9th lecture DOWNSTREAM PROCESSING

Every process industry that produces utile products, be it chemical, biochemical or purely bio-tech based, will have a systematic process layout which gives an overall idea about the step by step procedure involved in its large scale industrial production. Generally, the bioreactor or fermenter is considered as the basis for dividing the unit operations prior to it as upstream and following it as downstream.

UPSTREAM-PROCESSING starts from the preparation of fermentation, runs through cell propagation and product formation until the „cut" of microbial process. At this point we have the ready fermentation broth containing the desired product (see previous lectures)

DOWN-STREAM PROCESSING after the „cut" the product(s) will be isolated from the multicomponent broth and purified to marketable quality → this is the topic of this lecture.

In simple terms, the product isolation and purification steps carried out subsequent to product formation are called downstream processing. It is the most important part that decides the quality of the desired product obtained from the initial raw materials.

WHAT IS COMMON IN DOWNSTREAM TECHNOLOGIES?

- The product is in aqueous solution.
- Multiphase system: water, +solid, +oily, (+air bubbles)
- Complex system: many organic and inorganic substances, in solute, colloid and dispersed form

WHAT IS DIFFERENT?

- Wide range in product concentration: 100 ppm → 10%-ig
- Wide range in production scale: 100 g/year → 1.000.000 t/year
- Many different operations (more than in chemical industry)

OPERATIONAL SEQUENCE

There are no fixed operational sequences but general guide-lines:

1. Separation of cells If the presence of the biomass or cells causes trouble, they have to be removed. The very first step in downstream processing is solid-liquid separation, in which the cells (and other solids: medium pellets, $CaCO_3$, product crystals) are separated from the fermentation broth. This can be done by

- filtration,
- centrifugation,
- sedimentation,
- flocculation or
- gravity settling.

Each of these processes has unique principle by which it works to separate the solid content from liquid broth.

(1/b Cell disruption: only intracellular products)

If the product is stored inside the cells, the cells must be ruptured and the product must be freed. After releasing the product, we can proceed with product concentration and purifycation, as discussed next.

2. Concentration step(s) In fact the product in diluted broth may not be economical enough for efficient recovery. Enrichment of the product from the bioreactor effluents for increasing product concentration may reduce the cost of product recovery. If the desired product is

extra-cellular, we can proceed to product concentration. Here, any unwanted component which has notably different property from that of the product is removed. Water is usually the main impurity. Product concentration is carried out using the unit operations like

- Extraction
- Adsorption
- Membrane filtration
- Precipitation
- (evaporation, distillation)

3. Purification Once the product is isolated and concentrated, next is purification, which is the most costly procedure of all. In this, unwanted components which closely resemble the product in its physical and chemical properties are removed. Highly sensitive and sophisticated equipment is essential for product purification and hence it is considered to make up a significant fraction of downstream processing expenditure. Different types of chromatography are the highly popular and preferred methods.

Typical operations:

- all previous
- chromatography

4. Polishing The last part is product polishing in which the purified product is packed into a form which is stable, easily portable and convenient to use. Product sterilization and removal of trace contaminants are then performed to ensure the safety of the product. Products are purified to achieve the demands of the market/customers (standards, regulations, legal measures).

Typical operations:

- all previous
- crystallization
- drying (spray drying, lyophilisation)

PURIFICATION ↔ POLISHING

No strict distinction but different approach:

Purification: engineering approach, separation of impurities is optimized for minimal product loss.

Polishing: market approach, separation is optimized to fit the market demands even if a part of the product is lost.

However, biological or biotechnology products that are used in pharmaceutical industry demand for high grades of purity and quality to meet their standards. Here is where downstream processing becomes a crucial step in the overall process. When dealing with biological or biotech products, quality is more important than quantity. And the quality of the product is decided by the purity. This requirement has led to the increased focus on the significance of downstream processing in biotech industry.

LEVELS OF PURITY

- Human injection pharma products
- Human enteral pharma products
- Veterinary pharma products
- Food

- External pharma products
- Cosmetics (short → long contact)
- Technical – raw material for other products

The Pharmacopoeia quality is not always the best! (e.g. NaCl in dextrose.)

