CHAPTER 1

MICROBIAL CELL CULTURE AND ITS APPLICATIONS

6.1.1. Introduction

In your previous class you have studied that microorganisms like bacteria and fungi are cultured to obtain a number of products, which are beneficial for human beings. Some of the commonest examples are antibiotics, ethanol and enzymes. With the advent of biotechnology and genetic engineering techniques, microorganisms are also being used for the production of recombinant molecules such as insulin, hepatitis B vaccine, growth hormones and interferons. Microorganisms are also grown or cultured for production of biomass per se such as the single cell protein (SCP). Besides these, scientists culture microbes in their laboratories for research proposes. It is therefore very important to understand how microbes are cultured in the laboratory or in the industry for production of various important molecules.

A microbial culture works as a factory in which the metabolism of a microorganism is exploited to convert raw material into products. There is a maximal capacity for a single microbial cell to convert raw material into products in a given period of time. Hence, to produce a given amount of product within a given time, it is necessary to calculate the number of cells as well as doubling time of the particular bacterium used in the process. Like any other chemical reaction, which requires an appropriate temperature, pressure, pH and solvent, microbes also grow in an appropriate environment of pH, temperature, nutrients (provided by the growth medium) and the substrate (raw material), which is converted by the bacterium into the desired product.

This chapter deals with basic principles of culturing microbes in the laboratory, large-scale industrial productions, and their applications. An important point that should be kept in mind is that, the requirements for culturing microbes on a large or industrial scale are different from culturing them in a laboratory.

6.1.2. Microbial culture techniques

Nutrients for microbial culture:

All growing microorganisms require water, sources of energy, carbon, nitrogen, oxygen and mineral elements. For culturing microbes on a small scale such as in a laboratory, it is relatively simpler to devise nutritive medium using pure chemicals such that full composition of the medium is known. Such media are called **synthetic media**. Alternatively, we may use media commonly available in the market such as nutrient broth, typticase soya broth (TSB) or brain heart infusion (BHI) broth. These media contain highly complex components such as peptone,

beef extract, yeast extract or casein digest. Such media are called **semi-synthetic media**. These media are well suited for culturing most microbes in the laboratory. However, when one wants to culture microbes on a large scale for production of useful metabolites, one uses sources of nutrients, which are economical and available readily. In some cases, these nutrient sources or raw materials may need a pretreatment before use. Other considerations while selecting the growth medium are:

- Should yield maximum product or biomass per gram of the substrate used.
- Should be of consistent quality and available throughout the year.
- Cause minimum problems during preparation and sterilization.
- Give minimum problems during production process particularly aeration, agitation, extraction and purification of the product.

Carbon sources: Cereal grains, starch, cane molasses, glucose, sucrose and lactose are commonly used as carbon sources.

Nitrogen sources: Ammonium salts, urea, corn steep liquor or slaughterhouse waste are used as nitrogen sources.

The carbon and nitrogen sources mentioned above are cheap and easily available.

Trace elements: Trace elements like Fe, Cu, Zn, Mn, Mo are also needed in small quantities.

Growth factors: Some microorganisms cannot synthesize specific nutrients like some amino acids or some vitamins. Such growth factors are incorporated into the medium in adequate amounts as a pure compound or as a component of complex mixture.

Antifoaming agents: In most microbiological processes, foaming is a problem. Foaming may be due to the components of the culture medium or some molecules produced by the microbes. The most common cause of foaming is the presence of proteins in the culture medium. Excess foaming denatures proteins and provides hindrance to free diffusion of oxygen in the medium. Commonly used antifoams are fatty acids, such as olive oil or sunflower oil. Silicones are also used as antifoams.

Energy sources: The carbon sources such as carbohydrates, lipids and proteins in the culture medium are the major sources, which provide energy for the growth of microbes.

Water: Water is the major component of the culture media. When required for culturing microbes in the laboratory, single distilled or double distilled water would suffice. When assessing its suitability for large-scale microbial culture as in industry, we should consider the pH and dissolved salts. Water is also required for ancillary services such as heating, cooling and rinsing. Clean water of consistent composition is therefore a prerequisite for large-scale cultivation of microbes.

Culture Procedures

Sterilization procedures: When microbes are cultured in laboratory in 100 -1000 ml flasks, sterilization of the nutrient medium can be easily done in an autoclave at a pressure of 15 pounds psi (per square inch) for 15-20 minutes. However, when microbes are cultured on a large scale such as for fermentation processes in the industry, we need to sterilize thousands of liters of culture medium or the substrate. There is also the problem of sterilizing the huge culture vessel i.e. the fermentor. Steam is used almost universally for sterilization of the fermentation media. Medium may be sterilized *in situ* in the fermentor itself. But if the medium is sterilized in a separate vessel, the fermentor must be sterilized before sterile medium is added to it. The fermentor is sterilized by passing steam through its jacket or coils. The steam is also sparged into the vessel through all entries allowing it to exit slowly from the air outlet. Steam pressure is held at 15 lbs psi in the vessel for 20 minutes.

The air used in the fermentation process should also be sterilized. This is done by filter sterilization.

Aeration and mixing: When microbes are cultivated in the laboratory, aeration and mixing can be easily achieved by putting the flasks on shakers (shake culture). This may be further augmented by the use of baffle flasks **(Fig. 1)**. In large-scale bioreactors however, transfer of oxygen to microorganisms is particularly difficult because the microorganisms must be well mixed and the oxygen dispersed to achieve relatively uniform concentration. Many fermentor designs have mechanical stirrers to mix the medium, baffles to increase turbulence, which ensure adequate mixing. Forced aeration also provides mixing and the needed oxygen.

Equipment for microbial culture

In the laboratory, microbes can be cultured in test tubes or ordinary Erlenmeyer flasks. Such cultures are usually carried out in 100 -1000 ml volumes. Growth of microbes in the laboratory can be augmented by simple improvements in the design of the flasks or by the use of shakers.

Baffle flask: One of the simplest ways is to produce a V- shaped notch or indentation in the sides of the flask. Such flasks are called baffle flasks **(Fig. 1)**. This improves the growth of the microbes by improving the efficiency of oxygen transfer due to increased turbulence of the agitated culture medium.

Fig. 1. A Baffle flask.

Shakers: Continuous agitation of the culture medium also greatly improves the efficiency of the oxygen transfer and this improves the growth of the microbes. In the laboratory, this is done by the use of shakers **(Fig. 2)**. Shakers may be end-to-end type or rotatory type. These may be designed for use at the ambient temperature or in a controlled temperature environment (incubator shaker).

