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قسم التقنيات الاحيائية



التقنيات التخمرات

المرحلة الثالثة

الفصل الاول

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## Fermentation Technology/ Lec. 1

### Introduction to Fermentation Technology

The term 'fermentation' is derived from the Latin verb '*fervere*', to boil, which describes the appearance of the action of yeast on extracts of fruit or malted grain. The boiling appearance is due to the production of carbon dioxide bubbles caused by the anaerobic catabolism of the sugars present in the extract.

**Louis Pasteur**, who is known as the father of the fermentation process, in early nineteenth century defined fermentation as "life without air". He proved that existing microbial life came from preexisting life. There was a strong belief that fermentation was strictly a biochemical reaction. Pasteur disproved the chemical hypothesis.

Fermentation has come to have different meanings to biochemists and to industrial microbiologists. Its biochemical meaning relates to the generation of energy by the catabolism of organic compounds, whereas its meaning in industrial microbiology tends to be much broader as the term is extended to describe any process for the production of product by the mass culture of a microorganism.

- **Biochemists consider** fermentation as 'an energy-generating process in which organic compounds act both as electron donors and acceptors'; hence fermentation is 'an anaerobic process where energy is produced without the participation of oxygen or other inorganic electron acceptors.

- **Microbiologists consider** fermentation as 'any process for the production of a product by means of mass culture of microorganisms.

**Basically, the term fermentation technology denotes** microbial cell production and generation of products under either aerobic or anaerobic conditions. The secretion of metabolites from the inside of microbial cells to the surrounding medium and accumulation of the metabolite in the medium occurs as a consequence of the oxidation of monosaccharides, particularly glucose under both aerobic and anaerobic conditions. While energy is released when fats and proteins are degraded by cells, monosaccharides are the major energy and carbon source in microbial cultivation, for metabolic and economic reasons.

Successful development of a fermentation process requires major contributions from a wide range of other disciplines, particularly biochemistry, genetics and molecular biology, chemistry, chemical and process engineering, and mathematics and computer technology.

A typical operation of a fermentation process involves both **upstream processing (USP)** and **downstream processing (DSP)** stages as shown in the following Figure.

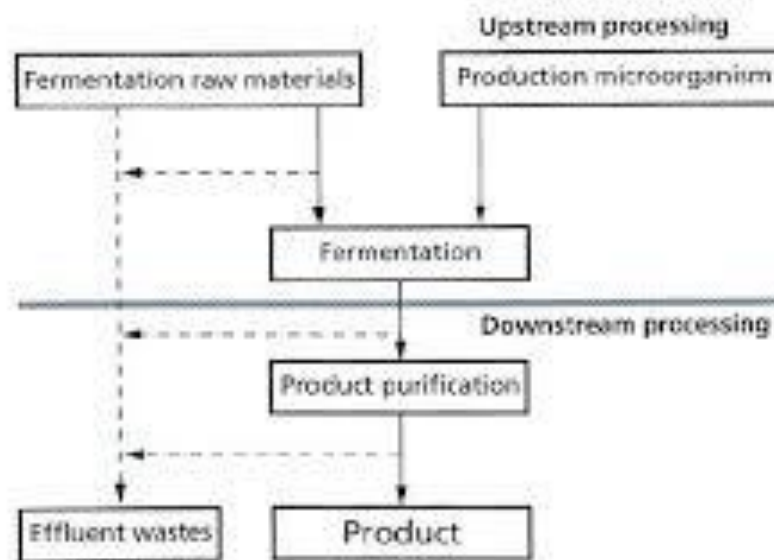


Fig. i Outline of a fermentation process.

**1. The upstream processing (USP)** is associated with all factors and processes leading to the fermentation, and consists of three main areas.

**a) The producer microorganism:** Key factors relating to this aspect are: the strategy for initially obtaining a suitable industrial microorganism, strain improvement to enhance productivity and yield, maintenance of strain purity, preparation of a reliable inoculum and the continuing development of selected strains to improve the economic efficiency of the process.

**b) The fermentation medium:** The selection of suitable cost-effective carbon and energy sources, and other essential nutrients, along with overall media optimization are vital aspects of process development to ensure maximization of yield and profit.

**c) The fermentation.** Industrial microorganisms are normally cultivated under rigorously controlled conditions developed to optimize the growth of the organism or production of a target microbial product. Fermentations are performed in large fermenters often with capacities of several thousand litres. These range from simple tanks, which may be stirred or

unstirred, to complex integrated systems involving varying levels of computer control. The mode of fermenter operation (batch, fed-batch or continuous systems), the method of its aeration and agitation, where necessary, and the approach taken to process scale-up have major influences on fermentation performance.

**2. Downstream processing (DSP)** includes all unit processes that follow fermentation. They involve cell harvesting, cell disruption, product purification from cell extracts or the growth medium, and finishing steps. Overall, DSP must employ rapid and efficient methods for the purification of the product, while maintaining it in a stable form. This is especially important where products are unstable in the impure form or subject to undesirable modifications if not purified rapidly. For some products, especially enzymes, retention of their biological activity is vital. Finally, there must be safe and inexpensive disposal of all waste products generated during the process. In any Fermentation, the central component of the system is obviously the fermenter, in which the organism is grown under conditions optimum for product formation; however, one must not lose sight of operations upstream and downstream of the fermenter. Before the fermentation is started the medium must be formulated and sterilized, the fermenter sterilized, and a starter culture must be available in sufficient quantity and in the correct physiological state to inoculate the production fermenter. The downstream processing involves extraction of the product and purification as normal chemical units of operation. The solids are separated from the liquid, and the solution and supernatant from separation unit may go further for purification after the product has been concentrated.

Regardless of the type of fermentation (with the possible exception of some transformation processes) an established process may be divided into six basic component parts:

- (i) The formulation of media to be used in culturing the process organism during the development of the inoculum and in the production fermentor.
- (ii) The sterilization of the medium, fermenters and ancillary equipment.
- (iii) The production of an active, pure culture in sufficient quantity to inoculate the production vessel.
- (iv) The growth of the organism in the production fermenter under optimum conditions for product formation.
- (v) The extraction of the product and its purification.
- (vi) The disposal of effluents produced by the process.

The interrelationships between the six component parts are illustrated in the following figure:

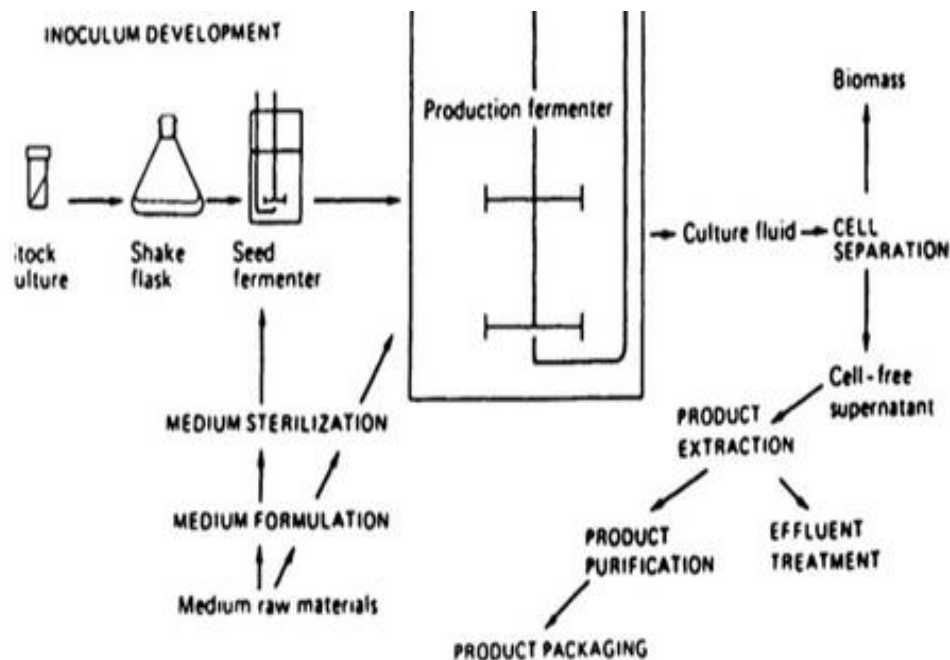


Figure: The basic component parts of a generalized fermentation process

## Fermentation products

Major fermentation products are in the area of chemicals, pharmaceuticals, energy, food and agriculture; most fermented products are formed into three types:

**1. Biomass:** the commercial production of biomass has been seen in the production of baker's yeast and production of single cell protein (SCP).

**2. Cell Products:** these products are categorized as either extracellular or intracellular. Some microbial products are primary metabolites, produced during active growth (the **trophophase**), which include amino acids, organic acids, vitamins and industrial solvents such as alcohols and acetone. However, many of the most important industrial products are secondary metabolites, which are not essential for growth, e.g. alkaloids and antibiotics. These compounds are produced in the stationary phase of a batch culture, after microbial biomass production has peaked (the **idiophase**).

**3. Modified Compounds (Biotransformation):** Almost all types of cell can be used to convert an added compound into another compound, involving many forms of enzymatic reaction including dehydration, oxidation, hydroxylation, amination, isomerisation, etc. These

types of conversion have advantages over chemical processes in that the reaction can be very specific, and produced at moderate temperatures. Examples of transformations using enzymes include the production of steroids, conversion of antibiotics and prostaglandins.

### **Industrial Microbial Strains**

The first task in the industrial fermentations is to find a suitable microorganism for use in the desired process. Microorganisms are used extensively to provide a vast range of products and services. They have proved to be particularly useful because of:

- 1- The ease of their mass cultivation.
- 2- Speed of growth.
- 3- Use of cheap substrates (which in many cases are wastes).
- 4- The diversity of potential products.
- 5- The ability to readily undergo genetic manipulation that opened up further possibilities for new products and services from the fermentation industries.

The industrial microorganism must carry out the fermentation process economically and therefore the selection of the culture to be used is **a compromise between the productivity of the organism and the economic constraints of the fermentation process.**

- Industrial microorganisms should ideally have the following important criteria that are considered in the choice of strains:

1. genetic stability
2. efficient production of the target product, whose route of biosynthesis should preferably be well characterized.
3. Limited or no need for vitamins and additional growth factors.
4. Utilization of a wide range of low-cost and readily available carbon sources.
5. Amenability to genetic manipulation.
6. Safety, non-pathogenicity and should not produce toxic agents, unless this is the target product.
7. Ready harvesting from the fermentation.
8. Ready breakage, if the target product is intracellular.
9. Production of limited byproducts to ease subsequent purification problems.

**Thermophilic properties** of the industrial microorganisms (use of an organism having an optimum temperature above 40°C) may be useful in a fermentation environment due to:

- 1- Reduces the cooling costs of a large-scale fermentation.
- 2- Having the possibility to produce thermostable products such as thermostable enzymes.

- The productivity of an organism is usually measured in its ability to convert substrate into product and to give a high yield of product per unit time.

- Fermentation industries often prefer to use established **GRAS** (generally regarded as safe) microorganisms, particularly for the manufacture of food products and ingredients. However, where pathogens and some genetically manipulated microorganisms (GMMs)

are used as the producer organism, additional safety measures must be taken. Special containment facilities are employed and it may be

possible to use modified strains ('crippled' strains) that cannot exist outside the fermenter environment. Examples of microorganisms classified as GRAS (generally recognized as safe):

- **Bacteria:** *Bacillus subtilis*; *Lactobacillus bulgaricus*; *Lactobacillus lactis*
- **Yeast:** *Candida utilis*; *Kluyveromyces lactis*; *Saccharomyces cerevisiae*
- **Fungi:** *Aspergillus niger*; *Aspergillus oryzae*; *Mucor javanicus*; *Penicillium roqueforti*

## **Fermentation Technology/ Lec. 2**

### **Industrial Microbial Strains**

**Source of Industrial microbial strains:** the specific industrial microorganisms employed are often acquired via:

1- Isolation from the natural environment.

2- Acquired from culture collections.

**\* Isolation of microbial strains**

The first stage in the screening for microorganisms of potential industrial application is their isolation. Isolation involves obtaining either pure or mixed cultures followed by their assessment to determine which carry out the desired product.

**Strategies** that are adopted for the isolation of a suitable industrial microorganism from the environment can be divided into two types, '**shotgun**' and **objective approaches**.

→ **Shotgun Strategy**: samples of free-living microorganisms, biofilms or other microbial communities are collected from animal and plant material, soil, sewage, water and waste streams, and particularly from unusual man-made and natural habitats. These isolates are then screened for desirable traits.

→ **Objective Strategy**: is to take samples from specific sites where organisms with the desired characteristics are considered to be likely components of the natural microflora. For example, when attempting to isolate an organism that can degrade or detoxify a specific target compound, sites may be sampled that are known to be contaminated by this material. These environmental conditions may select for microorganisms able to metabolize this compound. Subsequent isolation as pure cultures on solid growth media involves choosing or developing the appropriate selective media and growth conditions. Once isolated as pure cultures, strains must be screened for the desired property; production of a specific enzyme, inhibitory compound, etc. However, at this stage the level of activity or concentration of the target product per se is not of major concern, as strain development can normally be employed to vastly improve performance. Selected isolates must also be screened for other important features, such as stability and, where necessary, nontoxicity.

#### \* **Culture collections**

Microbial culture collections provide a rich source of microorganisms. There are almost 500 culture collections around the world; most of these are small, specialized collections that supply cultures or other related services only by special agreement. Others, notably national collections, publish catalogues listing the organisms held and provide extensive services for industrial and academic organizations.

In the UK for example, the National Culture Collection (UKNCC) is made up of several collections. They are housed in separate institutions and tend to specialize in bacteria, yeasts, filamentous fungi or algae of either industrial or medical importance; whereas in the USA



there is a main centralized collection, the American Type Culture Collection (ATCC), which holds all types of microorganisms.

→ The prime functions of a culture collection are:

- 1- To maintain the existing collection.
- 2- To continue to collect new strains.
- 3- To provide pure, authenticated culture samples of each organism.

Most of industrial microorganisms, irrespective of their origins, are subsequently modified by conventional strain improvement strategies, using mutagenesis or breeding programmer, to improve their properties for industrial use.

### \* **Strain improvement**

Natural isolates usually produce commercially important products in very low concentrations and therefore every attempt is made to increase the productivity of the chosen organism. Increased yields may be achieved by:

1. **Optimizing the culture medium and growth conditions**, but this approach will be limited by the organism's maximum ability to synthesize the product.
2. **Genetic modification**: The potential productivity of the organism is controlled by its genome and, therefore, the genome must be modified to increase the potential yield and this can be achieved via genetic engineering or random mutagenesis. Recombinants and mutants strains are often subjected to screening and selection to obtain strains whose characteristics are more specifically suited to the industrial fermentation process. However, such strains are **unlikely to survive well in nature**, as they often have altered regulatory controls that create metabolic imbalances. Also, they must then be maintained on specific media that select for, and help retain, the special characteristic(s).

→ Examples of some targets for strain improvement are given in below: Rapid growth; Genetic stability; Non-toxicity to humans; Ability to use cheaper substrates; Modification of submerged morphology; Elimination of the production of compounds that may interfere with downstream processing; Phosphate deregulation; Permeability alterations to improve product export rates.