CELL DISRUPTION

When the product is intra-cellular, one more step is added after cell separation. This is cell disruption, to release the product contained within. Cells are broken by mechanical, physical or chemical methods

How strong is the cell wall?

Animal cells burst in deionized water, the microorganisms do not – the cell wall resists the osmotic pressure.

How large is this pressure?

Physiological saline solution = 0,9% NaCl → ~1/6 Mol → ~ 1/3 osmol → p ~ 24/3 = 8 bar → pressure vessel

KINETICS OF CELL DISRUPTION

The outflow of inner product (P_i) can be described with a first order kinetic equation – it's independent from disruption method:

$$\frac{dP_i}{dt} = -kP_i$$

Amount of product inside the cell at t = 0 is $P_i = P_{i0}$ = maximal, later it decreases with t approaching zero. Integrating this equation from 0 to t we have the

$$\int_{P_{i0}}^{P_i} \frac{dP_i}{P_i} = -\int_0^t k dt$$

form.

$$\ln P_i - \ln P_{i0} = -k(t - 0)$$

transformed

$$P_i = P_{i0} \cdot e^{-kt}$$

Engineers are not interested in cell contained product but in recovered, liberated amount (R_k)

$$R_k = P_{i0} - P_i$$

Expressed as

$$R_k = P_{i0}\left[1 - e^{-kt}\right]$$

In many cases the sensitive products (i.e. proteins) decompose during the cell disruption procedure. This phenomenon should also be considered in kinetic description of disruption. This denaturation is also going on according a first order reaction kinetics:

$$\frac{dS}{dt} = -K_d S$$

Here (S) is the specific activity of product (activity/mg protein). Its value at t=0 is S_0, at a later t is S. S/S_0 expresses the ratio or percentage of active molecules from the whole amount. K_d is the kinetic constant of decomposition process. Repeating the previous integration the decomposition shows an exponential function:

$$S = S_0 e^{-K_d t}$$

The resultant yield is the product of the two parameters:

$$R_e = R_k S$$

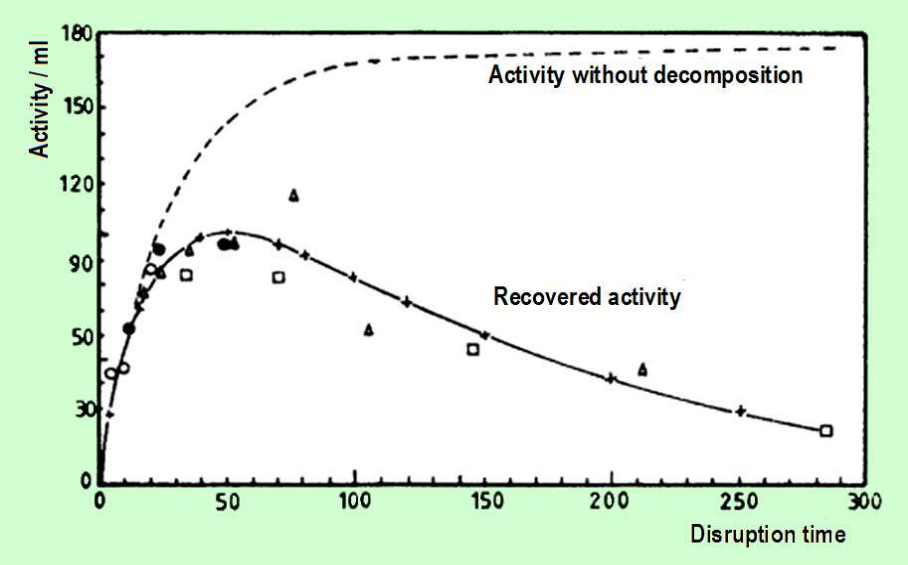
Substituting the forms:

$$R_e = P_{i0}\left[1 - e^{-kt}\right]\left[S_0 e^{-K_d t}\right]$$

Contracting the constants:

$$R_e = K\left(1 - e^{-kt}\right)e^{-K_d t}$$

There is an optimal process time when the resultant yield is maximal.