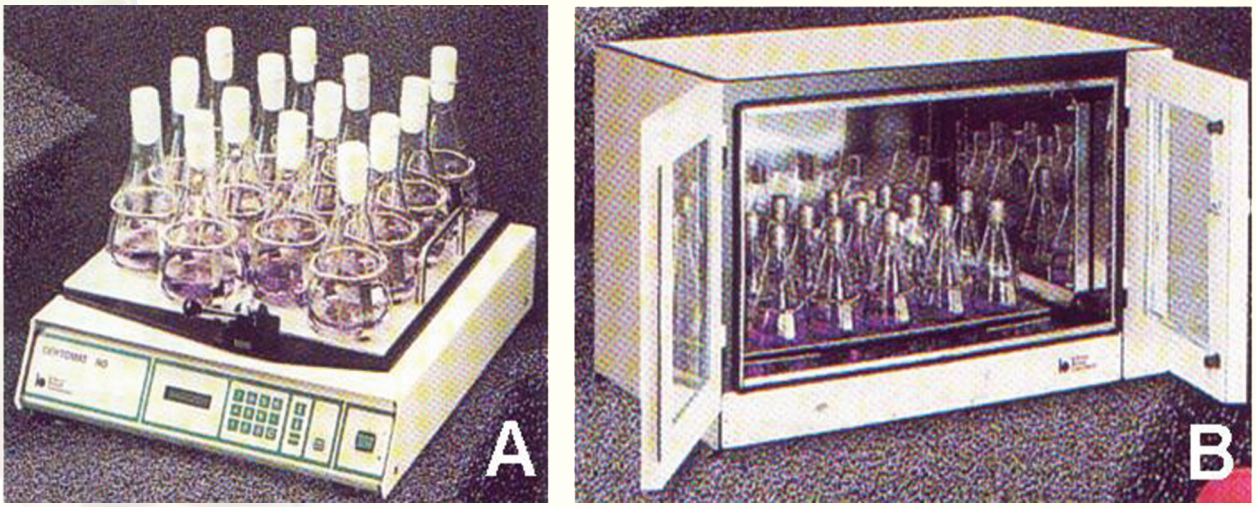

Fig. 2. (A) Shaker; (B) Incubator Shaker.

Fermentors (bioreactors): These are vessels, which are used for large-scale growth of microorganisms under a controlled environment. You have read about the basic design of a fermentor in your previous class in Unit I. It is a closed vessel with adequate arrangement for aeration, agitation, temperature and pH control, and drain or overflow vent to remove the cultured microbes along with their products. The vessel is capable of being operated aseptically for a number of days. These are also equipped with sampling ports, which allow withdrawal of the fermentation broth at regular intervals while the fermentation is in progress.

There are four main types of fermentor or bioreactor designs. The most common of these is a stirred tank bioreactor in which the culture medium is stirred with an impeller. In the bubble column reactor, the air is forced through a bottom sparger that creates enough agitation to ensure proper aeration. Basic features of a fermentor are shown in **Fig. 3.**

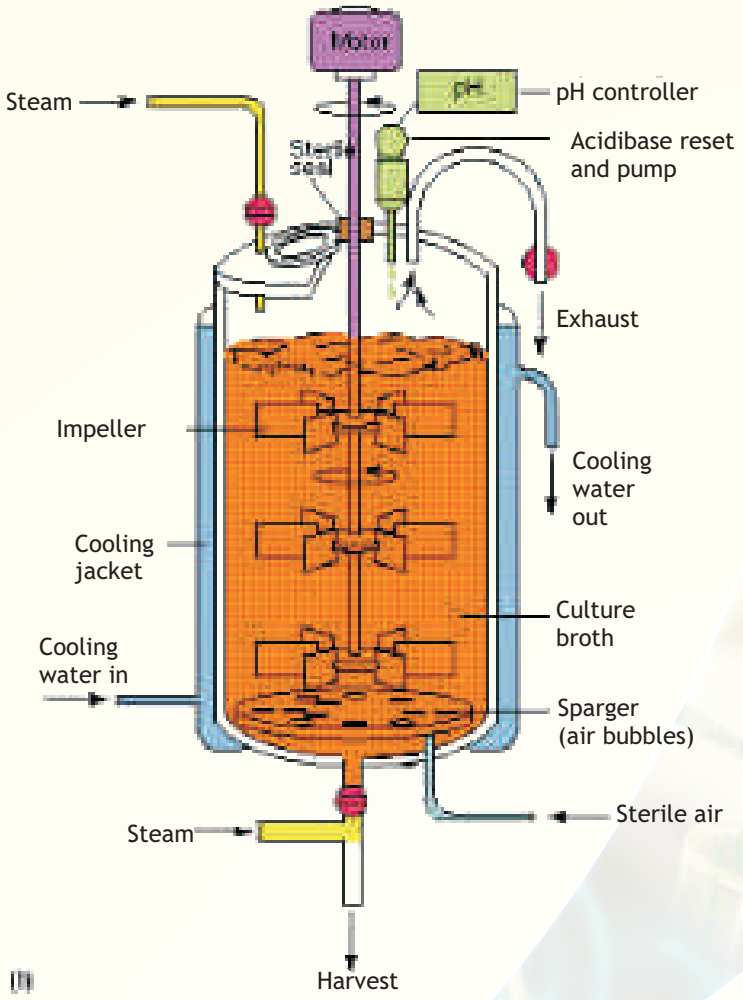

Fig. 3. Basic features of a fermentor (Bioreactor).

It should be noted that a high concentration of the microbial cells, as is achieved in a fermentor, could rapidly deplete the soluble oxygen in the medium, creating anaerobic conditions that may not be favorable to the growth of microorganisms and/or production of the desired products. So, forced aeration is done. The rapid growth of the microorganisms in the fermentor quickly alters the pH of the medium. So pH of the growing culture is continuously monitored and acid or alkali is added as required.

However, you should not harbor any notion that fermentors are used only for large-scale growth of microbes on an industrial scale **(Fig. 4)**. Small scale fermentors of capacity 10 -100 liters are also used in research laboratories These are used by the scientists in research, to optimize various parameters for the growth of microbes. The laboratory scale fermentors are also used by scientists, to produce enough quantities of metabolites from microbes for research purposes **(Fig. 4)**.

Fig. 4. (A) Laboratory-scale fermentor; (B) Industrial-scale fermentor.