### **Media for industrial fermentation**

The selection of suitable media for the industrial microbial process is vital aspects to ensure maximization of the product. In many instances, the basis of industrial media are waste products from other industrial processes, notably sugar processing wastes, lignocellulosic wastes, cheese whey and corn steep liquor. The development of media and specific conditions for the growth of microorganisms is a large part of industrial microbiology. Microorganisms can be grown in controlled environments with specific limitations to maximize the synthesis of desired products. In general, microorganisms require substrates for three main functions:

1. To synthesis new cell material
2. To synthesis extracellular products
3. To provide the maintenance energy

#### \* **Media formulation**

Most fermentations require liquid media, often referred to as broth, although some solid substrate fermentations are operated. The fermentation media should be:

- 1- Satisfied all the nutritional requirements of the microorganism and complete the technical objectives of the process.
- 2- Formulated to promote the synthesis of the target product, either cell biomass or a specific metabolite.

→ **Where biomass or primary metabolites are the target product:** the objective is to provide a production medium that allows optimal growth of the microorganism.

→ **Where secondary metabolite, such as antibiotics, is the target product:** their biosynthesis is not growth related. Consequently, for this purpose, media are designed to provide an initial period of cell growth, followed by conditions optimized for secondary metabolite production. At this point, the supply of one or more nutrients (carbon, phosphorus or nitrogen source) may be limited and rapid growth ceases.

Most fermentations, except those involving solid substrates, require large quantities of water in which the medium is formulated. General media requirements include:

- Carbon source.
- Nitrogen sources.
- Phosphorus and sulphur.

- Minor and trace elements.
- Vitamins, such as biotin and riboflavin ( required by some microorganisms)
- Oxygen: aerobic fermentations are dependent on a continuous input of molecular oxygen, and even some anaerobic fermentations require initial aeration of media, e.g. beer fermentations.
- Buffers or the pH is controlled by acid and alkali additions.
- Antifoam agents may be required.
- For some processes, precursor, inducer or inhibitor compounds must be introduced at certain stages of the fermentation.

The media adopted also depend on the scale of the fermentation. For small-scale laboratory fermentations, pure chemicals are often used in well-defined media. However, this is not possible for most industrial-scale fermentation processes, simply due to cost, as media components may account for up to 60–80% of process expenditure. Industrial-scale fermentations primarily use cost-effective complex substrates, where many carbon and nitrogen sources are almost indefinable. Most are derived from natural plant and animal materials, often byproducts of other industries, with varied and variable composition. Small-scale trials are usually performed with each new batch of substrate, particularly to examine the impact on product yield and product recovery.

→ The main factors that affect the choice of individual raw materials are as follows:

- 1- **Cost and availability:** ideally, materials should be inexpensive, and of consistent quality and year-round availability.
- 2- **Ease of handling in solid or liquid forms, along with associated transport and storage costs,** e.g. requirements for temperature control.
- 3- **Sterilization requirements and any potential denaturation problems.** A medium that is easily sterilized with minimum thermal damage is vitally important. Thermal damage not only reduces the level of specific ingredients, but can also produce potentially inhibitory byproducts that may also interfere with downstream processing.
- 4- **Formulation, mixing, complexing and viscosity characteristics** that may influence agitation, aeration and foaming during fermentation and downstream processing.
- 5- **The levels and range of impurities.** The higher levels of impurities may generate further undesired products during the process which require more costly and complex

recovery and purification steps downstream, and possibly increased waste treatment costs. **Carbon source** is required for all biosynthesis leading to reproduction, product formation and cell maintenance. In most fermentations it also serves as the energy source. Carbohydrates are traditional carbon and energy sources for microbial fermentations, although other sources may be used, such as alcohols, alkanes and organic acids. Animal fats and plant oils may also be incorporated into some media, often as supplements to the main carbon source. Carbon requirements may be determined from the **biomass yield coefficient (Y)**.

Some examples of carbon used in the industrial microbial processes: molasses, malt extract, starch and dextrin, sulphite waste liquor, cellulose, whey, alkanes and alcohols, hard animal fats and plant oils.

**Nitrogen sources:** Most industrial microbes can utilize both inorganic and organic nitrogen sources. Inorganic nitrogen may be supplied as ammonium salts, often ammonium sulphate and diammonium hydrogen phosphate, or ammonia. Organic nitrogen sources include amino acids, proteins and urea. Nitrogen is often supplied in crude forms that are essentially byproducts of other industries, such as corn steep liquor, yeast extracts, peptones and soya meal. Purified amino acids are used only in special situations, usually as precursors for specific products. Some examples of nitrogen sources used in the industrial microbial processes: corn steep liquor, yeast extracts, peptones and soya meal. Purified amino acids are used only in special situations, usually as precursors for specific products.

## **Types of Media**

Media used in fermentation can be broadly divided into three categories based on their composition: synthetic, semi-synthetic and complex.

### **1. *Synthetic Media (chemically defined media)***

Synthetic media are fully chemically defined. All the components are known, as is the specific concentration of each of the components. Such media are usually quite simple, containing a carbon/energy source, a nitrogen source, and a range of salts (usually inorganic). Synthetic media are useful in research and laboratory situations where experimental accuracy is paramount and data interpretation needs to be clear.

### **2. *Semi-synthetic Media***

Semi-synthetic media are largely chemically defined, as above, but include one or more poorly specified component(s) of variable but controlled composition, for example, yeast extract, which is particularly useful if a cell line requires a range of B vitamins. Such media are useful in research and laboratory situations where a particular organism has a requirement for a substrate that would be too expensive to supply in the pure form on a routine basis.

### **3. *Complex Media***

Complex media are largely composed of substances that are usually of plant or animal origin, and that have poorly defined and variable composition. These materials vary from batch to batch, and composition is influenced by time of year, location of origin, and small changes in production methods. Seasonal availability may change appreciably. They are usually relatively cheap, can be supplied in bulk and the source should be reliable. Substrates such as beet and cane molasses, corn steep liquor, soya bean meals and extracts, whey powders, hydrolyzed starches and a range of many different oils all fall into this category.

## **Fermentation Technology/ Lec. 3**

### **Culture systems**

In fermentation process, it is important that the mode of operation of the fermentor (culture system used) is selected and optimized carefully to maximize yield of product. Fermentation process can be carried out as: **Batch culture, Continuous culture and Fed batch culture.**

#### **Batch culture**

Batch culture involves adding all nutrients to the fermentor prior to inoculation; no nutrients are added to, or medium removed from the fermentor during the culture phase. This system is thus a closed system in terms of nutrition.

- ★ What problems might be caused by a nutritionally closed system (batch culture)?

Obviously in batch culture, enough nutrients must be added prior to inoculation to sustain growth throughout the complete culture system. This means that excess nutrient is initially present and this may inhibit or repress product formation. Towards the end of the growth phase, when most growth occurs and much of the product may be formed, the nutritional environment changes rapidly as nutrients become depleted.

- ★ If the biomass concentration ( $X$ ), cell numbers ( $N$ ) or another parameter directly proportional to biomass is monitored throughout the growth cycle of a unicellular organism in batch culture, a plot of biomass

against time can be made. As illustrated in the following Figure, the inoculated culture will pass through a number of phases:

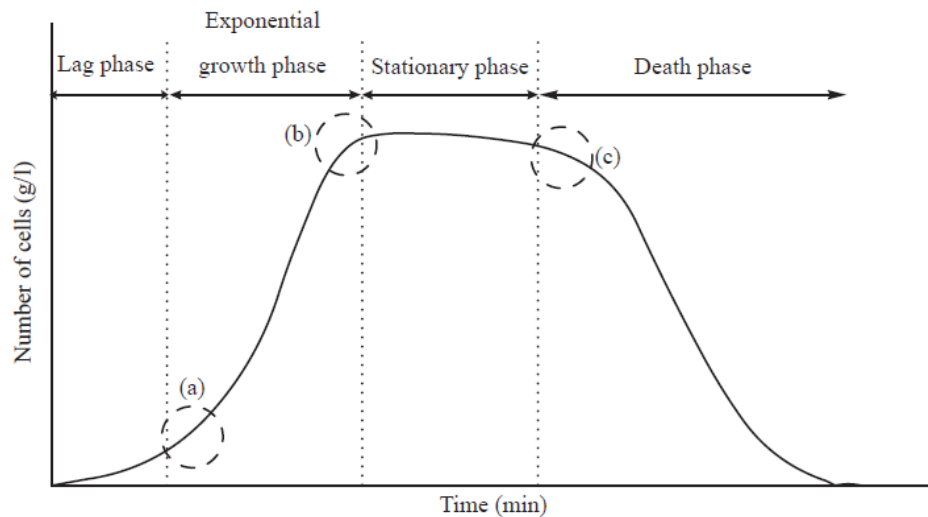


Figure: Growth curve of a batch culture. (a) Acceleration phase, (b) Retardation phase and (c) Declining phase.

### 1- Lag phase

The first period after inoculation, during which no growth takes place. This period may be considered as a time of adaptation (the microorganisms are adjusting to the new environment). In a commercial industrial process, the lag phase should be reduced or avoid as much as possible as it is non-productive.

★ Suggest how the lag phase could be minimized or avoided?

Lag phase occurs because organisms have to adapt to new conditions after inoculation. By preparing the inoculum up under identical conditions (medium composition, temperature and pH) as those in the production fermentor, and ensuring that at the time of inoculation the inoculum is in the rapid growth phase, the lag phase can be largely avoided.

### 2- Exponential growth phase

Once the lag phase has been completed, organisms enter the phase of rapid growth during which microorganisms multiply at a very rapid pace and thus the growth rate gradually increases. The cells grow at a constant and maximum rate. In this period, the cells number increase exponentially and therefore is known as the log, or exponential, phase.

For unicellular organisms that divide by binary fission (division of the cell into two equally sized daughter cells), growth in this phase will be exponential, as outlined below.

If the initial number of cells is  $N_0$ , then after 1 generation the number will be  $N_0 \times 2^1$ , after 2 generations  $N_0 \times 2^2$ , after 3 generations  $N_0 \times 2^3$  ... and so on.

Thus, in an exponentially growing culture, the number of organisms after a given time ( $N_t$ ) will be given by:

$$N_t = N_0 \times 2^n \text{ (where } n \text{ is the number of generations)}$$

Microbiologist usually measure the growth rate of an organism in such circumstances in terms of the doubling time ( $t_d$ ), or mean generation time (MGT), which is the time taken for a doubling in numbers or biomass to occur. However,  $t_d$  is inversely related to growth rate, and hence is not a suitable constant by which to model growth processes.

The specific growth rate constant  $\mu$ : is a direct measure of the rate of growth of an organism,

If  $N$  represents the number of organisms at a given time, then the increase in numbers with time (*e.g.*, the growth rate),  $(dN/dt)$ , can be expressed as:

$$dN/dt = \mu N$$

Where  $\mu$  is the specific growth rate constant (the rate of increase in biomass per unit of biomass). This equation holds true for biomass concentration ( $X$ )

$$dx/dt = \mu X$$

From equation, it can be shown that

$$\frac{\ln X_t - \ln X_0}{t_t - t_0} = \mu$$

Where  $X_0$  is the biomass concentration at an initial time ( $t_0$ ),  $X_t$  is the biomass concentration at a subsequent time ( $t_t$ ), and  $\ln$  denotes the natural logarithm.

In terms of  $\log_{10}$ , the equation becomes:

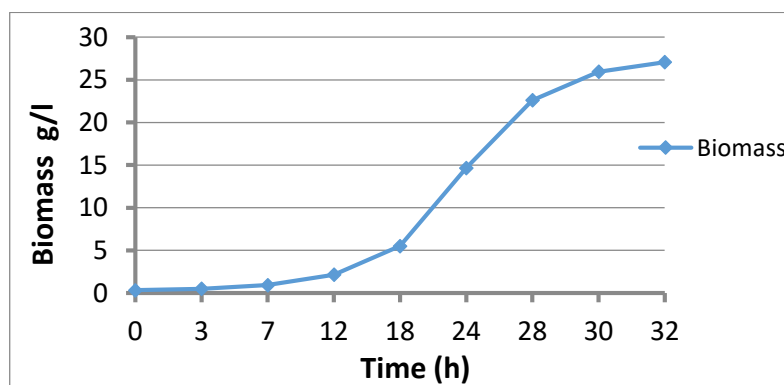
$$\frac{\log_{10} X_t - \log_{10} X_0}{t_t - t_0} = \frac{\mu}{2.303}$$

### **Exercise 1:**

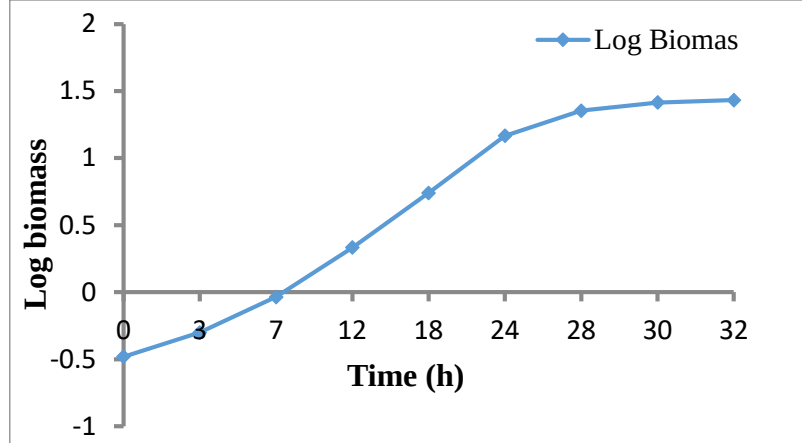
Below are given data relating to the increase in biomass with time in a batch culture. Calculate  $\mu$  from the data. Note that the units of  $\mu$  are  $\text{h}^{-1}$ .

| <u>Time (h)</u> | <u>Biomass (g/l)</u> | <u>Time (h)</u> | <u>Biomass (g/l)</u> |
|-----------------|----------------------|-----------------|----------------------|
| 0               | 0.33                 | 24              | 14.66                |
| 3               | 0.50                 | 28              | 22.60                |
| 7               | 0.92                 | 30              | 25.95                |
| 12              | 2.15                 | 32              | 27.08                |
| 18              | 5.50                 |                 |                      |

### **Solution:**



If the values for biomass concentration ( $X$ ) are converted to  $\log_{10}$  and plotted against time, a straight line portion of the graph will be apparent between 3-28 hours.



Choose a point at the start of this straight line portion ( $X_0$ ) and read off the time ( $t_0$ ). For instance if  $t_0 = 7$  hours,  $X_0 = 0.92$ . If  $t_t = 24$  hours,  $X_t = 14.66$

$$\frac{\log_{10}(14.66) - \log_{10}(0.94)}{24 - 7} = \frac{\mu}{2.303}$$

$$\frac{1.166 - (-0.027)}{17} \times 2.303 = \mu$$

$$\frac{1.193}{17} \times 2.303 = \mu$$

$$\text{Thus } \mu = 0.16$$

### **Exercise 2:**

Under *given conditions*, an organism grows with  $\mu = 0.45 \text{ h}^{-1}$ . Assuming exponential growth throughout and the same conditions, how long will it take for the population to increase from  $1.30 \times 10^4 \text{ cells/L}$  to  $2.56 \times 10^5 \text{ cells/L}$  under the same conditions?

$$\frac{\log (2.26 \times 10^5) - \log (1.30 \times 10^4)}{t_t - t_0} = \frac{0.45}{2.303}$$

$$\frac{t_t - t_0}{1.294} = \frac{2.303}{0.45}$$

$$t_t - t_0 = \frac{2.303}{0.45} \times 1.294$$

$$t_t - t_0 = 6.6 \text{ hours}$$

Thus it will take 6.6 hours for the biomass to increase by the stated amount.

