atmospheric pressure
- Cell disruption is primarily achieved by high liquid shear in the orifice and the sudden pressure drop upon discharge causes explosion of the cells

- Efficiency of disruption is independent of the cell concentration, but is a function of the pressure exerted, the number of cycles through the homogenizer and the temperature
- Example – the Manton & Gaulin homogenizer (APV – type mill) are used for pilot and production-scale cell disruption
- Disruption of yeast cell preparations typically requires 3 passes through the pressure cell at 650 bar, whereas wild-type *E. coli* generally needs 1100-1500 bar.

- Disadvantage of this method is that all intracellular materials are released and therefore, the product of interest is required to be separated from a complex mixture of proteins, nucleic acids and cell wall fragments. Some fragments of cell debris are not easily separated, making the solution difficult to clarify. In addition, 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 homogenizers have to be contained to prevent the escape of aerosols

D. Ultrasonic disruption:

- Here microscopic bubbles or cavities are generated by pressure waves
- It is performed by ultrasonic vibrators that produce a high-frequency sound with a wave density of ~20 KHz/sec. A transducer converts the waves into mechanical oscillations via a titanium probe immersed in the concentrated cell suspension
- This technique has limited usage as it generates heat which can denature thermolabile proteins
- Sonication is effective on a small scale, but is not used in large scale operations due to problems with transmission of power and heat dissipation

Non-mechanical methods

A. Autolysis:

- Used for the production of yeast extract and other yeast products
- Uses microbes' own enzyme; no foreign substances are introduced into the product
- Low cost method

B. Osmotic shock:

- Useful for releasing products from periplasmic space
- Achieved by equilibrating the cells in 20% (w/v) buffered sucrose, then rapidly harvesting & resuspending in water at 4°C

C. Enzymic digestion of cell wall:

- Lysozyme is useful for lysing Gram positive organisms. It hydrolyses β -1,4 glycosidic linkages within the peptidoglycan of bacterial cell walls
- Ethylene diamine tetraacetic acid (EDTA) chelates divalent cations that stabilize the structure of outer membranes and is therefore used to improve the effectiveness of lysozyme & other substances on Gram negative bacteria
- Snail gut enzymes containing a mixture of β -glucanases, are used to destruct yeast cell wall

D. Treatment with solvent and detergents:

- Organic solvents – acetone, butanol, chloroform, methanol – have been used to liberate enzymes and other substances from microorganisms by creating channels through the cell membrane
- Simple treatment with alkali or detergents, such as lauryl sulphate or Triton X-100, can also be effective