Types of microbial culture

Batch culture: Batch culture is a closed culture system, which contains an initial limited amount of nutrients. After the medium is inoculated with the bacterial inoculum, the organism will grow and show usual growth phases viz. lag, log (exponential), stationary and decline. You have read about these in your class XI. Growth results in the consumption of nutrients and excretion of microbial products. At stationary phase, the growth is zero. This means, that in such a culture, growing cells are exposed to continually changing environment due to gradual consumption of nutrients and the accumulation of metabolites. The cell density [X], the concentration of substrate [S] and cell-specific substrate turnover rate [QS] during such a system are shown in **Fig.5**. Culturing microbes in the laboratory, in an ordinary flask, is nothing but an example of batch culture.

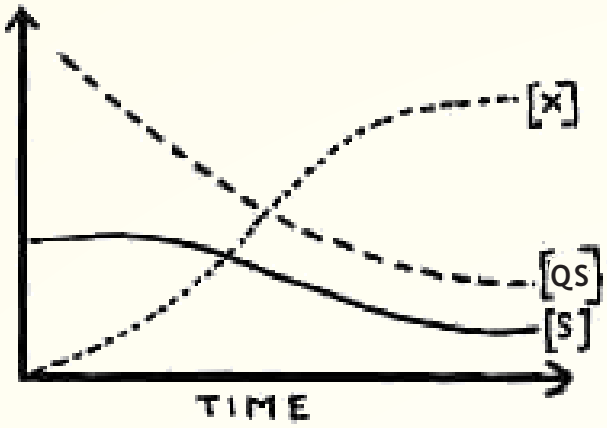

Fig. 5. Characteristic features of a batch culture.

Fed-batch culture: If a batch culture described above is continuously or sequentially fed with fresh medium without removing the growing culture, it is called fed-batch culture. In this system, substrate concentration remains constant and the cell density keeps increasing over time **(Fig. 6)**. This also means that over the period of time, the volume in the culture vessel goes on increasing.

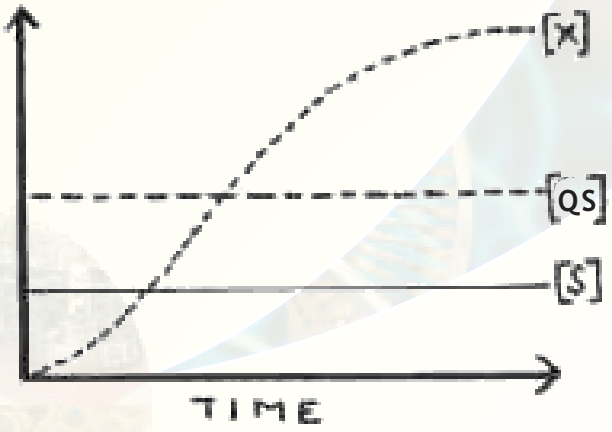

Fig. 6. Characteristic features of a fed-batch culture.

Continuous culture: This is a very interesting way of getting a continuous supply of microbial growth and/or products. The growth medium is designed in such a way that one of the nutrients is in limited quantity. Thus, during the exponential growth, as this nutrient is exhausted the growth will stop. However, just before the nutrient is fully exhausted, fresh medium containing the limited nutrient is added. This is repeated every time the limited nutrient is about to exhaust. This system is also fitted with an overflow device. This means that the added volume displaces out an

equal volume of culture from culture vessel. That is, formation of new biomass by the culture is balanced by the loss of culture from the vessel. In continuous culture, cells can be grown at a particular growth rate for an extended period of time. Most of the time, the chemical environment inside a continuous culture is constant. In a **chemostat**, constant chemical environment is maintained whereas in a **turbidostat** constant cell concentration is maintained. If medium is fed to such a culture at a suitable rate, a **steady state** is achieved eventually. At steady state, the cell growth and substrate consumption takes place at a fixed rate **(Fig. 7)**. Growth rate of cells remains constant during steady state operation. This means that at steady state, the concentration of cells, metabolites and other nutrients inside the reactor remain constant. That is, formation of new biomass by the culture is balanced by the loss of the culture from the vessel. Continuous culture is most suitable for production of biomass or metabolites. Continuous culture is widely used for production of both microbial biomass as well as their metabolites.

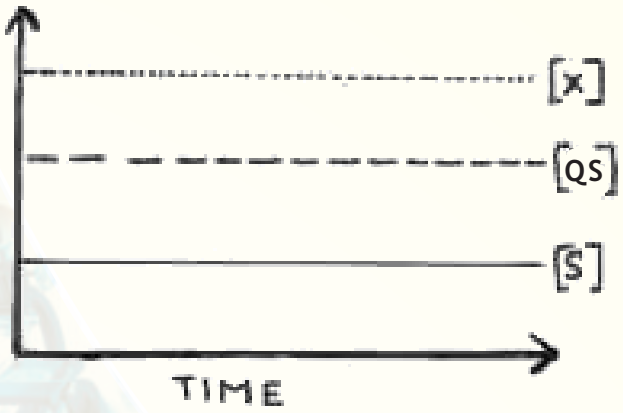

Fig. 7. Characteristic features of a continuous culture at steady state.

6.1.3. Measurement and kinetics of microbial growth

A clear understanding of microbial growth is necessary for proper utilization of biological processes for production of metabolites. In this section, the methods used for the measurement and quantitative evaluation of microbial growth will be discussed. There are four general patterns of microbial growth exemplified by bacteria, yeast, mold and viruses. All these microorganisms grow in different ways: bacteria grows by binary fission, yeast divide by budding, fungi divide by chain elongation and branching whereas viruses normally do not follow a regular growth pattern as they grow intracellularly in host cells.

Measurement of microbial growth

Microbial growth is defined as an orderly increase in all chemical components in the presence of suitable medium and environment. During the period of balanced growth, doubling of biomass is accompanied by doubling of other measurable properties of the microbe such as protein, DNA, RNA or intracellular water. In general, measurement of cell mass or cell number is used for

quantitative measurement of cell growth. The parameter that characterises microbial growth is the doubling time. It is the time required for the cell mass or number to double its original value during the balanced growth (i.e., log phase) of the organism.

Measurement of cell mass or number is one of the easiest ways to measure microbial growth.

(1) It is carried out by measuring the dry weight of the cell material in a fixed volume of the culture by removing the cells from the medium, and drying them till constant weight is obtained. For *E. coli*, the bacterium that is extensively used in laboratories, the dry cell weight of 1 billion cells is approximately 150 mg.