- ★ The growth rate ( $\mu$ ) of an organism is not fixed but can vary with environmental conditions such as temperature and concentration of particular substrates (nutrients). For example, at low concentrations, a given substrate can limit the growth.
- ★ The substrate concentration influences the total amount of biomass formed. The growth yield ( $Y$ ) is the factor which relates the quantity of biomass formed from utilization of a given quantity of substrate.



- **Biomass yield coefficient (Y):** an index of the efficiency of conversion of a substrate into cellular material.

As cells grow, there is a linear relationship between the amount of biomass produced and the amount of substrate consumed. This relationship is expressed quantitatively using the biomass yield  $Y_{xs}$ .

$$Y_{x/s} = \frac{\text{g cells produced}}{\text{g substrate consumed}} = \frac{X_t - X_0}{S_0 - S_t}$$

Where,  $X_0$  and  $S_0$  are biomass and substrate concentrations respectively, at initial time.  $X_t$  and  $S_t$  are biomass and substrate concentrations respectively, at a subsequent time.

### **Exercise 3:**

Below are given data relating to increase in biomass concentration of an organism with time and the concomitant decrease in a particular substrate with time. Calculate  $Y_{x/s}$  for growth of the organism on the substrate.

| <u>Time (h)</u> | <u>Biomass (kg/L)</u> | <u>Substrate concentration (kg/ L)</u> |
|-----------------|-----------------------|----------------------------------------|
| 0               | 0.33                  | 55.0                                   |
| 3               | 0.50                  |                                        |
| 7               | 0.92                  |                                        |
| 12              | 2.15                  |                                        |
| 18              | 5.50                  | 45.06                                  |
| 24              | 14.66                 |                                        |
| 28              | 22.60                 |                                        |
| 30              | 25.95                 |                                        |
| 32              | 27.08                 | 3.56                                   |

Solution:

$$Y_{x/s} = \frac{\text{g cells produced}}{\text{g substrate consumed}} = \frac{X_t - X_0}{S_0 - S_t}$$

$$Y_{x/s} = \frac{27.08 - 0.33}{55.0 - 3.56}$$

$$Y_{x/s} = \frac{26.75}{51.44}$$

$$Y_{x/s} = 0.52 \text{ kg dry biomass per kg substrate}$$

### **Exercise 4:**

An organism grows on a given substrate in given conditions with  $Y_{xs} = 0.45$  (kg dry biomass per kg substrate). How much biomass will be produced by the utilization of 10kg substrate under the given conditions?

$$Y_{x/s} = \frac{\text{g cells produced}}{\text{g substrate consumed}}$$

$$0.45 = \frac{X_t - X_0}{10}$$

$$0.45 \times 10 = X_t - X_0$$

$$X_t - X_0 = 4.5$$

If  $X$  represents the biomass concentration at a given time, then the rate at which substrate is utilized (the change in substrate concentration  $[S]$  with time) is given by

$$\frac{d[S]}{dt} = q^x$$

Where  $q^x$  is the metabolic quotient for the given substrate (**the rate of substrate utilization per unit of biomass**). It can be shown that

$$q = \frac{\mu}{Y_{xs}}$$

Thus, if  $Y_{xs}$  for an organism is known, the value of  $q$  at a given growth rate can be calculated.

#### **Exercise 5:**

Under given conditions 1kg dry biomass of an organism is produced from utilization of 2.2 kg of sucrose. What is the metabolic quotient ( $q$  sucrose) when the organism grows at a rate of  $\mu = 0.55 \text{ h}^{-1}$ ?

$$\begin{aligned} q &= \frac{\mu}{Y_{xs}} \\ q &= \frac{0.55}{1/2.2} \\ &= \frac{0.55}{0.45} \end{aligned}$$

Thus  $q = 1.22 \text{ kg substrate per hours per kg biomass}$

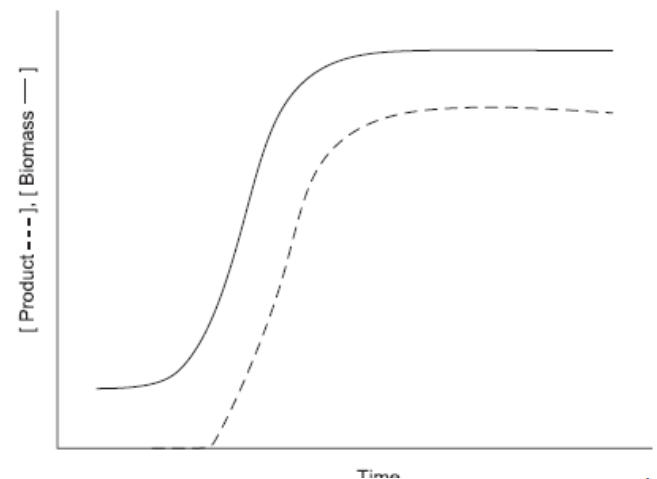
- **Batch productivity ( $P_p$ ) g product (h)<sup>-1</sup>:** The batch productivity is calculated by dividing the final concentration of the product over the total time of the batch.
- 3- **Stationary Phase:** as the microorganisms grow during the exponential phase, the nutrients become exhausted and / or growth inhibitors (toxic metabolite) accumulate. Therefore, population growth ceases, and the growth curve becomes horizontal. Increase in cell number due to cell divisions exactly balanced by a decrease in cell number due to death. Cell death may result from Nutrient limitation & Toxic waste accumulation (e.g. acid buildup from fermentation); as well as O<sub>2</sub> depletion, critical population level reached
- 4- **Death phase:** Stationary phase, in a standard bacterial growth curve, is followed by a die-off of cells, called Death phase (or decline phase). It is the period in which the cells are dying at an exponential rate. Some of the reasons are: continued accumulation of wastes, loss of cell's ability to detoxify toxins, etc.

Table: Summary of the growth phases shown in Growth curve of a batch culture

| Growth phase | Rate of growth | Comments                                                                                                |
|--------------|----------------|---------------------------------------------------------------------------------------------------------|
| Lag          | Zero           | Innoculum adapting with the changing condition (temperature, pH)                                        |
| Acceleration | Increasing     | Trivial                                                                                                 |
| Exponential  | Constant       | Population growth changes the environment of the cells                                                  |
| Retardation  | Decreasing     | The effect of changing conditions appear                                                                |
| Stationary   | Zero           | One or more nutrients are exhausted to the threshold level of the cell                                  |
| Decline      | Negative       | The duration of stationary phase and the rate of decline are strongly dependent on the kind of organism |
| Death phase  | Negative       | Cells lyse due to lack of nutrient                                                                      |

The batch culture growth curve gives a good indication of when to stop the fermentation.

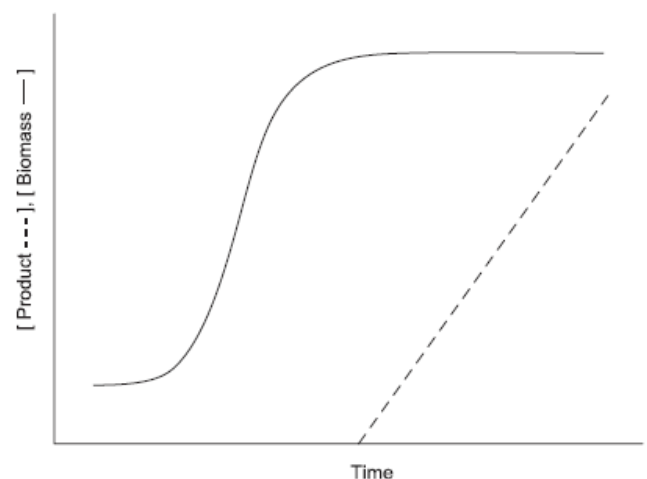
Growth-associated products (primary metabolites) are produced during the exponential phase with their formation decreasing when growth ceases. Typically, the rate of product formation directly relates to the rate of growth. The fermentation can be terminated at the end of the exponential growth phase before the cell enters stationary phase. This growth phase is sometimes referred to as the trophophase.



*Figure:*

*Growth associated product formed during the period of active culture growth*

Non-growth-associated products (e.g., classic secondary metabolites) have a negligible rate of formation during active cell growth. These secondary metabolites are produced as the cells enter stationary phase; this can also be described as idiophase. Most antibiotics are produced as secondary metabolites. The fermentation can then be stopped just before the cells enter the death phase.



*Figure:*

*Nongrowth associated product formed during the period of nonculture growth (stationary phase)*

## Fermentation Technology/ Lec. 4

### Continuous Culture

Continuous culture involves removing medium (culture) from the fermentor continuously and replacing this with fresh medium, usually at the same rate (so that the working volume remains constant). This is an open system.

The rate of flow of medium into a system of continuous culture is known as the dilution rate ( $D$ ). When the number of cells in the culture vessel remains constant over time (steady state), the dilution rate is said to equal the rate of cell division in the culture, since the cells are being removed by the outflow of medium and are being replaced by an equal number through cell division in the culture (formation of new biomass by the culture is balanced by the loss of cells from the vessel).

If the working volume of the fermentor is  $V \text{ m}^3$  and the flow rate in and out  $F \text{ m}^3\text{h}^{-1}$  then the dilution ( $D$ ) is given by:

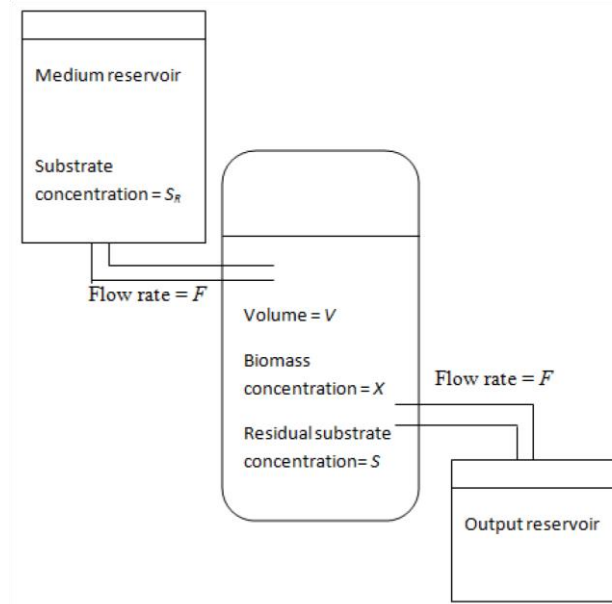
$$D = \frac{F}{V}$$

The unit of  $D$  is thus per hour ( $\text{h}^{-1}$ ).

➤ Two control methods are used in continuous culture fermentation, namely, chemostat and turbidostat.

#### ❖ chemostat:

In the chemostat the growth of the cells is controlled by the availability of the growth limiting chemical component of the medium (usually carbon or nitrogen source). This component is present in the inflowing medium at a concentration low enough to limit the growth rate of the organism.



**Figure: chemostat mode of continuous culture**

In such a system, the rate of change in biomass concentration in the culture is given by the increase in biomass due to growth minus the loss of biomass carried out in the outflow. The increase in biomass per unit volume due to growth is given by  $\mu X$  and decrease due to loss in the outflow is  $DX$  ( $D$  is the dilution rate of the system). Thus in such a continuous system: rate of increase in biomass concentration:

$$\frac{dX}{dt} = \mu X - DX$$

Once the continuous system has been operating for a while a steady state can be achieved, in which the biomass concentration remains constant

$$\frac{dx}{dt} = 0$$

(The biomass concentration is constant, so the rate of increase is zero) The amount of biomass lost from the fermentor by dilution (diluted out in outflowing medium) is replaced exactly by growth, so the

$$\mu X - DX = 0$$

The value of  $x$  is constant with time, so:

$$\mu = D$$

Thus, in the steady state the growth rate is equal to the dilution rate.

In the steady state the nutritional environment (nutrient concentration) also remains constant, making it easier to optimise conditions for product formation.

★ The relationship between  $\mu$  and  $D$ :

1. If the cells growing in the vessel at a rate greater than the dilution rate ( $\mu > D$ ), then the ratio of  $dx/dt$  will be positive. Thus the biomass concentration in the vessel increases.
2. If the growth rate of the cells less than the dilution rate ( $\mu < D$ ), and the proportion of  $dx/dt$  will be negative. The concentration of cells decrease to zero, and the cells will be washed out of the vessel at a rate greater than they are being produced, resulting in a decrease in biomass concentration.
3. If  $\mu = D$ , the concentration of cells in the vessel remain constant over a period of time and the ratio of  $dx/dt = 0$ , the steady state will be established.

★ When the growth or bacterial division inside culture vessel stopped with continuous mixing, the probability of leaving the microorganism of the container be equal, and this process is called **washing (Wash-out)**, and is defined as the rate of microorganisms leaving the container when the growth ceased with the constant flow of substrate into the culture vessel and is expressed in the relationship:

$$- dx/ dt = DX$$

### ❖ Changes in substrate concentration

The rate of change in the substrate concentration in chemostat culture is given by:

$$\text{Rate of change in residual substrate} = \text{rate of substrate input by inflow} - \text{rate of substrate output in outflow} - \text{rate of substrate consumption by organisms}$$

Or

$$\frac{dS}{dt} = DS_R - DS - \frac{\mu X}{Y}$$

Where  $S_R$  is the concentration of substrate in inflow and  $S$  is the residual substrate concentration in the fermenter and the outflow. The equation for the rate of substrate consumption by organisms is derived from equation  $q = \frac{\mu}{Y_{XS}}$

Rearranging equation gives

$$\frac{dS}{dt} = D(S_R - S) - \frac{\mu X}{Y_g}$$

In the steady state the concentration of residual substrate does not change with time, but is constant.

$$\frac{dS}{dt} = 0, \quad \text{and so} \quad D(S_R - S) = \frac{\mu X}{Y_g}$$

And

$$\frac{D}{\mu} (S_R - S) Y_g = X$$

Since in the steady state

$$\mu = D, \quad \frac{D}{\mu}$$

The biomass concentration in the fermentor and the outflow ( $X$ ) is given by:

$$X = (S_R - S) Y_g$$

This equation signify that the biomass formed = the growth yield multiplied by the substrate used. In chemostat culture the substrate used is  $S_R - S$  (the difference between the substrate concentrations in the inflow and outflow).