METHODS OF CELL DISRUPTION

ULTRASOUND

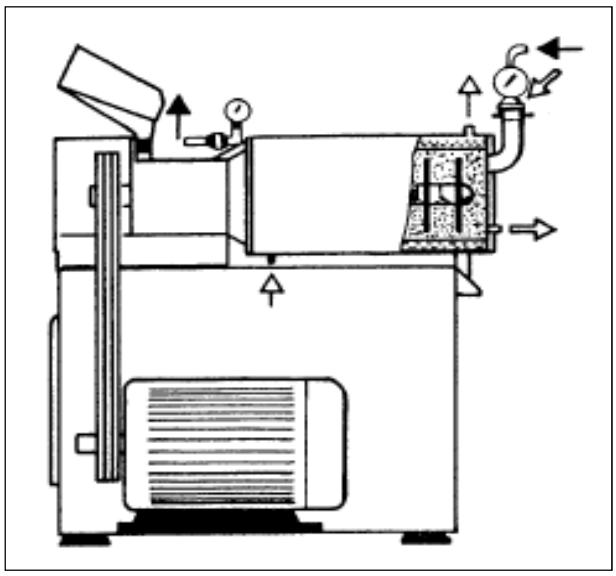
A common laboratory-scale method for cell disruption applies ultrasound (typically 20–50 kHz) to the sample (*sonication*). In principle, the high-frequency is generated electronically and the mechanical energy is transmitted to the sample via a metal probe that oscillates with high frequency. The probe is placed into the cell-containing sample and the high-frequency oscillation causes a localized low pressure region resulting in cavitation and impaction, ultimately breaking open the cells. Acoustic cavitation is the formation, growth, and implosive collapse of

bubbles in a liquid. Although the basic technology was developed over 50 years ago, newer systems permit cell disruption in smaller samples (including multiple samples under 200 µL in microplate wells) and with an increased ability to control ultrasonication parameters.
Disadvantages include:

- Heat generated by the ultrasound process must be dissipated (cooling)
- High noise levels (most systems require hearing protection and sonic enclosures)
- Yield variability
- Free radicals are generated that can react with other molecules.
- Labor size only

BEAD MILLS

Cell disruption in bead mills is regarded as one of the most efficient techniques for physical cell disruption. Various designs of bead mills have been used for microbial cell disruption. These mills consist of either a vertical or a horizontal cylindrical chamber with a motor-driven central shaft supporting a collection of offcentred discs or other agitating element. The chamber is filled to the desired level with steel or 0,1-2 mm abrasion-resistant glass beads (ballotini) which provide the grinding action. The charge of grinding beads is retained in the chamber by a sieve-plate. The horizontal configuration of the mill is known to give a better efficiency of disruption relative to the vertical one.



Advantages:

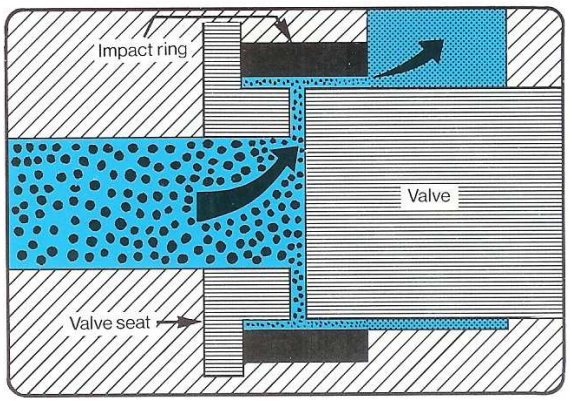
- continuous operation possible
- scale up possible (up to 600-1000 litre grinding space)

Disadvantages:

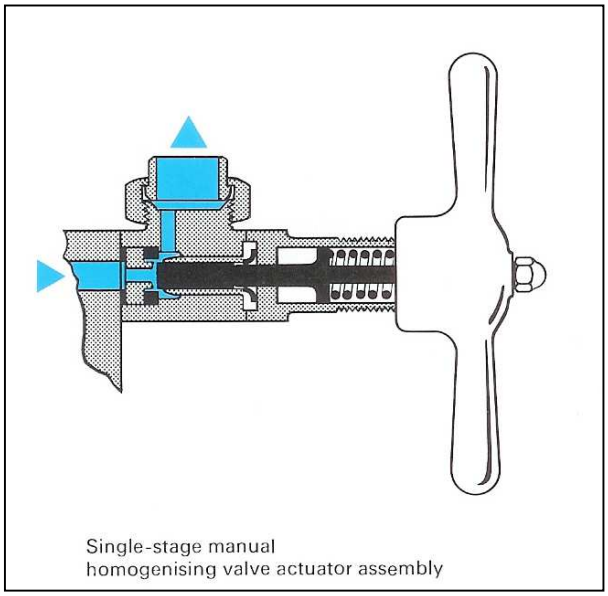
- Large energy consumption (needs cooling)

HIGH PRESSURE HOMOGENISATORS

Among the liquid shear disruption devices, the high-pressure Manton-Gaulin APV type homogenizer is probably the most widely used. The high-pressure homogenizer consists of a positive displacement piston pump with one or more (usually three) plungers. The cell suspension is drawn through a check valve into the pump cylinder and, on the pressure stroke (extreme high pressure, 200 - 600 - 1000 bar), is forced through an adjustable discharge valve with restricted orifice.



High-pressure homogenizer discharge valve unit.



The discharge pressure is controlled by a handwheel assembly, which, through a springloaded valve rod, positions the valve, in relation to the valve seat. During disruption, the cell suspension passes between the valve and its seat and impinges on an impact ring.
Disruption mechanisms:

- Flow shear, large speed differences within a thin liquid film
- Collision, in consequence of sudden changes in direction of movement cells hurtle into the impact ring.

Continuous disruption: effectivity of disruption can be calculated from the volume flow rate through the homogenizer (m^3/h^{-1}), the concentration of cell slurry (kg yeast/m^3 suspension), and the number of passes.

Advantages:

- Possible continuous operation
- Possible scale up, large Manton-Gaulin APV homogenizers capable of processing up to 53 m^3/h^{-1} at 55.2 MPa are available

Disadvantages:

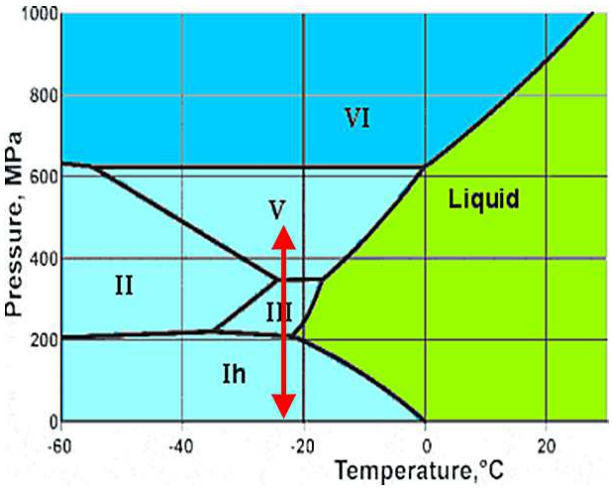
- Robust construction: single stage (200 – 600 bar) and double valves (600 -1000 bar)
- Danger of clogging
  In addition to yeasts and bacterial cell disruption, the high-pressure homogenizer has been used for the disintegration of mycelial organisms such as *Aspergillus niger*, it has not been found very suitable for this purpose. Problems were encountered with *Aspergillus sp*. due to the blockage of the homogenizing valve. Small mycelial pellets gave rise to clogging of the homogenizer valve.

X-PRESS (freeze-press)

Freeze-pressing of microbial cell suspensions can be used to disrupt the cells. Examples of the freeze-pressing equipment include the Hughes press in which a frozen paste of cells is forced through a narrow slit or orifice, at temperatures of about -25°C.
The frozen cell suspension is pressed trough an orifice.
How is it possible?