(2) Cell growth is also measured by measuring the absorbance of cell suspensions in a spectrophotometer. This principle is based on the fact that small molecules scatter light proportionate to their concentration. When light passes through a suspension of bacteria, there is a reduction in light transmitted as a consequence of scattering. Thus, with different cell concentrations, the absorbance at a particular wavelength will be proportional to the cell concentration. If you have a standard graph, plotted with absorbance versus cell concentration, the cell concentration of the unknown microbial sample can be calculated by measuring the absorbance at the same wavelength.

Apart from the methods described above, other methods are also used for measuring cell growth. Some of these are: measurement of wet weight of cells, turbidity measurements, ATP measurement, viable plate count **(colony forming units or cfu)** and use of Coulter counter. Coulter counter is an electronic instrument and is used for direct counting of microbial cells in suspension. In Coulter counter, the cells are made to pass in a single file through electrical field and the electrical impedance thus generated is recorded.

Growth kinetics and specific growth rate

In order to understand microbial growth kinetics, we shall take bacterial **binary fission** as the example where each cell division produces two identical daughter cells. The time taken for the cell to divide is called **generation time**. The generation time is also known as **doubling time (t_d)**, because the population of cells doubles over this time. For example, one cell of *E.coli* put in a nutritive medium will divide every 20 minutes. After one hour of growth (i.e. after three generations), one cell will have become eight (1 to 2, 2 to 4, 4 to 8). Because cell number doubles with each division, the increase in cell number over time is **exponential** or **logarithmic**.

A typical bacterial growth curve is depicted in **Fig. 8,** about which you have already learnt in Unit I (Chapter 1) of the textbook for class XI. In the lag phase, the microbial population remains almost constant. It is however a period of intense metabolic activity as the bacteria adapt to the culture conditions. Once the cells have adapted to the culture, cell division occurs with increasing frequency until the maximum growth is reached. This is called the log phase. At this

point **exponential growth** occurs and cell biomass or cell number increases at a constant rate.

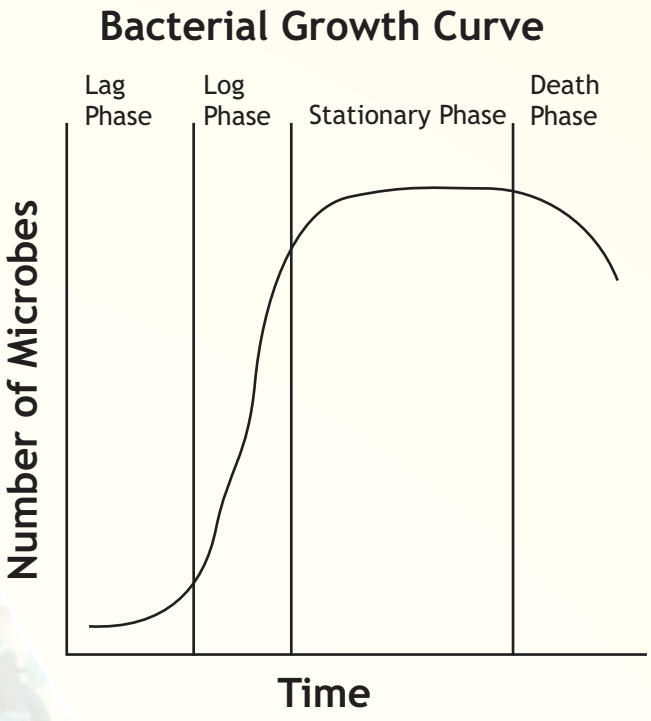

Fig. 8. A typical bacterial growth curve.

Mathematically, exponential growth can be described by two methods:

One related by biomass (X) and the other by cell numbers (N).

Let us first consider the exponential growth as related to biomass.

Balanced growth is like a chemical reaction where substrate is getting converted into product i.e. cell biomass in this case. Thus, growth behaves like an auto-catalytic reaction and the rate of growth will be proportional to the cell biomass present at that time. This can be described as follows:

rate of change of biomass $dX / dt = \mu X$ Eq.------- 1

where

X : concentration of biomass (g/L)

t : time (h)

μ : specific growth rate ($hour^{-1}$)

The equation 1 can be rearranged to estimate the specific growth rate (μ) as:

The specific growth rate $\mu = 1 / X \cdot dX / dt$ Eq.------- 2

Specific growth rate is an index of rate of growth of the cells in particular environment. In other words, specific growth rate is characteristic of the microorganism and is a function of the growth environment including temperature, pH, medium composition and levels of dissolved oxygen.

During the period of true exponential growth, equation 1 can be integrated to provide the following equation:

$$X_t = X_0 e^{\mu t} \qquad \text{Eq. ------- 3}$$

where

X_t: biomass conc. after time t

X_0: biomass conc. at the start of the exponential growth

e : base of the natural logarithm

Taking natural logarithm:

$$\ln X_t = \ln X_0 + \mu t \qquad \text{Eq. -------- 4}$$

This equation is of the form of straight-line y = mx + c.

Thus, for an exponentially growing culture, a plot of natural log of biomass concentration against time (i.e. a semilog plot) will give a straight line with the slope equal to μ.

or $\mu = (\ln X_t - \ln X_0) / t$

Please note that if $\log_{10}$ values were plotted instead of natural log the slope would be equal to μ/2.303.

$$\mu = 2.303 (\text{Log } X_t - \text{Log} X_0) / t \qquad \text{Eq. -------- 5}$$

If we consider a situation where at time zero, the cell biomass is X_0, then after exponential growth equivalent to one doubling time (t_d), the microbial biomass will become $2 X_0$.

i.e. $X_t = 2X_0$ when $t = t_d$

Substituting these parameters in Equation 3 gives:

$$2X_0 = X_0 e^{\mu t d} \qquad \text{Eq. ---------- 6}$$

Taking natural logarithm gives:

$$\ln 2X_0 = \ln X_0 + \mu t_d$$

or $$\mu t_d = \ln 2$$

Thus,

$$t_d = 0.693 / \mu \qquad \text{Eq. ---------- 7}$$

Where t_d is the doubling time of the culture.

By calculating μ from the graph or from Equation 5, and substituting it in Equation 7, we can calculate the doubling time of the culture. From the Equation 7, we can also say that doubling time and specific growth rates are inversely related. Higher the doubling time, lower will be the specific growth rate and vice versa. Thus, the microbial culture having high specific growth rate will have low doubling time.

Specific growth rate of microbial cells, in general, is calculated during the balanced growth of cells particularly during the exponential phase of growth. It signifies growth capacity of the culture in the particular environment. Specific growth rates of microbial cells change during different phases of batch growth, having a maximum value during the exponential phase. It is also affected by growth temperature, medium composition and other environmental parameters that affect the microbial growth.