The output of biomass from a continuous culture system is given by the rate at which medium passes out of the outflow (e.g. the flow rate,  $F$ ) multiplied by the concentration of biomass in that outflow. Thus:

$$\text{Output} = FX$$

As  $D = \frac{F}{V} \implies F = DV,$

Therefore, output =  $DVX$

The productivity of this system (output per unit volume) is thus

$$\text{Productivity } \frac{DVX}{V} = DX$$

★ In a continuous culture, if the substrate is depleted below the level that supports the growth rate dedicated by the dilution rate, the following sequence of events takes place:

- a) The growth rate of cells will be less than the dilution rate and they will be washed out of the vessel at a rate greater than they are being produced, resulting in a decrease of biomass concentration both within the vessel and in the overflow
- b) The substrate concentration in the vessel will rise because fewer cells are left in the vessel to consume it.
- c) The increased substrate concentration in the vessel will result in the cells growing at a rate greater than the dilution rate and biomass concentration will increase.
- d) The steady state will be re-established.

Thus, a chemostat is a nutrient limited self-balancing culture system, which may be maintained in a steady state over a wide range of sub-maximum specific growth rates.

★ Suggest how a continuous culture could be operated to avoid (i) washout and (ii) wastage of unused nutrient?

- (i) If the biomass is recovered from the output stream (for example by centrifugation) and returned to the fermentor, washout will be prevented. Incidentally, with biomass feedback, the fermentor can be operated at higher dilution rates and with higher outputs than would otherwise be possible.
- (ii) Wastage of unused nutrient can be overcome by recycling the medium from the output stream after separation of the biomass. This also saves water. Such procedure may not be possible if the medium contains antagonists to growth or product formation.

### Exercise:

An organism grows in a continuous chemostat culture of 50 m<sup>3</sup> working volume, with sucrose as the growth limiting nutrient, at  $D = 0.45 \text{ h}^{-1}$ . The steady state biomass concentration is 3.75 kg dry biomass m<sup>-3</sup> and sucrose concentration is 1.1 kg m<sup>-3</sup>. The sucrose concentration in the incoming medium is 10.0 kg m<sup>-3</sup>.

- (i) What is the metabolic quotient?
- (ii) What would the sucrose concentration in the input need to be in order for the output to be 35 kg biomass h<sup>-1</sup>?

**Solution:**

- (i) The metabolic quotient  $q = \mu / Y_g$

The growth rate is equal to  $D$ , So  $\mu = 0.45 \text{ h}^{-1}$

$Y_g$  can be calculated from equation  $X = (S_R - S)$

$$3.75 = (10.0 - 1.10)$$

$$Y_g = \frac{3.75}{(10.0 - 1.10)} = 0.42 \text{ kg biomass per kg sucrose}$$

Thus, the metabolic quotient  $q = \frac{0.45}{0.42} = 1.07$  kg sucrose per hour per kg biomass

(ii) The output of the system is given by  $DVX$

Thus, for output of 50 kg biomass h<sup>-1</sup>,  $35 = 0.45 \times 50 \times X$

$$X = \frac{35}{0.45 \times 50} = 1.56$$

For  $X$  to be  $1.56 \text{ kg m}^{-3}$ , according to the equation  $X = Y_g(S_R - S)$

$$1.56 = 0.42(S_R - 1.10)$$

$$S_R = \frac{1.56}{0.42} + 1.10 = 4.81 \text{ kg m}^{-3}$$

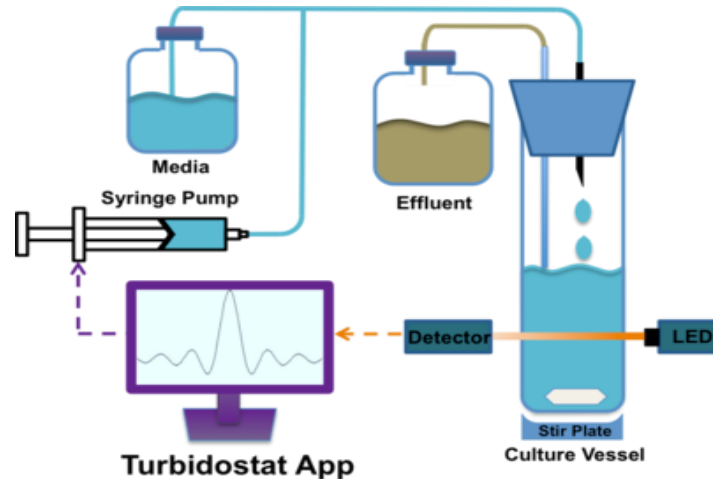
Thus for the output to be  $35 \text{ kg h}^{-1}$ , the incoming sucrose concentration would need to be  $4.81 \text{ kg m}^{-3}$ .

### ★ Turbidostat

In the Turbidostat, the concentration of cells in the culture is kept constant by controlling the flow of medium such that the turbidity of the culture is kept within certain, narrow limits. This may be achieved by monitoring the biomass with a photoelectric cell and feeding the signal to a pump supplying medium to the culture such that the pump is switched on if the biomass exceeds the set point and is switched off if the biomass falls below the set point.

Systems other than turbidity may be used to monitor the biomass concentration such as  $\text{CO}_2$  concentration or pH in which case it would be more correct to term the culture a biostat.

The chemostat is the more commonly used system because it has the advantage over the biostat of not requiring complex control systems to maintain a steady state. However the biostat may be advantageous in continuous enrichment culture in avoiding the total washout of the culture in its early stages.



➤ The advantages of continuous culture system are:

- 1- None productive periods (lag phase, growth phase and down time) are avoided when the systems are up and running, giving high outputs.
- 2- The chemostat system has constant nutritional environment, making it easier to optimise conditions and thus maximize output.

➤ The disadvantages continuous cultures are:

- 1- The long culture period
- 2- The complexity of operation
- 3- The failure of the system if the dilution rate is faster than the maximum growth rate of the organism
- 4- At high dilution rates a significant proportion of the substrate remains unused (is wasted)

➤ Applications of continuous culture

Continuous fermentations are particularly well suited for the production of biomass and growth-associated primary metabolites. It has been applied most widely in the production of microorganism as food/ feed protein (single cell protein or SCP).



## Fermentation technology/ Lec 5

### Fed-Batch Culture

In 1973 Yoshida and his co workers introduced the term fed-batch culture to describe batch cultures which are fed continuously, or sequentially, with medium, without the removal of culture fluid.

**Fed-batch culture** is a batch culture in which a fresh aliquot of one or more nutrient is continuously or periodically fed into the fermentor during the fermentation period without the removal of the culture fluid. In this way, nutrients can be added at the same rates as they are used up, so excess of nutrient (and any inhibition resulting from this) can be avoided.

- ★ A fed-batch culture is established initially in batch mode and is then fed according to one of the following feed strategies:
  1. The same medium used to establish the batch culture is added, resulting in an increase in volume.
  2. A solution of the limiting substrate at the same concentration as that in the initial medium is added, resulting in an increase in volume.
  3. A concentrated solution of the limiting substrate is added at a rate less than in (1) and (2), resulting in an increase in volume.
  4. A very concentrated solution of the limiting substrate is added at a rate less than in 1, 2 and 3, resulting in an insignificant increase in volume.

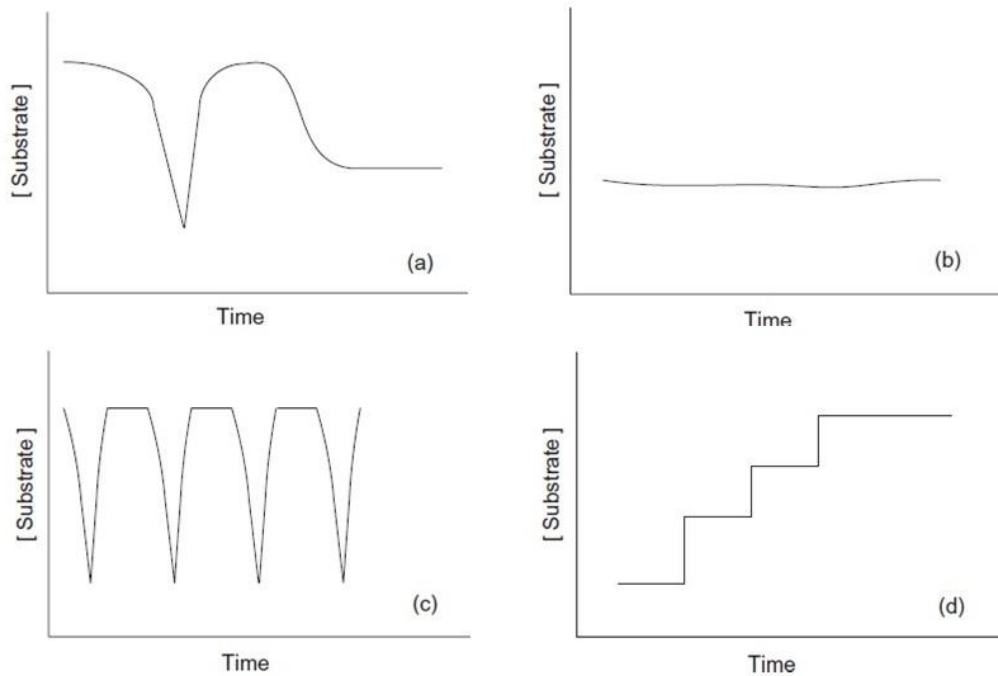
Fed-batch systems employing strategies 1 and 2 are described as **variable volume**, whereas a system employing strategy 4 is described as **fixed volume**. The use of strategy 3 gives a culture intermediate between the two extremes of variable and fixed volume.

- ❖ **Fixed Fed batch culture:** fixed volume fed-batch fermentation is one where the limiting substrate is fed without diluting the culture. The volume within the bioreactor is maintained at around the same level by feeding a super-concentrated medium containing the limiting substrate. Advantage can also be taken of the loss of volume via evaporation, since the volume can be kept constant by addition of an equal volume of liquid feed.
- ❖ **Variable fed-batch culture:** A variable fed-batch fermentation is one where the addition of the feed alters the working volume in the bioreactor. The feed can be the same medium as that used in the vessel at the beginning of the process, or it can be a concentrated solution of the limiting substrate. This allows the

organism to continue growing at its maximum specific growth rate resulting in a higher concentration of final biomass.

The addition of the feed can be over a short or long period, starting immediately after inoculation or at a predetermined point during the run. The feeding strategy can be continuous over a long period of time or incremental, with the addition of fixed volumes at given time points.

Examples of feeding regimes in fed-batch processes, (a) Variable feeding regime; (b) Continuous feeding regime; (c) intermittent feeding regime; (d) incremental feeding regime



**Figure:** Different feeding regimes in fed-batch processes, (a) Variable feeding regime;

(b) Continuous feeding regime; (c) intermittent feeding regime; (d) incremental feeding regime

The addition of fresh medium and nutrients have the effect of increasing the culture volume however this feeding strategy allows the organism to:

1. Grow at the desired specific growth rate,
2. Minimising the production of unwanted by-products,
3. Allowing the achievement of high cell densities and product concentrations.

❖ Fed-batch culture is controlled by *feed-back control* and *control without feed-back*.

1. Feed-back control– The fermentation process is controlled by monitoring process parameters like dissolved oxygen content, carbon dioxide to oxygen ratio, pH, concentration of substrate, and concentration of the product.
2. Control without feed-back– The substrates and nutrients are added at regular intervals.

❖ **Examples of some applications of fed-batch culture:**

- I. Production of baker's yeast is mostly by fed-batch culture, where biomass is the desired product. Yeast producers observed in the early 1900s that when the concentration of the malt carbon source (primarily

maltose) was too high within the culture medium, the yeast cells used this for the formation of the by-product ethanol, and not biomass.

However, if the concentration of malt was too low, yeast growth would be restricted and the final biomass yield would be reduced. To overcome this problem the feeding of the malt was carefully controlled during the exponential growth phase, and maintained at optimal concentration, minimising ethanol production and maximising biomass production

- II. An example of this system is in the production of alkaline protease (an enzyme used in biological detergents) by species of the bacterium *Bacillus*: batch feeding of nitrogen sources (ammonia, ammonium ions or amino acids) keeps these substrates at low concentrations and thus avoids the repression that these nitrogen sources exert on protease synthesis.
- III. The production of penicillin, a secondary metabolite, is also by fed-batch method. Penicillin process has two stages: an initial growth phase followed by the production phase called the '**idiophase**'. The culture is maintained at low levels of biomass and phenyl acetic acid, the precursor of penicillin, is fed into the fermenter continuously, but at a low rate, as the precursor is toxic to the organism at higher concentrations. Today, molasses is the main carbon source, and is added incrementally during the process to just balance the oxygen supply capability of the fermenter system.  
  
This is an example of the balancing act that is possible using fed-batch, namely, the carefully metered feeding of an inducer or toxic intermediate at rates that still permit active growth and synthesis to proceed. It is easy to see how difficult this kind of balance would be to achieve in batch.
- IV. *Pichia pastoris*, another commercially important organism widely used in the production of recombinant proteins, also requires a careful feeding strategy. Initially the feed consists of a substrate to achieve high cell density quickly, followed by very careful feeding of methanol, the usual inducer of the recombinant gene expression. If the methanol is present in high concentrations it can be toxic for the cells and therefore its presence must be carefully controlled.

#### ❖ Reasons and advantages of Fed-batch Control

1. **Controlling the concentration of the limiting substrate to prevent the repressive effects of high substrate concentration and avoids catabolite repression.**
2. **By careful feeding strategy the organism's growth rate and subsequent oxygen demand can be controlled.** This was one of the original aims of developing a bakers' yeast fedbatch process: to balance the oxygen transfer rate of a given fermenter with the rate of nutrient feeding in order to minimise substrate flow to the ethanol.
3. **High cell density (up to ten times greater) can be achieved by use of fed-batch over batch culture.** Batch culture limits the final cell growth due to no extra carbon being added during the run, and the carbon that is present at the beginning of the bioprocess results in catabolite repression and inhibition of growth. Using fed-batch with a careful feeding strategy, *E. coli* and *Pichia* can achieve very high cell densities of over 100 gL<sup>-1</sup>.
4. **Avoiding the toxic effects of some medium components**
5. **Extend the product formation phase**

6. **Increased production of non-growth-related secondary metabolites.** Many secondary metabolites are produced from intermediates and end products of primary metabolism. Others are formed after introduction of key precursors after the growth phase, as is seen in penicillin production with phenylacetic acid or phenoxyacetic acid, precursors of penicillins G and V respectively, being added prior to the stationary phase to allow the formation of penicillin.
7. **Reduction of broth viscosity.** This is particularly important in filamentous fungal fermentations, or where the product is highly viscous, such as the polysaccharide products of *Sphingomonas elodea* – gellan gum; and *Xanthomonas campestris* – xanthan gum. The addition of fresh medium during the fermentation run, leads to the broth being ‘diluted’, and a brief viscosity drop, allowing better aeration and agitation within the system.

## Solid state fermentation

Not all microbial production processes involve culture of the production organism in liquid medium; instead the organism can be grown on the surface of piece of a solid substrate. This solid substrate (or solid state) fermentation SSF is an established traditional technology in many countries, producing commodities such as edible mushrooms, fungal fermented foods, fungal ripened cheeses and soy sauce. Before the development of processes in liquid culture, citric acid and some microbial enzymes were produced this way.