In this process phase and consequent volume changes of ice contribute to disruption.
If the pressure is high enough → 2000 – 6000 bar → the ice gets compressible = deformable.
The first triple point: -22 ˚C, 211,5 MPa
Relative density of crystal forms:

Ice-1 → Ice-3
0,92 → 1,14
volume reduction: -19%
Ice-3 → Ice-5
1,14 → 1,23
volume reduction : -7%



PHASE DIAGRAM OF ICE

In addition, solid shear due to crystalline ice is important. Cell breakage in the press yields cell wall membrane preparations that are relatively intact and may be a good method for isolation of membrane-associated enzymes.
Advantages:

- High efficiency
- No denaturation, decay
- Very concentrated cell cake can be disrupted

Disadvantages:

- Batch operation only
- No scale up
- Heavy construction

PHYSICAL METHODS OF CELL DISRUPTION

DRYING:

- *hot air drying* is not effective and denaturating.
- *air drying* at 25-30 °c for 2-3 days causes autolysis.
- *slow vacuum drying* of pastes (esp. bacteria) in a desicator causes autolysis.
- *lyophilisation* (freeze drying) of cell suspensions is particularly useful for labile enzymes.
- *solvent drying* dehydration with water-miscible solvents at low temperatures (e.g. acetone, gives acetone powder)

FREEZING – THAWING
The freeze-thaw method is commonly used to lyse bacterial and mammalian cells. The technique involves freezing a cell suspension in a dry ice/ethanol bath or freezer and then thawing the material at room temperature or 37 °C. This method of lysis causes cells to swell and ultimately break as ice crystals form during the freezing process and then contract during thawing. Multiple cycles are necessary for efficient lysis, and the process can be quite lengthy. However, freeze/thaw has been shown to effectively release recombinant proteins located in

the cytoplasm of bacteria and is recommended for the lysis of mammalian cells in some protocols.

CHANGE OF PHYSICAL CONDITIONS

Heat shock – in aqueous medium (0,5-1 hour at 60-80-100 °C, mind the heat denaturation!)

Osmotic shock – with neutral compounds (sugars, sugar alcohols, glycerol) not with salts. "Saturate" the cells with sugar, than transfer them into pure water.

Solvent treatment

- drying with acetone than dissolve the cell membranes with ether
- Autolysis of yeasts with toluene

Detergent treatment

They penetrate into the cell membrane and destroy its structure.

- Both cationic and anionic detergents
- Bile acids

Decompression

Henry's low:

$$c^* = \frac{1}{H(t)} \cdot p_i$$

At high pressure a lot of gas is dissolved in the liquid (even inside the cells).
With a sudden pressure drop the solubility drops, too - the gas forms bubbles everywhere (like in sodas) and explodes the cells.

ENZYMATIC METHODS

Enzymatic cell lysis, which is attractive in terms of its delicacy and specificity for just the cell wall structure, is restricted by the cost of the enzyme which is lost into the extract. Enzymatic cell lysis of bacteria using lysozyme has been successful for the isolation of enzymes.
Sensitivity of microorganisms to various lytic enzymes varies greatly with the type, the phase of growth and fermentation conditions.

Specific enzymes hydrolyzing the cell wall:

bacteria	- lysozyme
yeasts	- mannanase (Yeast Lyase, *Cytophaga sp*.)
moulds	- chitinase, cellulase
plant cells	- cellulase, pectinase

Multicomponent preparates:

snail enzyme	- gastric juice
induced enzymes of *Trichoderma sp*	

In some cases, *autolysis* of microbial cells without any foreign enzyme may be possible. Autolysis of yeasts can be promoted with toluene. Although autolysis is, in general, slower

than other disruption methods, it is volume independent and so 100 m3 of ceils may be lysed as fast as 100 cm3.

Conclusion

A cell disruption process cannot be considered in isolation from further downstream processing. This is because the cell disruption operation affects the physical properties of the cell slurry such as viscosity, density, particle size, and settleability of suspension, which in turn affect subsequent processing. The choice of appropriate cell disruption equipment and the extent of treatment in an equipment is an important decision in order to facilitate further downstream processing of the disrupted material.