Let us now study the second approach, which examines growth in relation to cell number, where number of the cells at the start of the exponential growth is N_0.

Let us take an example where $N_0 = 1$.

As the cell divides, we shall have:

No. of cell division	0	1	2	3	n
No. of cells	1	2	4	8	2^n
Mathematically	N_o	$N_o \times 2$ $N_o 2^1$	$N_o \times 2 \times 2$ $N_o 2^2$	$N_o \times 2 \times 2 \times 2$ $N_o 2^3$	- $N_o 2^n$

Thus, after a period of exponential growth time (t), the number of cells (Nt) will be given by:

$$N_t = N_0 2^n \qquad \text{Eq.-------- 8}$$

Where,

n = the number of the divisions

N_0 = the initial cell number

Taking logarithm

$$\text{Log } N_t = \text{Log } N_0 + n \text{ Log } 2$$

Thus, the number of divisions (n) that have taken place is given by:

$$n = (\text{Log } N_t - \text{Log } N_0) / \text{Log } 2$$

$$= (\text{Log } N_t - \text{Log } N_0) / 0.301$$

$$= 3.3 (\text{Log } N_t - \text{Log } N_0) \qquad \text{Eq.-------- --- 9}$$

The mean generation time or doubling time (t_d) i.e. the time taken to undergo single generation that doubles the population is:

t_d = Total growth time / number of divisions

$$= t / n \qquad \text{Eq.---------- 10}$$

The specific growth rate and doubling times of the organism decide the medium requirements and fermentation batch time for the production of biochemical molecules, and thus, are important parameters for large-scale production process.

Example: Calculate the generation time (doubling time or t_d) of a bacterial population in which the number of bacteria increases from 10^4/ml to 10^7/ml during four hours of exponential growth.

Answer: First calculate the number of divisions the population must have undergone to increase from 10^4 to 10^7 in 4 hours.

Using the equation 9: $n = 3.3 (\text{Log } 10^7 - \text{Log } 10^4)$

$= 3.3 (7 - 4)$

$= 10$

Using the equation 10: $t_d = 240$ minutes / 10

= 24 minutes

6.1.4. Scale-up of microbial processes

In your class XI text book (Chapter I: Introduction to Biotechnology), you were introduced to the early attempts on large-scale production of acetone from the bacterium *Clostridium acetobutylicum*. This chapter also brings out a number of products, which are produced from microbes for which a large-scale cultivation of microbes is necessary. You were also introduced to

the quantitative approach of producing just 1 liter of curd at home. Imagine the issues involved when one has to produce thousands of liters of curd in dairy industry.

Direct production of microbes on a large or commercial scale has the risk of not only large investments, but also producing products, which may not be of appropriate quality so that there are problems in their commercialisation. To avoid these risks, the manufacturers try to validate laboratory process on an intermediate scale before attempting commercial production. This step is carried out in a **pilot plant**, which is a mini version of the commercial plant.

Before setting up a commercial scale plant the results, which are derived from pilot plant, are theoretically extrapolated to the commercial scale in terms of equipment size (fermentor / bioreactor), utilities like steam, water, electricity, fittings etc, labour (technical and non-technical) and market surveys. All these data are compiled into a techno-economic report to assess the feasibility of the project. Such a report is also important for seeking financial assistance for the project from banks or other financial institutions.

The following example will give you an idea about the importance of scale-up calculations for the bioprocess industry in terms of reactor size:

Recombinant insulin is produced at 100 mg/L by *E. coli* at a cell concentration of 1 g/L. Calculate the volume of reactor (size of the fermentor) needed to produce 1 Kilogram of insulin in the following conditions:

(a) When the cell concentration is 1 g/L and insulin production is 100 mg /L.

(b) When the cell concentration is 50 g/L and insulin production is 100 mg /L.

(c) When the cell concentration is 50 g/L and insulin production is 500 mg /L.

Answer:

(a) Insulin production is 100 mg/L; so fermentor volume needed for 1 Kg of insulin is 1 Kg / 100mg = 1000, 000mg/100,g = 10,000mg = 10,000L.

So we need 10,000-litre fermentor to produce 1 Kilogram of insulin in one batch.

(b) In this case the cell concentration is increased to 50 g/L; so insulin production per liter will be 50 X 100 = 5000 mg = 5 g / L;

Thus, to produce 1 Kilogram of insulin we need 1 Kilogram / 5 g = 1000 g / 5g = 200 g. So, if the cell concentration is increased 50 times, we need 200-litre reactor to produce 1 Kilogram of insulin.

(c) In this case cell concentration is high (50 g/L) and the insulin concentration is also very high (500 mg/L). Thus insulin yield per liter of culture is 500 X 50 = 25, 000 mg / L which is 25 gram/L. Thus, to produce 1 Kilogram of insulin we need 1 Kilogram / 25

gram1000g/25g = 40g thus, we need a 40 liter reactor to produce 1 kilogram of insulin.

Please notice that the reactor volume decreases when we have high cell density or high concentration of insulin production as in case of (c). Such calculations are useful in deciding the reactor size required to produce a desired amount of the metabolite. Usually 30% extra space is recommended in the fermenter vessel for air, froth etc.

6.1.5. Isolation of microbial products

Once the fermentation is complete, it is necessary to recover the desired metabolite. Minimally, this will involve separation of the cells from the fermentation broth. But it may also include, purification of the metabolite with or without cell disruption; cell disruption will be necessary if the metabolite is intracellular. Such operations are referred to as **downstream processing**. The steps involved in isolation of the desired microbial product are: (1) separation of cells from the fermented broth, (2) cell disruption if the product is intracellular or concentration of the broth if the product is extracellular (3) initial purification of the metabolite, (4) metabolite-specific purification in which the metabolite of interest is purified to a high degree, and (5) polishing of the metabolite (bringing it to 98 -100% purity) where it is further concentrated and formulated for use.

Fig. 9 and 10 depict the steps involved in isolation of microbial products or metabolites of extracellular and intracellular origin respectively. The important steps are: separation of microbial cells (biomass / pellet) from the fermentation broth, concentration, metabolite-specific purification and final purification. Isolation of cells from the fermented broth is, in general, carried out by either centrifugation or ultra filtration. Some cells rapidly settle out of suspension once aeration and agitation of the fermented broth ceases. The settling of cells may also be assisted by the addition of certain flocculating agents. Where cell settling does not occur, cell removal can be effected by centrifugation. An alternative to centrifugation is ultra filtration. The term ultra filtration describes processes in which particles significantly greater in size than the solvent are retained when the solution is forced through a membrane of very fine pore size, usually less than 0.5 mm. Microbial cells can be concentrated using ultra filtration so that the fermented broth is separated from cells.