### ★ What is solid state fermentation (SSF)?

**“Solid state fermentation involves the growth of microorganisms on solid, normally organic, materials in the absence or near absence of free water. The substrates used are often cereal grains, bran, legumes and lignocellulosic materials, such as straw, wood chippings, etc”.**

### ★ What advantage does SSF has over fermentation in liquid culture?

The major advantage is that SSF can make use of substrates that are not easily converted to liquid form, for example starchy or pectinaceous materials which tend to form viscous solutions when heated and cellulosic materials. The conversion of such materials to liquid medium can involve time, effort and expense.

Solid state fermentation involves the growth of microorganisms on moist solid particles, in situations in which the spaces between the particles contain a continuous gas phase and a minimum of visible water.

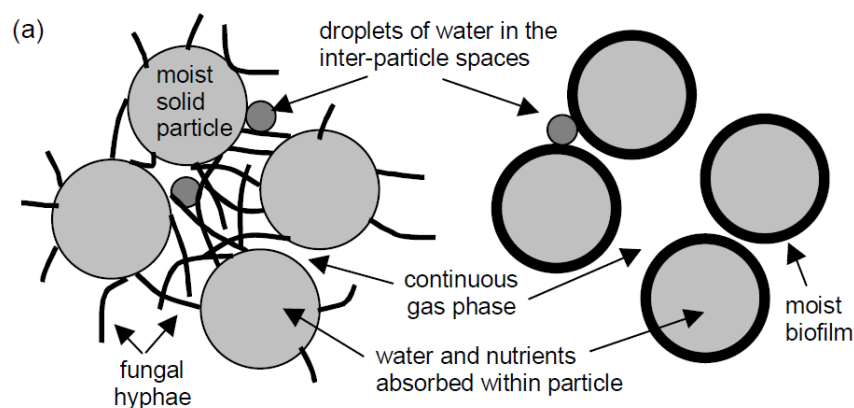


Table: Advantages and disadvantages of solid-substrate fermentations

| Advantages                                | Disadvantages                                 |
|-------------------------------------------|-----------------------------------------------|
| Potentially provide superior productivity | Slower microbial growth                       |
| Low-cost media                            | Problems with heat build-up                   |
| Simple technology                         | Bacterial contamination can be problematic    |
| Low capital costs                         | Difficulties often encountered on scale-up    |
| Reduced energy requirements               | Substrate moisture level difficult to control |
| Low waste-water output                    |                                               |
| No problems with foaming                  |                                               |

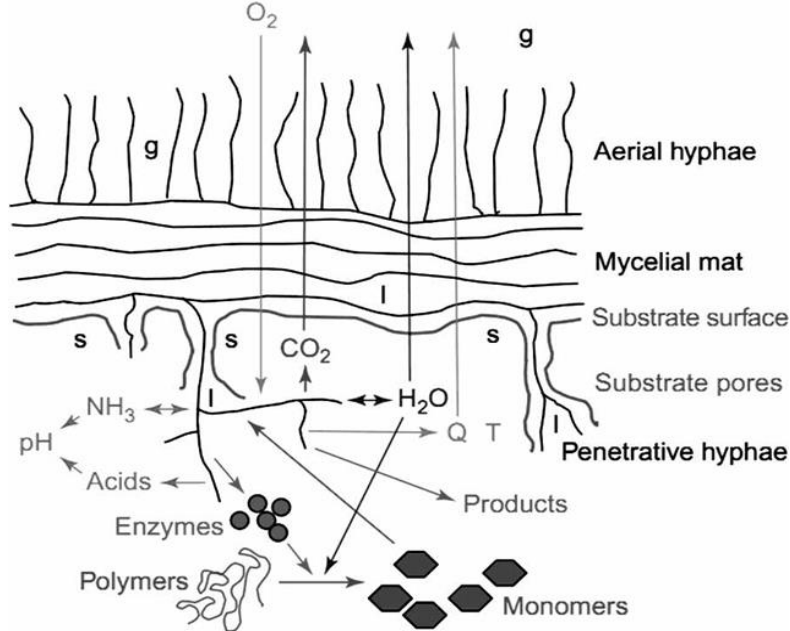
The microorganisms associated with solid-substrate fermentations are those that tolerate relatively low water activity down to  $A_w$  values of around 0.7.

- The majority of SSF processes involve filamentous fungi, although some involve bacteria and some involve yeasts
- The majority of SSF processes involve aerobic organisms.

### Substrates used in SSF

- The substrates used in SSF processes are often products or byproducts of agriculture, forestry or food processing.
- Examples of substrates used are cereal grains, bran, legumes and lignocellulosic materials, such as straw, wood chippings, etc.
- ★ Solid-substrate fermentations lack the sophisticated control mechanisms that are usually associated with submerged fermentations. Their use is often hampered by lack of knowledge of the intrinsic kinetics of microbial growth under these operating conditions.
- ★ Control of the environment within the bioreactors is also difficult to achieve, particularly the simultaneous maintenance of optimal temperature and moisture. However, in some instances, solid-substrate fermentations are the most suitable methods for the production of certain products. For example, most fungi do not form spores in submerged fermentations, but sporulation is often accomplished in solid-substrate fermentations. This method is successfully employed in the production of *Coniothyrium minitans* spores for the biocontrol of the fungal plant pathogen, *Sclerotinia sclerotiorum*.

### Environmental parameters that influence solid-substrate fermentations



A scheme of some microscale processes that occur during solid-state fermentation (SSF)

### 1- Water activity, $A_w$

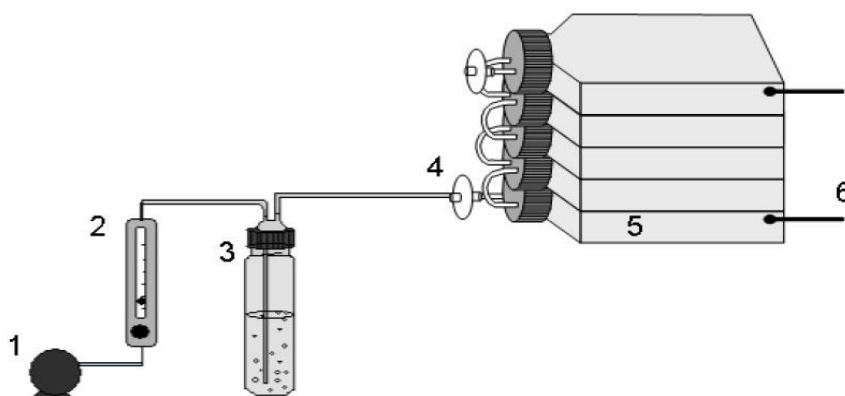
Water is lost during fermentation through evaporation and metabolic activity. This is normally replaced by humidification or periodic additions of water. If moisture levels are too low, the substrate is less accessible, as it does not swell and microbial growth is reduced. However, if the moisture levels are too high there is a reduction in the porosity of the substrate, lowering the oxygen diffusion rates and generally decreasing gaseous exchange. Consequently, the rate of substrate degradation is reduced and there is also an increased risk of microbial contamination.

### 2- Temperature

Heat generation can be more problematic than in liquid fermentations and has a major influence on relative humidity within the fermentation. The temperature is largely controlled by aeration and/or agitation of the substrate.

### 3- Aeration

Most solid-substrate fermentations are aerobic, but the particular requirements for oxygen depend upon the microorganism(s) used and the specific process. Rates of aeration provided are also closely related to the need to dissipate heat,  $\text{CO}_2$  and other volatile compounds that may be inhibitory. The rate of oxygen transfer is greatly influenced by the size of the substrate particles, which determines the void space. Oxygen transfer within this void space is closely related to the moisture level, as the oxygen dissolves in the water film around the substrate particles. However, as mentioned above, if excess water fills the void spaces, it has a detrimental effect on oxygen transfer.



1- Air pump 2- Rotameter 3- humidification glass column  
4- air filter 5 plates column 6- temperature and humidity probes

## The Current and Potential Applications of SSF

### 1- Production of enzymes by SSF

Enzyme production is one of the most important applications of SSF. It has advantages over submerged fermentation such as high volumetric productivity, low cost of equipment involved, better yield of product, lesser waste generation and lesser time consuming processes etc.

### 2- Production of organic acids under SSF

Organic acids are the most common ingredients of food and beverages. Fermentation plays a key role in the production of organic acids. Some of the acids produced using SSF such as citric acid, lactic acid, gallic acid, fumaric acid, linoleic acid, and kojic acid.

### 3- Secondary metabolites production

Solid-state fermentation can be used for production of secondary metabolites such as antibiotics and the fungal secondary metabolite Gibberellic acid.

Antibiotics: Many antibiotics such as penicillin, **cephamycin** C, neomycin, iturin, cyclosporin A, cephalosporins are produced by SSF

### 4- Production of biofuel

Ethanol is the most widely used biofuel today. Although it is easier to produce ethanol using submerged fermentation, SSF is preferred due to its lower water requirement, smaller volumes of fermentation mash, prevention of end product inhibition, and disposal of less liquid water, which decreases pollution problems. Cellulosic materials are receiving major attention for ethanol production because of their abundant availability.

### Exo-polysaccharides production

Exo-polysaccharides such as succinoglycan, xanthan are the future products of SSF. *Agrobacterium* spp. has been used for succinoglycan production on an industrial scale as they are non-pathogenic and produce good yield of polysaccharide.

Succinoglycan was produced using *Agrobacterium tumefaciens* on agar medium, spent malt grains, ivory nut and grated carrot in a horizontal bioreactor.

Similarly, *Xanthomonas campestris* was grown on a variety of solid substrates such as spent malt grains, apple pomace and citrus peels for xanthan gum production. Fermentation was done in rotating culture bottles.

### 5- Production of poly unsaturated fatty acids (PUFA)



Poly unsaturated fatty acids (PUFA) have to be supplied in diet, as they are not produced in the body. Submerged and solid-state fermentation can be used for PUFA production.

## **6- Production of poly gamma glutamate (PGG)**

Poly gamma glutamate (PGG) is an anionic, water-soluble, and highly viscous polypeptide. PGG is used as thickener, humectant, drug carrier, heavy metal absorber and feed additive. Gamma linoleic acid (GLA) is the most extensively studied PUFA by SSF.

### **Bioreactors used for solid-substrate fermentations**

Most solid-substrate fermentations are batch processes, although attempts are being made to develop semi-continuous and continuous systems. Some processes do not require bioreactors; they simply involve spreading the substrate onto a suitable floor. Those processes employing vessels exhibit considerable variations. A few anaerobic processes, such as silage production, require no mechanisms for agitation or aeration. However, the majority are aerobic fermentations, requiring aeration and occasional or continuous agitation. The following types of bioreactors are commonly used in the SSF:

#### **1) Rotating drum fermentors:**

Comprising a cylindrical vessel of around 100 L capacity mounted on its side onto rollers that both support and rotate the vessel. These fermentors are used in enzyme and microbial biomass production. Their main disadvantage is that the drum is filled to only 30% capacity, otherwise mixing is inefficient.

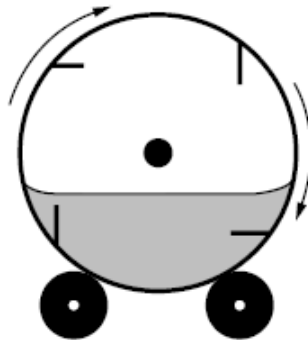


Figure: Rotating drum fermentor

#### **2) Tray fermentors:**

Which are used extensively for the production of fermented oriental foods and enzymes. Their substrates are spread onto each tray to a depth of only a few centimeters and then stacked in a chamber through which humidified air is circulated. These systems require numerous trays and large volume incubation chambers of up to 150m<sup>3</sup> capacity.

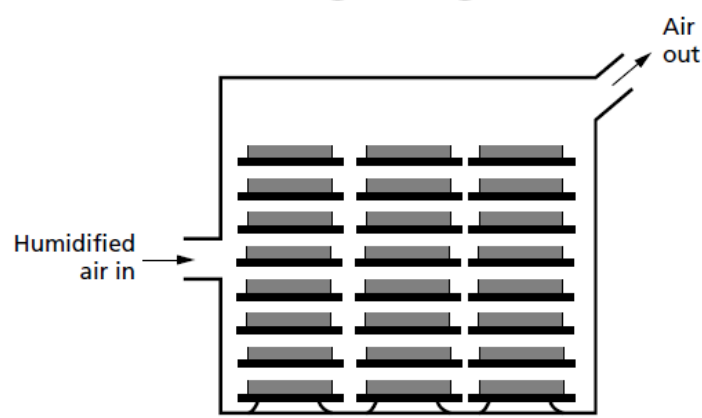


Figure: Tray fermentor

### 3) Bed systems:

As used in commercial koji production, consisting of a bed of substrate up to 1 m deep, through which humidified air is continuously forced from below.

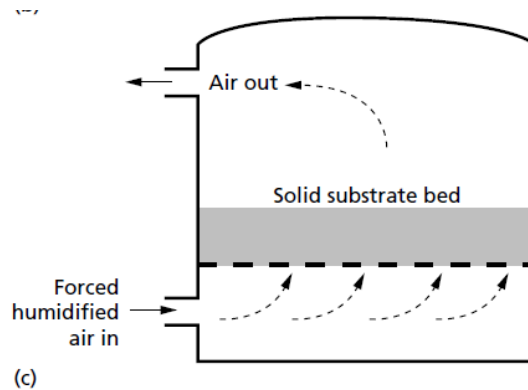


Figure: A bed System.

### 4) Column bioreactors:

Consisting of a glass or plastic column, into which the solid substrate is loosely packed, surrounded by a jacket that provides a means of temperature control. These vessels are used to produce organic acids, ethanol and biomass.

### 5) Fluidized bed reactors:

Which provide continuous agitation with forced air to prevent adhesion and aggregation of substrate particles. These systems have been particularly useful for biomass production for animal feed.

## Fermentation Technology/ Lec. 7

### Fermenters

The Fermenter is the heart of any biochemical process in which microbial cells are used for manufacture of a wide range of useful biological products.

**“Fermenter: is an apparatus that maintains optimal conditions for the growth of microorganisms, used in large-scale fermentation and in the commercial production of different compounds such as antibiotics and hormones”**

**The main function of a fermenter** is to provide a suitable environment in which an organism can efficiently produce a target product that may be cell biomass, a metabolite or bioconversion product.

Fermenters are tanks made from an appropriate grade of stainless steel. For industrial processes, they range in volume from about 1000 liters ( $1 \text{ m}^3$ ) to about 1 million liters ( $1000 \text{ m}^3$ ). Conventional fermenters are cylindrical vessels with dished (domed) top and bottom. Often circular toughened glass window (sight glass) is placed at the top of the fermenter (sealed into the fermenter wall with an airtight seal), so that the contents of the fermenter can be viewed during operation.

## **Fermenter design and construction**

Most fermentations are now performed in closed vessels designed to exclude microbial contamination. The performance of any bioreactor depends on many functions including:

1. Biomass concentration must remain high enough to show high yield.
2. Sterile conditions must be maintained for pure culture system.
3. Effective agitation is required for uniform distribution of substrate and microbes in the working volume of the bioreactor.
4. Heat transfer is needed to operate the bioreactor at constant temperature, as the desired optimal microbial growth temperature.

Most fermenters are designed to maintain high biomass concentrations, which are essential for many fermentation processes, whereas control strategies largely depend on the particular process and its specific objectives. The performance of any fermenter depends on many factors, but the key physical and chemical parameters that must be controlled are agitation rate, oxygen transfer, pH, temperature and foam production.

### **➤ In designing and constructing a fermenter a number of points must be considered:**

1. The vessel should be capable of being operated aseptically for a number of days and should be reliable in long-term operation and meet the requirements of containment regulations.
2. Adequate aeration and agitation should be provided to meet the metabolic requirements of the micro-organism. However, the mixing should not cause damage to the organism.
3. Power consumption should be as low as possible.
4. A system of temperature control should be provided.
5. A system of pH control should be provided.
6. Sampling facilities should be provided.
7. Evaporation losses from the fermenter should not be excessive.
8. The vessel should be designed to require the minimal use of labour in operation, harvesting, cleaning and maintenance.
9. Ideally the vessel should be suitable for a range of processes, but this may be restricted because of containment regulations.
10. The vessel should be constructed to ensure smooth internal surfaces, using welds instead of flange joints whenever possible.

11. The vessel should be of similar geometry to both smaller and larger vessels in the pilot plant or plant to facilitate scale-up.

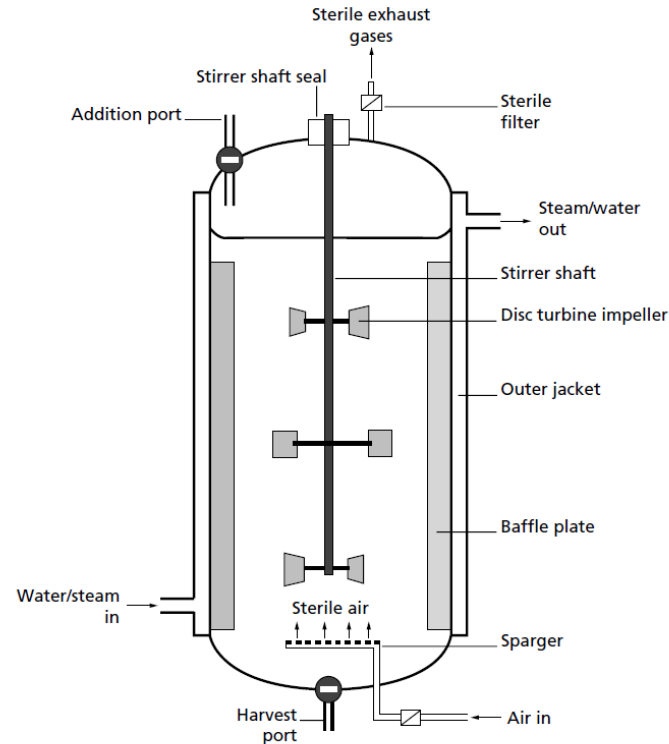


Figure: Diagram of a fermenter with three multi-bladed impeller

#### ❖ Fermenter body construction

Construction materials differ with small scale, pilot and large scale. Any vessel used should not have any corners and smooth surface is essential. Fermenters must withstand repeated sterilization and cleaning, and should be constructed from non-toxic, corrosion-resistant materials.

The material of construction is selected on the basis that it displays the following physical properties: ***it must be chemically inert such that it does not leach elements into the medium or chelate elements (principally metal ions) from the medium.*** So long as the materials of construction meet these requirements then the choice of material for the bioreactor is dictated by the scale at which the process is to be operated, the process itself and economic considerations.

★ In small scale for vessel construction glass or stainless steel may be used. Glass is useful because it gives smooth surfaces, is non-toxic, corrosion proof and it is usually easy to examine the interior of the vessel.

Two basic types of fermenter are used:

**Type I** – glass vessel round or flat bottom with top plate. It can be sterilized by autoclaving and the largest diameter is 60cm.

**Type II** – glass vessel flat bottom with top and bottom stainless steel plate. This type is used in *in situ* sterilization process and the largest diameter 30cm.

★ For pilot and large scale process, stainless steel (>4% chromium), mild steel (coated with glass or epoxy material), wood, plastic or concrete may be used as vessel construction material. Any materials used will have to be assessed on their ability to withstand pressure sterilization and corrosion and on their potential toxicity and cost.

### ❖ Agitation

Agitation of suspended cell fermentations is performed in order to mix the three phases within a fermenter:

- The liquid phase contains dissolved nutrients and metabolites.
- The gaseous phase is predominantly oxygen and carbon dioxide.
- The solid phase is made up of the cells and any solid substrates that may be present.

Mixing should produce homogeneous conditions and promote nutrient, gas and heat transfer. Heat transfer is necessary during both sterilization and for temperature maintenance during operation. Efficient mixing is particularly important for oxygen transfer in aerobic fermentations, as microorganisms can take up oxygen only from the liquid phase. Transfer into liquid from the gaseous phase is enhanced by agitation. It prolongs retention of air bubbles in suspension, reduces bubble size to increase the surface area for oxygen transfer, prevents bubble coalescence and decreases the film thickness at the gas-liquid interface.

### ❖ Aeration

Aeration of a fermentation medium has two functions:

- The supply of oxygen to the production organism.
- The removal of CO<sub>2</sub> formed as a result of aerobic respiration of the production organism.

Oxygen is supplied from air compressed by a compressor and stored in tanks. It is passed into the fermenter through a flow-meter/regulating valve system (to regulate the flow rate), then a sterilizing filter. It is introduced at the bottom of the fermenter through a sparger, which is an arrangement of pipework or a hollow plate performed with small holes so that the air stream is introduced into the medium as **small bubbles**.

**Q:** Explain why air is introduced into the fermenter as small bubbles?

Aeration involves the exchange of gases between the air and the medium. This exchange occurs at the interface between the two phases (the bubbles' surface). The smaller the bubbles are the greater their surface-volume ratio becomes, and the more efficient gas exchange will be.

The bubbles of air rise to the surface of the medium and take up space as they do so. At the surface the bubbles break up (disengage) releasing the gases they contain. To allow for these processes, fermenters are not filled completely, but a space (the head space) of about 20% of the capacity of the vessel is left free of medium. The

actual volume of the medium in the fermenter is called the working volume. Gases released into the headspace pass out of the fermenter through an air outlet at the top of the headspace. The air outlet is usually fitted with a sterilizing filter to prevent aerosols of the production organism contaminating the workplace as well as to maintain asepsis within the fermenter.

Aeration capacity of a fermenter under given conditions is a measure of the maximum rate at which oxygen can be dissolved into the medium. The rate at which the growing culture requires (uses) oxygen is called the oxygen demand. For aerobic organisms the aeration capacity should exceed the oxygen demand. Otherwise oxygen depletion (starvation) will occur, inhibiting growth and product formation.

**Q:** will the oxygen demand be constant throughout the growth cycle in a fermentation process? Explain your answer.

The oxygen demand is directly proportional to the metabolic rate of the cells in a culture and the cells concentration. Thus, oxygen demand increases in line with biomass (microbial cell) concentration in the rapid phase of growth and slows down as the growth rate slows towards the stationary phase.

In order to increase the aeration capacity of the system, the medium can be agitated (stirred) by impellers driven by a motor. The motor is placed directly above or below the fermenter and it drives a shaft which passes into the center of the fermenter where impellers are attached to it. There may be just one impeller near the base in a small fermenter or several in large fermenters. The aeration capacity in a stirred fermenter is proportional to the stirrer speed as well as the air flow rate and internal pressure.

Baffles in the form of narrow rectangular plates can be fixed to the internal walls of stirred fermenters. They increase turbulence and thereby increase aeration efficiency, although this does increase the energy required for stirring.

## **Fermentation Technology/ Lec. 8**

### **Types of bioreactors**

#### **1- Stirred tank bioreactor**

By far, the most common type of bioreactor in use today is the STR (stirred tank reactor). This essentially consists of a vessel with an aspect ratio of around 3: 1, and a mixing system typically driven through the head plate, although with some steam in-place systems the mixing will be driven through the base. The head plate will have ports that allow for the addition of probes, reagents and gas as well as the removal of samples.

Stirred reactor vessels for laboratory experiments of volume up to 20 litres are usually made of glass. For larger volumes, construction is made of stainless steel

- The height: diameter ratio of the vessel can vary between 2:1 and 6:1, depending largely on the amount of the heat to be removed, and the stirrer may be top- or bottom driven.
- All tanks are fitted with baffles, which prevent a large central vortex being formed as well as improve mixing.
- Four baffles are used for vessels less than 3 meters in diameter, and six to eight baffles are used in larger vessels.
- The width of the baffle is usually between  $T/10$  and  $T/12$ , in which  $T$  is the tank diameter.

Height of vessel to diameter:

$$\frac{H}{D_t} = 2:1 \quad \text{and} \quad 6:1$$

Typically, 75% of the designed volume is used as

working volume, in a fermentation vessel about 75% of the total CSTR volume is filled with liquid, the remaining 25% is used for gas space. If foaming takes place, there is no chance of immediate contamination.

**Spargers** should always be located near the bottom of the vessel with a distance  $D_i/2$  below the agitator, where  $D_i$  is the diameter of the impellers.

If heat removal is a problem, as it can be in large bioreactors greater than  $100\text{m}^3$ , up to 12 baffles can be used, through which coolant passes.

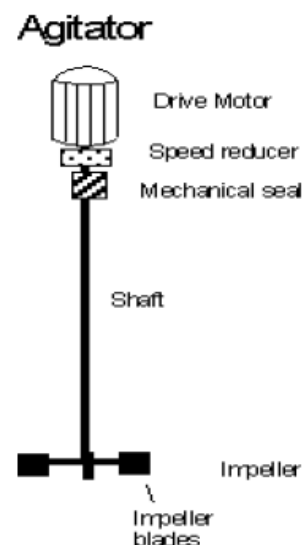
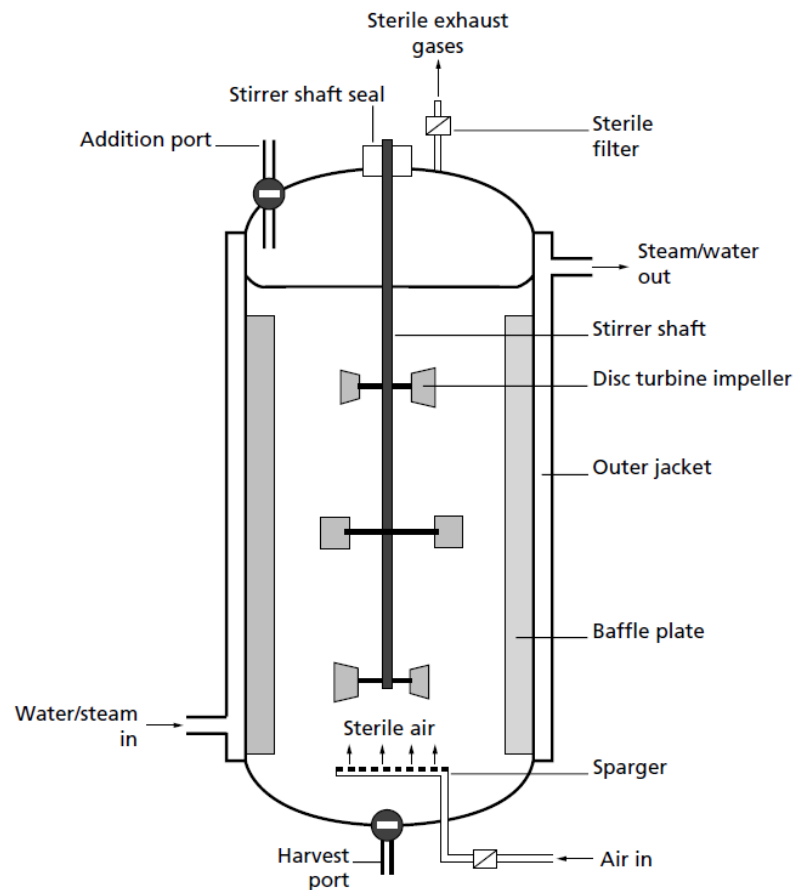
Careful consideration has to be given to agitator design within a bioreactor because it controls the operation of the bioreactor.

**The number of agitators** mounted on the shaft will be dependent on the height of liquid in the vessel. For specification of the correct number of agitators on the shaft,

- If the height of liquid in the vessel is equal to the tank diameter, one agitator is required.
- If the height of liquid is two or three times of the tank diameter ( $H_2T$  or  $3T$ ), additional agitators should be mounted on the shaft, separated by a distance  $w$ ; then  $w_T$ , where  $T$  represents tank diameter. Installation of multi-sets of impellers improves mixing and enhances mass transfer.

## 2- Airlift Bioreactors

In an airlift fermenter, mixing is accomplished without any mechanical agitation (Motor, driveshaft and impellers) as it replace by a constant flow of gas introduced into a riser tube. The airlift vessel may be baffled to improve mixing.



These vessels provide very gentle mixing, and so are particularly suited to cells that are too shear sensitive to be mixed by an impeller. Airlift bioreactors are used for tissue culture because the tissues are shear sensitive and normal mixing is not possible.

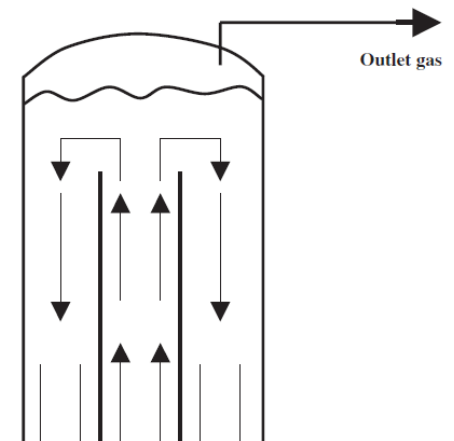
**There are many forms of airlift bioreactor.** In the usual form, air is fed into the bottom of a central draught tube through a sparger ring, so reducing the apparent density of the liquid in the tube relative to the annular space within the bioreactor. The flow passes up through the draught tube to the head space of the bioreactor, where the excess air and the by-product, CO<sub>2</sub>, disengage. The degassed liquid then flows down the annular space outside the draft to the bottom of the bioreactor.

Cooling can be provided by either making the draught tube an internal heat exchanger or with a heat exchanger in an external recirculation loop.

**In applications** of airlift bioreactor there are various types of fermenter. The most common airlift bioreactors are pressure cycle, internal and external loop bioreactors.

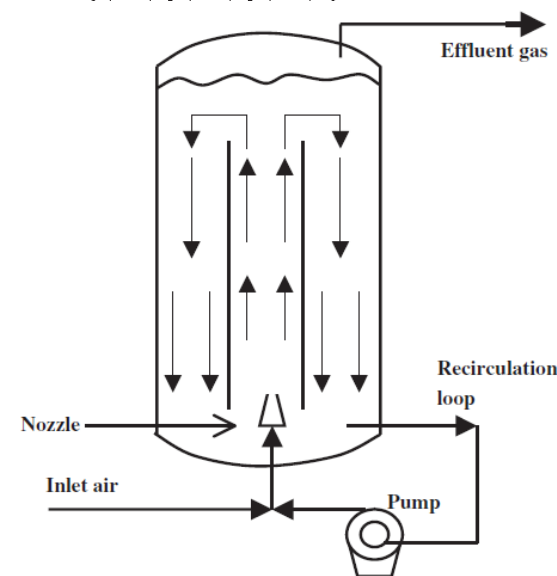
### ★ Airlift Pressure Cycle Bioreactors

The gas is circulated by means of pressurised air. In airlift bioreactors, circulation is caused by the motion of injected gas through a central tube, with fluid recirculation through the annulus between the tube and the tower or vice versa. The following Figure shows an airlift bioreactor with an internal loop cycle of fluid flow.