The clarified fermentation liquor will contain microbial metabolites and extra cellular enzymes. Several methods are available for recovery of metabolites such as precipitation, solvent extraction and ion exchange chromatography.

Different downstream operations are available for concentration as well as purification of the metabolite. But it is always advisable to use lesser number of steps to achieve desired purity of the metabolite or product. This is because, more the number of steps involved, more will be the cost of the production and lower would be the yield.

Most of the antibiotics are secreted into the medium, so their isolation mainly involves steps

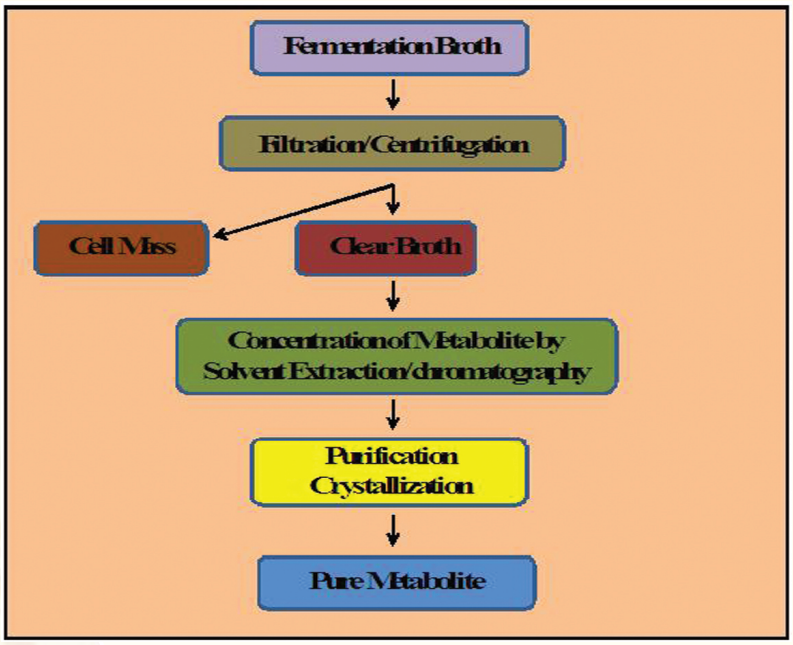

Fig. 9. Isolation of an extracellular microbial product (cell mass is discarded). Example: Streptomycin from *Streptomyces gresius*.

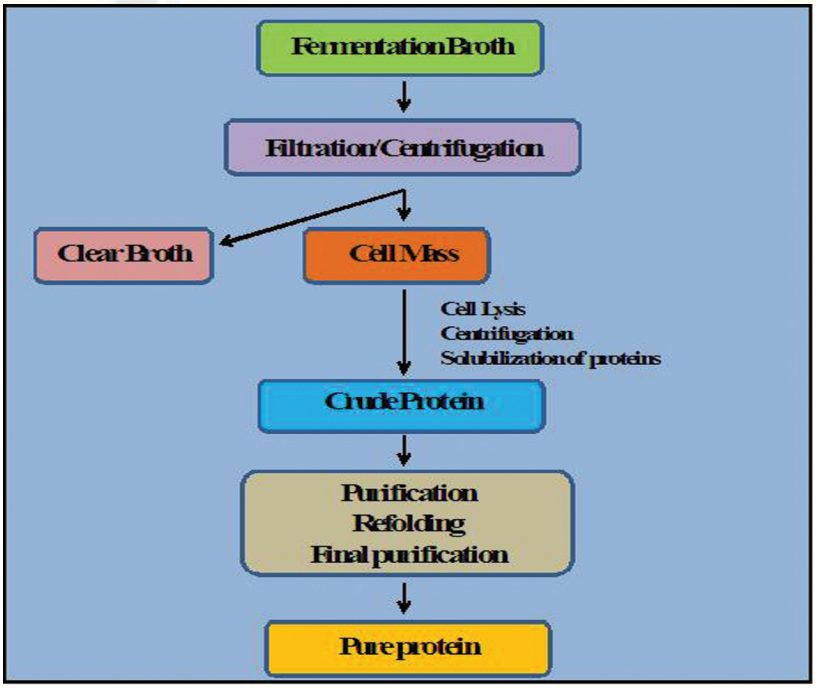

Fig. 10. Isolation of an intracellular microbial product (clear broth is discarded). Example: Recombinant insulin (Humulin®) from *E. coli*.

depicted in **Fig. 9**.

Most of the recombinant proteins expressed in *E. coli* accumulate intracellularly as protein aggregates. So their isolation and purification involve steps as described in **Fig. 10**. Once the pure metabolite is obtained, a stabilized formulation is made using several ingredients known as excipients.

6.1.6. Strain isolation, improvement and preservation

By this time, you must have realized that one of our major purposes to culture microbes is to produce useful products. But, do you think that all microbes produce useful or novel products? The answer is no. So, we have to search for those microbes, which produce substances of our interest. Where do we get these microorganisms?

As you know, microbes are wide spread in natural habitats especially in soil and in aquatic environments. They are also found in extreme environments namely arctic waters and hot springs. These natural habitats are our source of microbes. Once we have isolated microbes of desired interest, we can further improve their desired traits using a variety of methods.

Strain isolation:

The sample containing the microbes (e.g. soil) is put in a nutritive medium and allowed to grow in shake cultures. The growth conditions (e.g. temperature) or nutrients in the medium are provided such that these favour the growth of microbes of our interest. This is called **enrichment technique**. The enriched culture can further be sub-cultured by taking a small inoculum and putting it into fresh medium. In this way, the growth of the desired organisms improves successively. Further screening is done using a method where the organism will show its desired properties. For example, if we are looking for a microorganism, which produces an antibiotic, we may detect it by growing the culture on an agar plate in the presence of that bacterium against which antimicrobial activity is desired. Immunological methods are also available in which the microbes producing products are detected using **specific antibodies**. Molecular biology has made available a variety of **probes**, which enable the detection of organisms capable of producing specific products. Recently some of these methods have been adapted to robotic automation resulting in enormous throughput screening of microbes for newer / novel molecules.

Strain improvement:

Strain isolation procedure described above only identifies a strain, which has the capability or potential to produce a desired molecule. It does not ensure that it produces molecule in sufficient quantities to be economically viable. Techniques of classical genetics and genetic engineering are used to improve the desirable characteristics of the strain.

Mutation Selection: This is one of the oldest methods of strain improvement. The strain is exposed to chemical (e.g. nitrosoguanidine or NTG) or physical (e.g. UV rays) mutagens and the mutants having improved characteristics are selected. It is often necessary to carry out multiple successive mutations before we get the desired results. One of the classical examples of strain improvement using this methodology is the production of antibiotic penicillin. Several successive mutations were necessary to develop a strain of *Penicillium chrysogenum* capable of producing nearly 100 times the concentration of penicillin produced by the original strain *(Penicillium notatum)*, thus making production of penicillin commercially feasible.

Genetic Engineering Techniques: Until the recent breakthroughs in the techniques of genetic engineering, a bacterium could produce only substances coded for in its genome. Genetic engineering techniques about which you have learnt in the unit V (Chapter 1) allow totally new properties or capabilities to be added to the microorganisms giving rise to **recombinant strains.** Using these techniques, microorganisms may be manipulated to, synthesize or secrete enhanced quantities of biomolecules, facilitate production of novel compounds or allow utilization of cheaper substrates. Using these techniques, the microorganisms may also be utilized to produce plant, animal or human proteins. Some of the valuable human proteins which are being produced in microorganisms using this technology include recombinant human insulin (Humulin), hepatitis B surface antigen, human growth hormone and interferons. These proteins can now be produced in large quantities. Consequently the cost of the therapies which make use of these proteins viz. insulin (diabetes), hepatitis B surface antigen (vaccination against hepatitis B virus), human growth hormone (growth retardation) and interferons (immunotherapy) has been reduced considerably.

The tools, which are used for genetic engineering viz. the restriction enzymes, cloning and expression vectors and introduction of recombinant DNA into host cells, have been discussed fairly in detail in the unit V (Chapter 1). However there are many practical problems, which must be taken care of before a foreign **(heterologous)** gene may be expressed in a microorganism to make it commercially viable. For example, when a foreign gene is introduced into a host bacterium, it may not be expressed there. This problem is overcome by placing foreign gene under regulatory controls recognized by the host microorganism. To maximize production of foreign protein, the **expression vector** used is such that it replicates to **high copy number** and is stable. The foreign gene should ideally be linked to a strong promoter that has high affinity for RNA polymerase. The foreign gene may also be put under the control of a regulatory switch such that production of recombinant protein does not occur until required.

When a eukaryotic gene (e.g., plant, animal, human) is expressed in prokaryotic (bacterial) host, there are additional problems to be tackled. The non-coding region of eukaryotic gene must be excised. This requires use of reverse transcription of mRNA into cDNA. Additionally, the recombinant protein may not be secreted into the medium or its incorrect folding and accumulation intracellularly may generate **inclusion bodies**. All these problems make

downstream processing difficult and costly. Thus, an alternative would be to use a eukaryotic expression host. For this purpose, *Saccharomyces cerevisiae* has been quite popular because it is safe and scientists have long experience of using this yeast in industrial fermentations. Detailed information on biochemistry, physiology and genetics of this yeast is also known. Moreover, this yeast can be manipulated genetically rather easily. However, product yields are relatively low at 1-5% of the total protein. Other yeasts like *Pichia pastoris* has a number of advantages: it has strong inducible promoters; it is capable of making post-translational modifications similar to those performed by human cells; downstream processing is simpler as *Pichia* does not secrete its own proteins into the fermentation medium.

Metagenomics

In the last few years, another approach has been developed to identify and select microbial genes synthesizing novel molecules. This approach directly utilizes the large number of microbial genomes present in an environmental niche, for example in soil, in water such as ocean or in human gut. These genomes are contributed by both the culturable and the non-culturable variety of microbes and together constitute what has been termed as **metagenome**. The collective DNA is extracted from a sample of soil, water or any other environmental niche. It is subjected to restriction digestion using restriction endonucleses and the fragments are cloned as described in unit V (Chapter 1) of this book. The clones are then screened for presence of a variety of molecules. The clones expressing novel molecules or molecules with improved characteristics are used for large-scale production by fermentation techniques described in this chapter.

The metagenomic approach not only give the scientists an opportunity to cast a wider net on microbial resource present in the environment to fish out genes of their interest, it also gives them the opportunity to analyze the genomes of the microbes without culturing these in the laboratory. Thus, it is really a very useful approach to study those microbes, which are difficult to culture in the laboratory or have never been cultured in the laboratory as yet, and analyze these to see if they carry any genes, which may be exploited for human use. A typical procedure depicting metagenomic approach is shown in **Fig. 11**.

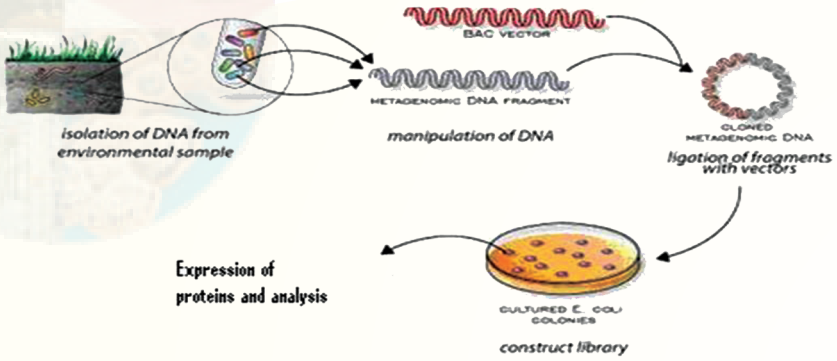

Fig. 11. A typical procedure depicting metagenomics approach .

Strain preservation:

Once a strain producing a novel or desired product has been obtained, it must be appropriately preserved for future use. If not done properly, the strain may be lost through loss of viability or even show decline in the production of the product for which it was isolated.

Storage on agar: Cultures are grown on agar slants or stabs & stored at 5 to -20 °C. These must be sub-cultured at approximately 6-month interval. The time of sub-culture may be extended to 1 year if cultures are covered with sterile mineral oil.

Storage in liquid nitrogen: The culture is grown and a cryoprotective agent like glycerol (10-30%) is added. These are dispensed in sealed ampoules & frozen in liquid nitrogen. (-176 to -196 °C).

Lyophilisation: Lyophilization or freeze-drying involves freezing of a culture followed by drying under vacuum. This results in sublimation of cell water. Lyophilised culture may remain viable for 5-10 years or more.