### Loop Bioreactor

A modified type of airlift system with gas and liquid flow patterns in which a pump transports the air and liquid through the vessel. Here, an external loop is used, with a mechanical pump to remove the liquid. Gas and circulated liquid are injected into the tower through a nozzle. The following Figure shows an airlift bioreactor that operates with an external recirculation pump.



### 3- Tower fermenters

As the name suggests, Tower fermenters are vessels characterized by a high height-to diameter ratio, anywhere from 6: 1 to 15: 1. They are aerated by gas sparging via a simple sparger usually located near the fermenter base. These systems can be operated continuously by the creation of settling zones by using baffles, which allow the product to be taken off and the cells returned to the main body of the vessel. Tower fermenters are used for continuous fermentation of beer, yeast and SCP.

### 4- Fluidized bed bioreactor

Fluidized bed bioreactors (FBB) have received increased attention in the recent years due to their advantages over other types of reactors. Most of the FBBs developed for biological systems involving cells as biocatalysts with three phase systems (solid, liquid and gas). The FBBs are generally operated in co-current up flow with

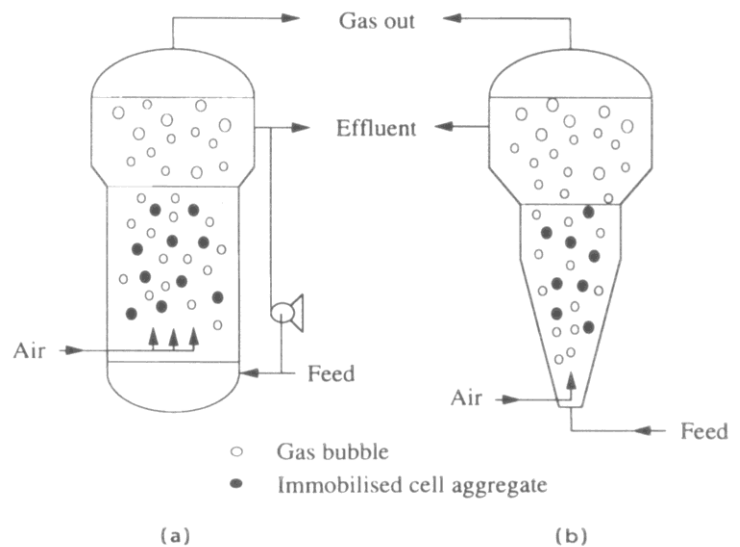


liquid as continuous phase. Usually fluidization is obtained either by external liquid re-circulation or by gas fed to the reactor.

In the case of immobilized enzymes, the usual situation is of two-phase systems involving solid and liquid but the use of aerobic biocatalyst necessitates introduction of gas (air) as the third phase. Basically, the particles used in FBBs can be of three different types:

- (i) inert core on which the biomass is created by cell attachment.
- (ii) Porous particles in which the biocatalyst is entrapped.
- (iii) Cell aggregates/ flocs (self-immobilization).

In comparison to conventional mechanically stirred reactors, FBBs provide a much lower attrition of solid particles. FBBs can be operated with smaller size particles without the drawbacks of clogging, high liquid pressure drops, channeling and bed compaction. The smaller particle size facilitates higher mass transfer rates and better mixing.



## Fermentation Technology/ Lec. 9

### **Fermenter control and monitoring**

Fermentation systems must be efficiently controlled in order to optimize productivity and product yield and ensure reproducibility. The key physical and chemical parameters involved largely depend on the bioreactor, its mode of operation and the microorganism being used. They are primarily aeration, mixing, temperature, pH and foam control.

Control and maintenance at optimum levels inside the reactor is mediated by sensors (electrodes), along with compatible control systems and data logging.

❖ There are three main classes of sensor

1. Sensors which penetrate into the interior of the fermentor. *e.g.* pH electrode, dissolved oxygen electrodes.
2. Sensors which operate on samples which are continuously withdrawn from the fermentor. *e. g.* exhausted-gas analysers.
3. Sensors which do not come into contact with the fermentation broth or gases, *e.g.* tachometers.

Internal sensors that are in or above the fermentation medium (pH, oxygen, foam, redox, medium analysis and pressure probes) should be steam sterilizable and robust. Sensors which do not come into direct contact with any internal component of the bioreactor do not need sterilization; for example, load cells, agitator shaft power and speed meters, and external sensors used to analyze samples regularly withdrawn from the bioreactor.

**Table:** The sensors used in fermenter monitoring and control

| Sensor                                                     | Measurement                                                                                       |                                                                                                                                                                 |                                             |
|------------------------------------------------------------|---------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------|
|                                                            | Physical                                                                                          | Chemical                                                                                                                                                        | Biological                                  |
| Electrodes                                                 | Temperature<br>(thermistor, resistance thermometer, thermometer)                                  | Dissolved oxygen<br>Dissolved carbon dioxide<br>Nutrients<br>(biosensors, e.g. for glucose)<br>pH<br>Metal ions<br>Foam level detection<br>Acid/alkali addition | Biosensors for biologically active products |
| Meters                                                     | Air flow rate in and out<br>Agitation shaft power<br>Speed of agitation, e.g. impeller tachometer |                                                                                                                                                                 |                                             |
| Transducers                                                | Pressure<br>Liquid flow                                                                           |                                                                                                                                                                 |                                             |
| Mass spectra                                               |                                                                                                   | Directly on-line or off-line nutrients and in flow and exhaust gases                                                                                            | Products                                    |
| Spectrophotometers<br>(determination on-line and off-line) |                                                                                                   |                                                                                                                                                                 | Biomass                                     |

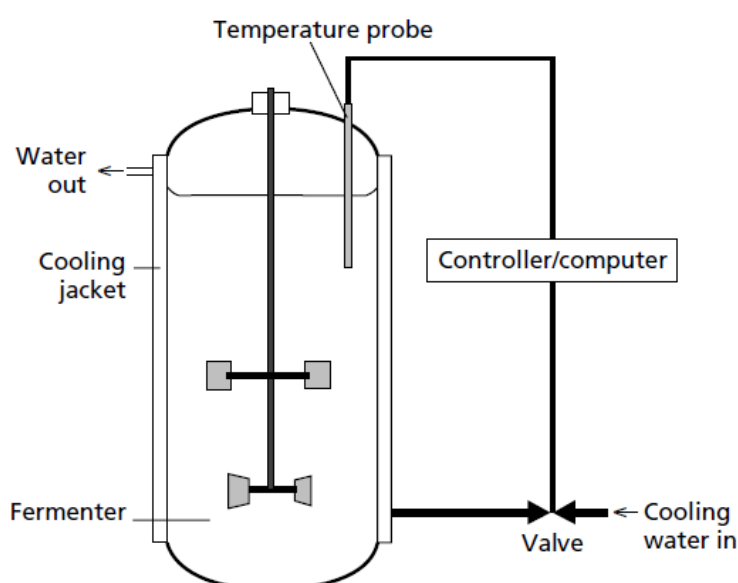
During fermentation of microorganisms in a fermenter, parameters such as pH, temperature, dissolved oxygen concentration and nutrient concentration will tend to vary.

In order to maintain optimal conditions for growth and product formation such variables can be monitored and controlled as follow:

## 1. Temperature

Temperature control is usually essential, and fermenters are normally equipped for both heating and cooling. If the ambient temperature is less than required fermentation temperature then the contents of the fermenter will require heating and *vice versa*. As organisms grow, exothermic metabolic reactions may cause the temperature to rise, and cooling may be necessary.

Temperature control involves measuring the medium temperature via resistance thermometers and thermistors (temperature –sensitive probe) linked to automatic heating or cooling systems. This system activates pumps to circulate hot or cold water around the fermenter jacket or coil as required.



**Figure:** A scheme for controlling fermenter temperature

On laboratory scale little heat is normally generated and extra heat has to be provided by placing fermenter in a thermostatically controlled bath, or by use of internal heating coils or by a heating jacket through which water is circulated or a silicone heating jacket. When certain size has been exceeded, the surface area covered by the jacket becomes too small to remove the heat produced by the fermentation. When this situation occurs internal coils must be used and cold water is circulated to achieve correct temperature.

## **2. pH**

Control of pH is usually a major factor as many fermentations yield products that can alter the pH of the growth media. Fermentation media often contain buffering salts, usually phosphates, but their capacity to control pH can be exceeded and addition of acid or alkali may be required. The pH can be maintained at the desired value by their automatic addition in response to changes recorded by the pH electrode. Many fermentations produce acid and adjustment of the pH can be made with ammonium hydroxide, which may also act as a nitrogen source.

## **3. O<sub>2</sub> and CO<sub>2</sub>**

Levels of dissolved O<sub>2</sub> and CO<sub>2</sub> are determined using O<sub>2</sub> and CO<sub>2</sub> electrodes which must be in direct contact with the medium and so is sealed through the fermentor wall. The electrode is linked to a control unit.

## **4. Foam**

Foam production in bioreactors is often a major problem, particularly in aerated fermentations. Formation of foam is due to the presence of surface-active agents, especially proteins, which produce stable foams in the headspace of the fermentor. If not controlled, this can lead to contamination and blockage of air filters.

There are basically three methods used to control foam production:

1. Media modification
2. Mechanical foam-breaking devices
3. The automatic addition of chemical antifoam agents.

Chemical foam control involves addition of chemicals (antifoams) which lower the surface tension of the medium and so cause bubbles (foam) to collapse.

The ideal antifoam should have the following properties:

1. Readily and rapidly dispersed with rapid action.
2. High activity at low concentrations.
3. Prolonged action.
4. Non-toxic to fermentation microorganisms, humans or animals.
5. Low cost.
6. Thermo stability.

7. Compatibility with other media components and the process, i.e. having no effect on oxygen transfer rates or downstream processing operations.

Natural antifoams include plant oils (e.g. from soya, sunflower and rapeseed), deodorized fish oil, mineral oils and tallow. The synthetic antifoams are mostly silicon oils, poly alcohols and alkylated glycols. Some of these may adversely affect downstream processing steps, especially membrane filtration.

Vegetable oils must be used where the product is destined for food use, and they have the added advantage that many organisms can also use them as source of nutrient.

The antifoam can be dosed (metered) via foam probe which is fitted in the headspace at a certain level. When foam touches the probe, the control unit linked to it activates a pump to pump in antifoam until the foam subsides and no longer makes contact with the probe.

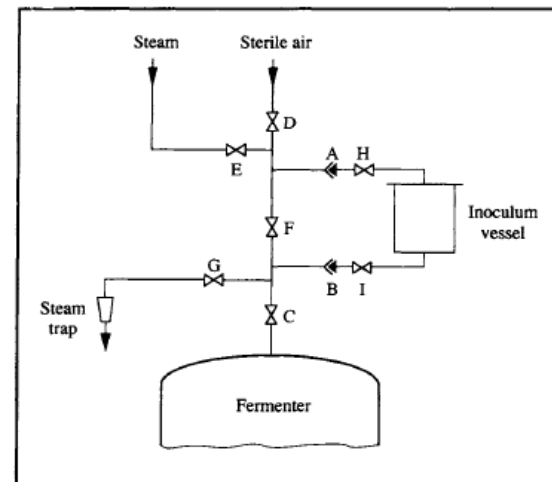
Mechanical foam control uses a device called a mechanical foam breaker which is positioned at the top of the fermenter.

## Fermenter Inoculation and Sampling

Consideration must be given in design of fermenters for aseptic inoculation and sample removal.

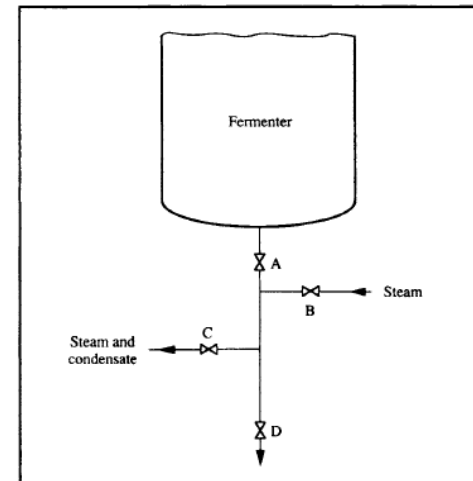
**Inocula** for large-scale fermentations are transferred from smaller reactors; to prevent contamination during this operation, both vessels are maintained under positive air pressure. The simplest aseptic transfer method is to pressurise the inoculum vessel using sterile air; culture is then effectively blown into the larger fermenter.

An example of the pipe and valve connections required for this type of transfer is shown in the following Figure. The fermenter and its piping and the inoculum tank and its piping including valves H and I are sterilised separately before culture is added to the inoculum tank. With valves H and I closed, the small vessel is joined to the fermenter at connections A and B. Because these connectors were open prior to being joined, they must be sterilised before the inoculum tank is opened. With valves D, H, I and C closed and A and B slightly open, steam flows through E, F and G and bleeds slowly from A and B. After about 20 minutes' steam sterilisation, valves E and G and connectors A and B are closed; the route from inoculum tank to fermenter is now sterile. Valves D and C are opened for flow of sterile air into the fermenter to cool the line under positive pressure. Valve F is then closed, valves H and I are opened and sterile air is used to force the contents of the inoculum tank into the fermenter. The line between the vessels is emptied of most residual liquid by blowing through with sterile air. Valves D, C, H and I are then closed to isolate both the fermenter and the empty inoculum tank which can now be disconnected at A and B.



**Sampling ports** are fitted to fermenters to allow removal of broth for analysis. An arrangement for sampling which preserves aseptic operation is shown in the following Figure.

Initially, valves A and D are closed; valves B and C are open to maintain a steam barrier between the reactor and the outside environment. Valve C is then closed, valve B partially closed and valve D partially opened to allow steam and condensate to bleed from the sampling port D. For sampling, A is opened briefly to cool the pipe and carry away any condensate that would dilute the sample; this broth is discarded. Valve B is then closed and a sample collected through D. When sampling is complete, valve A is closed and B opened for re-sterilisation of the sample line; this prevents any contaminants which entered while D was open from travelling up to the fermenter. Valve D is then closed and valve C reopen.



## Fermentation Technology/ Lec. 10

### Downstream processing

#### “The Recovery and Purification of Fermentation Products”

The efficient and economic fermentation of a product is not the end of a successful production process; the product must be recovered from the fermentation medium, purified if necessary and processed and packaged.

**Downstream processing:** encompasses all process following the fermentation which involved the extraction and purification of the product from the fermentation culture.

The extraction and purification of fermentation products may be difficult and costly. Ideally, one is trying to obtain a high-quality product as quickly as possible at an efficient recovery rate using minimum plant investment operated at minimal costs. Recovery costs of microbial products may vary from as low as 15% to as high as 70% of the total manufacturing costs. Obviously, the chosen process, and therefore its relative cost, will depend on the specific product.

To ensure good recovery or purification, speed of operation may be the overriding factor because of the labile nature of a product.

➤ The choice of recovery process is based on the following criteria:

1. The intracellular or extracellular location of the product.
2. The concentration of the product in the fermentation broth.
3. The physical and chemical properties of the desired product.
4. The intended use of the product.
5. The minimal acceptable standard of purity.
6. The magnitude of bio-hazard of the product or broth.

7. The impurities in the fermenter broth.
8. The marketable price for the product.

The target product may be recovered by processing the cells or the spent medium depending upon whether it **is an intracellular or extracellular product**. The main objective of the first stage for the recovery of an extracellular product is the removal of large solid particles and microbial cells usually by centrifugation or filtration. In the next stage, the broth is fractionated or extracted into major fractions using ultrafiltration, reverse osmosis, adsorption/ion-exchange/gel filtration or affinity chromatography, liquid-liquid extraction, two phase aqueous extraction or precipitation. Afterwards, the product-containing fraction is purified by fractional precipitation, furthermore precise chromatographic techniques and crystallization to obtain a product which is highly concentrated and essentially free from impurities. Other products are isolated using modifications of this flow-stream.

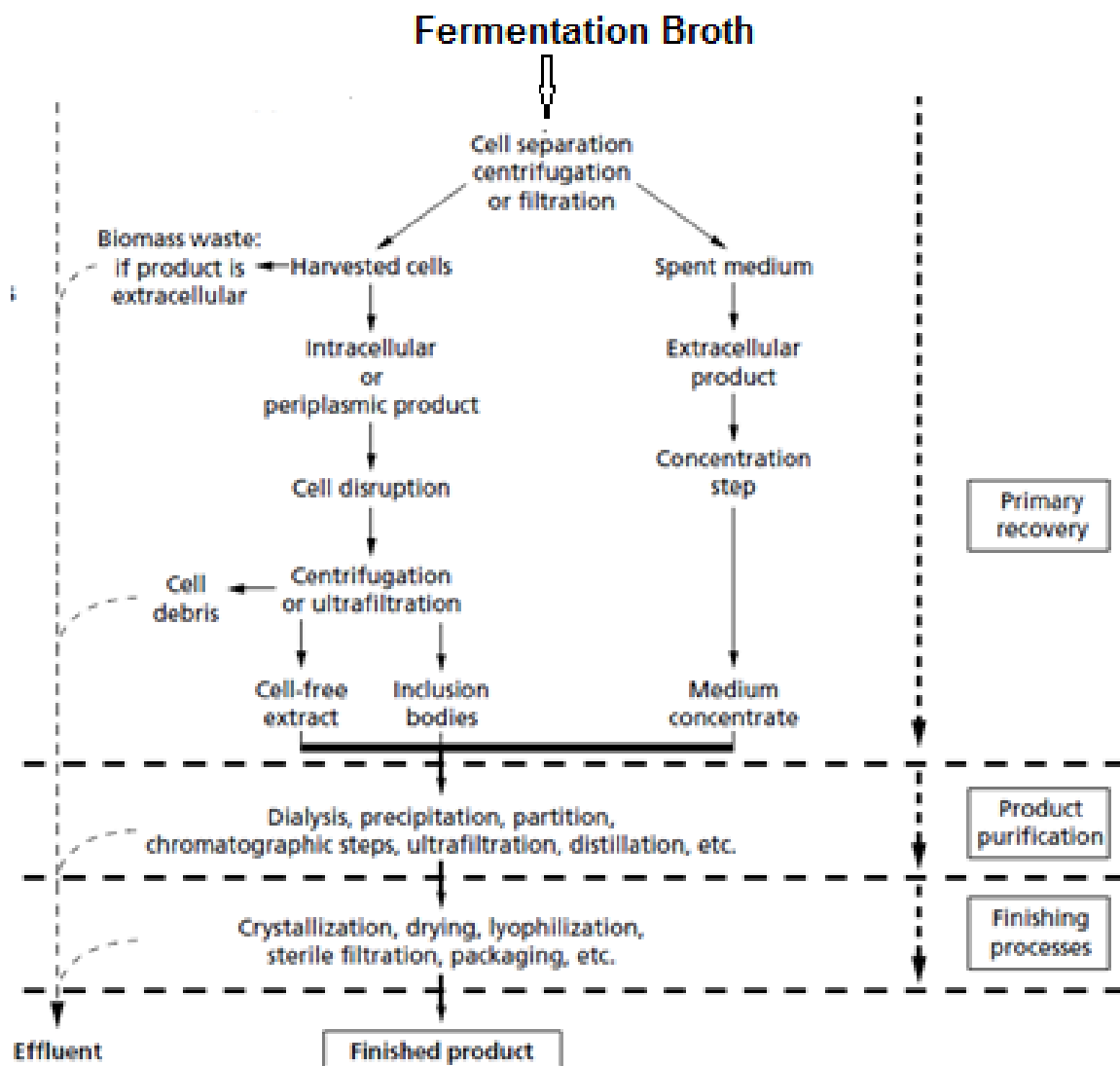


Figure: An outline of downstream processing operations

The following figure shows the typical unit processes that usually used in downstream processing:

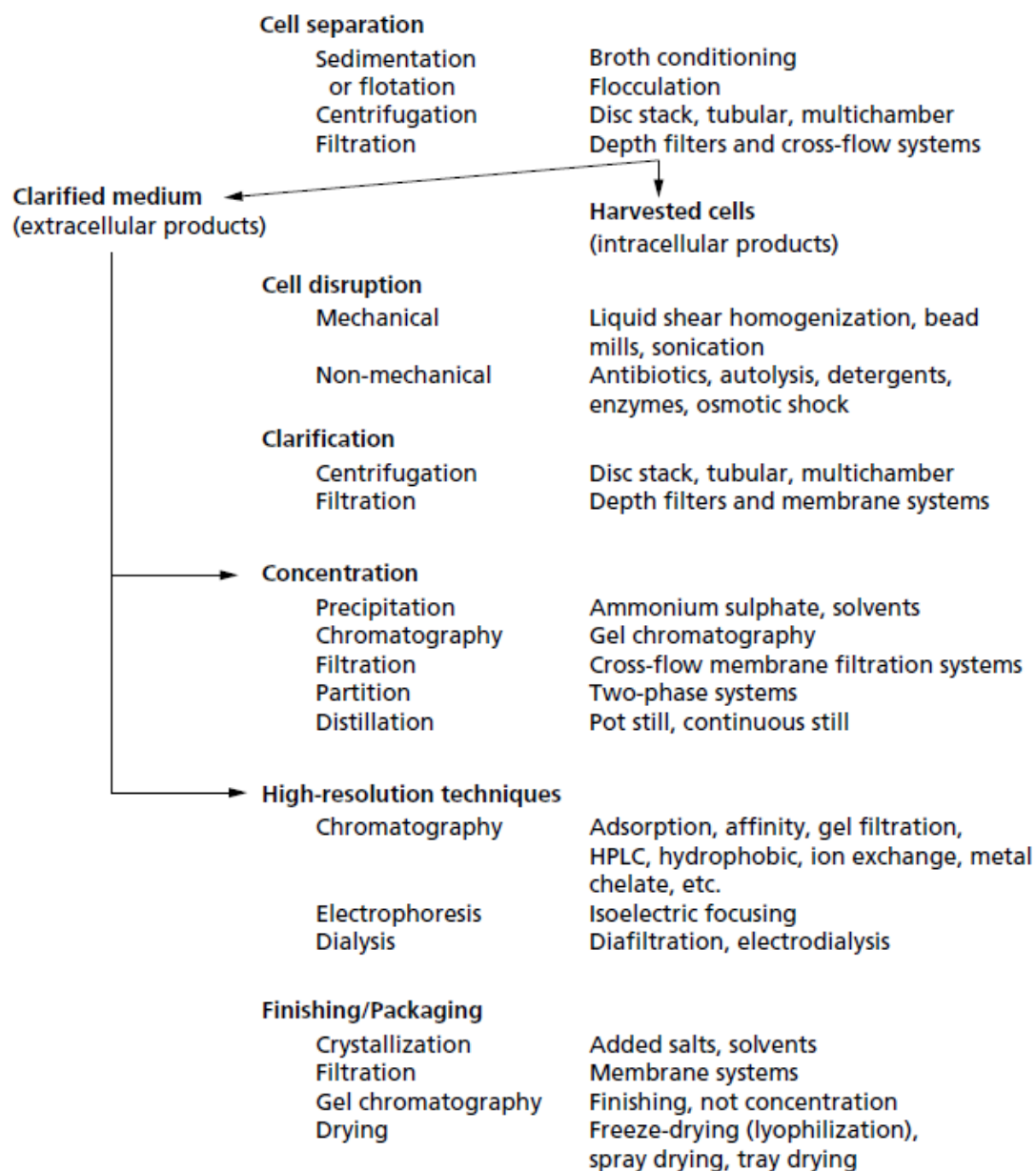


Figure: Typical unit processes used in downstream processing

Microbial cells and other insoluble materials are normally separated from the harvested broth by filtration or centrifugation. Because of the small size of many microbial cells it will be necessary to consider the use of filter aids to improve filtration rates, while heat and flocculation treatments are employed as techniques for increasing sedimentation rates in centrifugation. Some potential developments in cell recovery include the use of electrophoresis and dielectrophoresis to exploit the charged properties of microbial cells, ultra-sonic treatment to improve flocculation characteristics and magnetic separations. All these techniques suffer from high cost and scale-up difficulties and currently are not appropriate technologies. Of more current interest is the use of two-phase liquid extraction. Though still most appropriately used for separation of selected soluble components, it is easy to scale up and uses conditions which are gentle on the product.

### ★ The level of purity

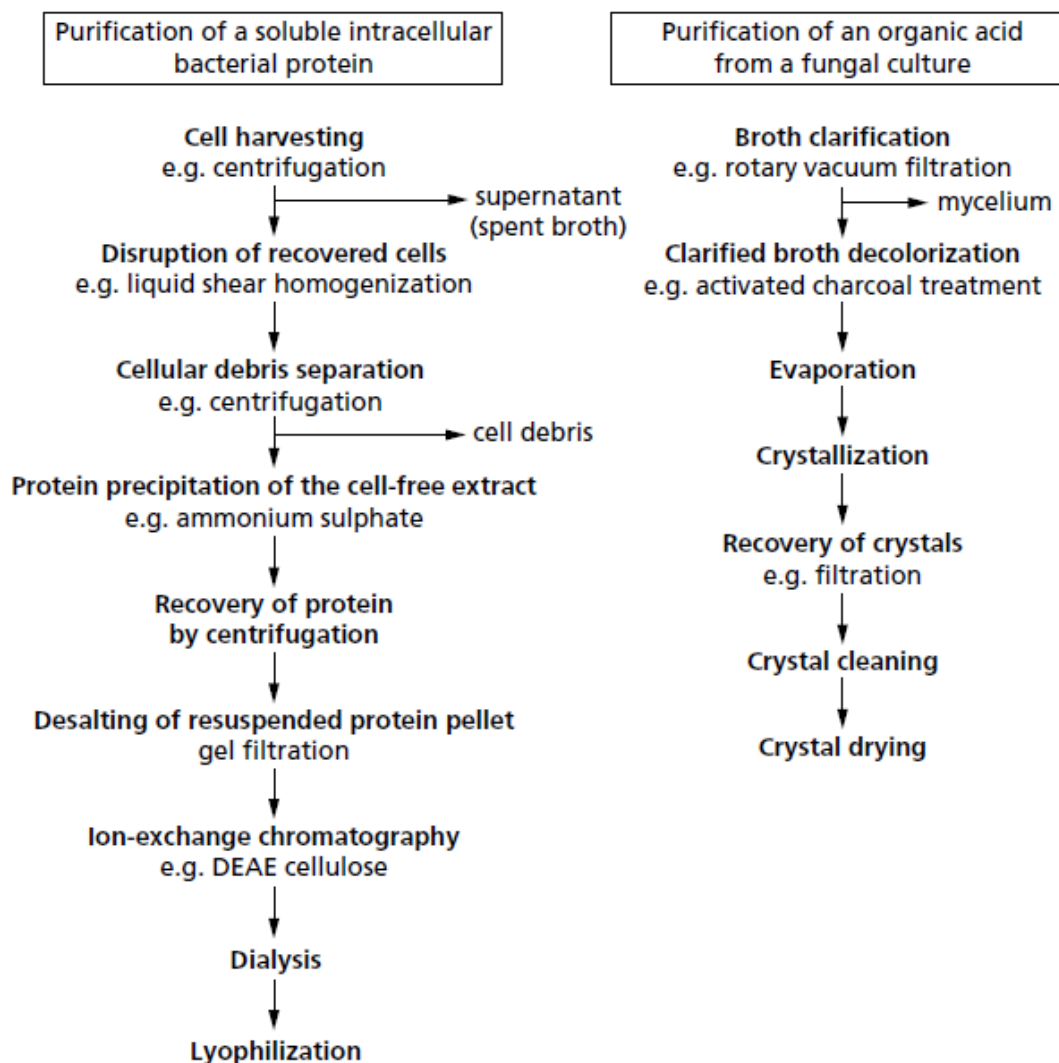
The level of purity that must be achieved is usually determined by the specific use of the product. Often, a product's purity will be defined by what is not present rather than what is. Purity of an enzyme, for example, is expressed as units of enzyme activity per unit of total protein. Not only is it important to reduce losses of product mass, but in many cases retention of the product's biological activity is vitally important.



It may be possible to modify the handling characteristics of the broth so that it can be handled faster with simpler equipment making use of a number of techniques:

1. Selection of a micro-organism which does not produce pigments or undesirable metabolites.
2. Modification of the fermentation conditions to reduce the production of undesirable metabolites.
3. Precise timing of harvesting.
4. pH control after harvesting.
5. Temperature treatment after harvesting.
6. Addition of flocculating agents.
7. Use of enzymes to attack cell walls.

Following are two examples of the **purification strategies** that can be used for the recovery of two products; **intracellular and extracellular products**.



★ Product recovery in the large-scale

The major problem currently faced in product recovery is the large-scale purification of biologically active molecules. For a process to be economically viable large-scale production is required, and therefore large-

scale separation, recovery, and purification. This then requires the transfer of small-scale preparative/analytical technologies (e.g. chromatographic techniques) to the production scale whilst maintaining efficiency of the process, bio-activity of the product and purity of the product so that it conforms with safety legislation and regulatory requirements.

- ★ The one factor that distinguishes processing in fermentation industries from that of chemical industries is that in fermentation processes the concentration of product at the final stage of synthesis (end of fermentation) is always relatively low.
- ★ Usually, the number of steps is kept to a minimum. This is not only because of cost, but because even though individual steps may obtain high yields, the overall losses of multistage purification processes may be prohibitive.
- ★ With solid state fermentation, the product can be extracted with water or other solvent from residual solids,

#### ★ **Recovery of volatile products**

If the product is volatile (*e.g.* ethanol), it may be possible to distil it directly from the harvested culture without pretreatment. Distillation can be carried out at reduced pressure (to lower the temperature required for volatilization) and continuous stills are available. It may be possible to distil the product directly from the fermentor at reduced pressure during fermentation, so preventing accumulation of product, and any inhibition high product levels may cause.