Culture Collections Centers

Cultures may be deposited to culture collection centers. These centers safely maintain cultures for years. The cultures are also made available to prospective investigators. With the advent of the modern biotechnology and the associated commercial and financial implications, the culture collection centers are governed by stringent rules & regulations to protect the intellectual property rights of the depositors. Some of the well-known culture collection centers of international repute are ATCC (American Type Culture Collection, USA), NCIB (National Collection of Industrial Bacteria, UK) and DSM (Deutsche Sammlung von Mikroorganismen and Zelkulturen, Germany). The National culture collection of India is called **MTCC (Microbial Type Culture a Collection and Gene Bank)** and is located at Institute of Microbial Technology, Chandigarh. Recently another National culture collection centre named **NBAIM (National Bureau of Agriculturally Important Microorganisms)** has been established in India at Mau in Uttar Pradesh (U.P.)

6.1.7. Applications of microbial culture technology

Microbial cultures have immense potential for production of very useful compounds. Once the microbial culture is established, depending on its metabolic activity it can be used for the production of numerous compounds. In general, microbial cultures can be exploited primarily in six different ways for the production of metabolites. They are listed below:

1. Production of whole microbial cells (for food, vaccines).
2. Production of primary metabolites (acids, alcohol).
3. Production of secondary metabolites (antibiotics).
4. Biotransformation reactions (enzymatic, steroid).
5. Exploitation of metabolism (microbial leaching, biodegradable waste treatment).
6. Synthesis of recombinant proteins (therapeutic proteins).

The most ancient use of microbial cultures is for the production of fermented foods such as curd and cheese where the whole bacteria are used as starter cultures. A complete list of such foods has been given in Unit I (Chapter 1) of the textbook for the class XI. The whole microorganisms are also used for preparations such as bacterial vaccines, e.g. vaccines for typhoid and tuberculosis. Single cell protein (SCP) is another example where the whole microorganisms are used a source of protein. Production of alcohol and acids are examples of primary metabolic products, whereas antibiotics are examples of secondary metabolites produced by different microorganisms. Microbial metabolism has also been exploited for the microbial production of vitamins. Extraction of metals from ores and treatment of liquid waste are also examples where microbial metabolism is used to convert unsuitable substrates to useful products.

Table 1. Some microbial species used for producing commercial products

Microorganisms	Products
Saccharomyces cerevisiae	Ethanol
Aspergillus niger	Citric Acid
Penicillium chrysogenum	Penicillin
Streptomyces griseus	Streptomycin
Corynebacterium glutamicum	L-Lysine
Propionibacterium shermanii	Vitamin B12
Aspergillus oryzae	Amylases
Leuconostoc mesenteroides	Dextran
Escherichia coli (via recombinant technology)	Insulin, growth hormones and interferons
Saccharomyces cerevisiae (via recombinant technology)	Hepatitis B surface antigen
Alcaligenes eutrophus	Poly 3-hydroxybutyrate (PHB)

One of the most important uses of microorganisms in recent years has been as hosts for production of recombinant proteins using genetic engineering techniques. Expression of human insulin in *Escherichia coli* and hepatitis B surface antigen in Yeast for making hepatitis B vaccine are the most notable examples of applications of microbes for human use. Some specific examples of products derived from microbial cultures have been given in **Table 1**.

6.1.8. Biosafety issues in Microbial Technology

Till date, all the products of the biotechnology industry have been found to be safe. Nevertheless, for all biotechnology processes, safety is of paramount importance. The main areas of consideration for safety aspects specific to biotechnology are:

- Potential of genetically modified organisms (GMO) or recombinant strains to infect humans, animals and plants to cause diseases.
- Toxicity and allergy associated with the use of microbially produced biomolecules especially the recombinant molecules.
- Other medically relevant implications like increasing the environmental pool of antibiotic resistant microorganisms or transfer of antibiotic resistant genes.
- Problems associated with the disposal of spent microbial biomass.
- Safety aspects associated with contamination, infection or mutation of process strains.

When the microbes are to be used in industrial processes, it is preferred to use only those microorganisms, which are included in the **'GRAS' (generally regarded as safe)** category. Also, when microbes are to be used as hosts for production of recombinant molecules, this should be carried out using GRAS organisms.

The main fear associated with the genetically engineered microorganisms (GMO) or recombinant strains is that they could escape from the laboratory into the environment with unpredictable and perhaps catastrophic consequences. It was believed that such released microorganisms could upset the balance of nature or that foreign DNA in the new microorganism could alter the metabolic activity of the host microbe in unpredictable and undesirable ways. Guidelines have been established to ensure safe working practices and levels of containment based on potential hazards. Many of the GRAS organisms found in nature have been genetically modified so that these may be used as host for production of recombinant biomolecules. At present, all new releases of genetically modified organisms are evaluated by expert committees on a case by case basis for various safety aspects before approval for their use is granted.

Besides these, whenever we are required to culture microbes whether for research or for industrial applications, we must follow **good microbiological practices (GMP)**. These include:

i. Persons must wash their hands with germicidal soap after handling viable microorganisms.

i. Eating, drinking, smoking etc. are not permitted in the working area.

ii. Food is to be stored outside the work area in cabinets / refrigerators designated for this purpose only.

iii. Mouth pipetting is strictly prohibited.

iv. All procedures are carried out in a way, so as to minimize splashes and generation of aerosols.

v. After work with viable microorganisms, work surfaces are decontaminated thoroughly.

vi. It is recommended that laboratory coats / aprons should be worn while working.

vii. All cultures, stocks or other waste are decontaminated and autoclaved before disposal.

Review Questions

1. While culturing microbes in the laboratory in a flask, what measures do you suggest to enhance their growth?
2. Differentiate between:
   a. Batch and Fed batch culture.
   b. Chemostat and turbidostat.
3. How is a continuous culture better than batch or fed batch cultures?
4. Explain what is meant by steady state in relation to the growth of microbial cultures?
5. How is the large quantity of air, required in industrial fermentors, sterilized?
6. What problems must be tackled while expressing a eukaryotic gene in a prokaryotic host?
7. How would you ensure that production of a recombinant molecule does not occur until required?
8. What problems make the downstream processing of recombinant proteins difficult and costly?
9. How is *Pichia pastoris* better expression host compared to *Saccharomyces cerevisiae*?

10. How metagenomic approach helps to identify newer/novel genes?
11. Suggest two methods of preserving microbial strains.
12. What is lyophilisation?
13. Explain what is meant by GMP, GRAS?
14. What are the functions of the microbial culture collections?
15. Enlist five good laboratory practices, which need to be followed while working with microbes.
16. Why lyophilised cultures of microbes remain viable for several years?

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