UNIT- II

- **SCREENING OF MICROORGANISMS (PRIMARY & SECONDARY SCREENING)**

In microbial technology, microorganisms hold the key to the success or failure of a fermentation process. It is therefore important to select the most suitable microorganisms to carry out the desired industrial process. The most important factor for the success of any fermentation industry is of a production strain. It is highly desirable to use a production strain possessing the following four characteristics:

- It should be high-yielding strain.
- It should have stable biochemical/ genetical characteristics.
- It should not produce undesirable substances.
- It should be easily cultivated on large-scale.

Definition of screening:

Detection and isolation of high-yielding species of microorganisms form the natural sources material, such as soil containing a heterogeneous microbial population is called Screening.

OR

Screening may be defined as the use of highly selective procedures to allow the detection and isolation of only those microorganisms of interest from among a large microbial population.

Thus to be effective, screening allows the discarding of many valueless microorganisms, while at the same time allowing the easy detection of the small percentage of useful microorganisms that are present in the population. The concept of screening will be illustrated by citing specific examples of screening procedures that are or have been commonly employed in industrial research programs. During screening programs except crowded plate technique a natural source such as soil is diluted to provide a cell concentration such that aliquots spread, sprayed or applied in some manner to the surface of the agar plates will yield well isolated colonies (30-300).

The screening programmes generally consists of-

1. Primary screening. 2. Secondary screening

1. Primary screening:

It is carried out just to detect and isolate the species from mixed population that posses desired capacity. It is generally carried out by crowded plate technique. A natural source such as soil is diluted to provide cell concentration such that amount spread, applied in some manner to the surface of agar plate which will yield colonies not touching neighboring colonies. The following are some examples of primary screening-

A) Screening of Antibiotic producing organisms

B) Screening of organic acid and Amine producing organisms

C) Screening of organisms producing Vitamins, Amino acids and Growth Factors extracellularly

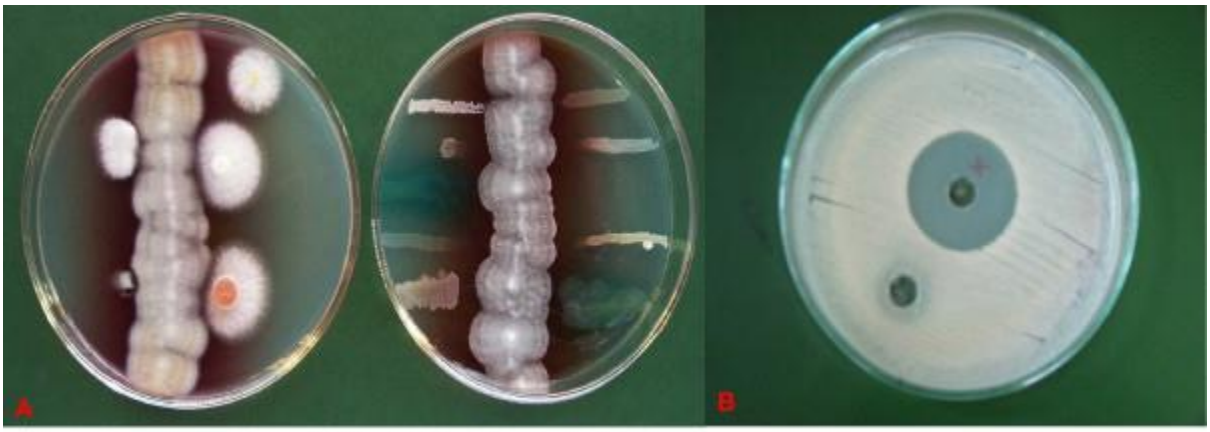
A) Screening of Antibiotic producing organisms: It is generally done by crowded plate technique. The diluted source is spread on the agar plate and the organisms are allowed to grow in incubator. After incubation the ability of particular colony to produce antibiotic is indicated by development of inhibition of growth around the colony.

(Primary screening carried out by this technique does not necessarily select antibiotic producing organisms, because inhibition around the colony may be due to change in pH of surrounding medium by the colony and rapid utilisation of nutrients in the surrounding area.)

Thus, further testing again is required to prove that the inhibitory activity associated with a microorganism can really be attributed to the presence of an antibiotic.

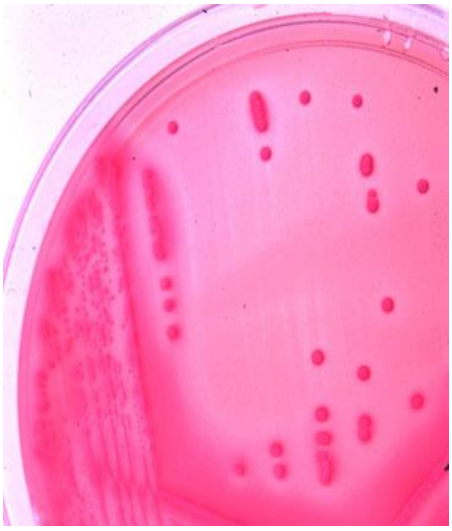
The crowded plate technique has limited application, since usually we are interested in finding a microorganism producing antibiotic activity against specific microorganism and not against the unknown microorganism that were by chance on the plate in the vicinity of an antibiotic producing organism. Antibiotic screening is improved, therefore by the incorporation into the procedure of a **"Test organism"** that is an organism used as an indicator for the presence of specific antibiotic activity.

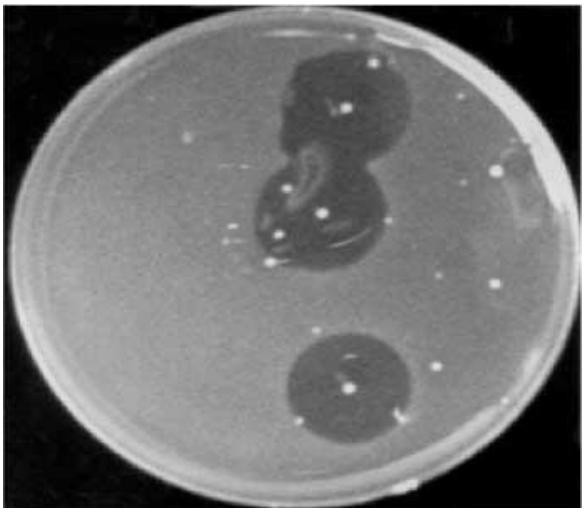
Dilutions of soil or of other microbial sources are applied to the surface of agar plates so that well isolated colonies will develop. The plates are incubated until the colonies are a few millimeters in diameter and so that antibiotic production will have occurred for those organisms having this potential. A suspension of test organism is then sprayed or applied in some manner to the surface of the agar and the plates are further incubated to allow growth of the test organism. Antibiotic activity is indicated by zones of inhibited growth of the organism around antibiotic producing colonies. In addition a rough approximation of the relative amount of antibiotic produced by various colonies can be gained by measuring in mm the diameters of the zones of inhibited test organism growth. Antibiotic producing colonies again must be isolated and purified before further testing.



A: Primary Screening for antimicrobial metabolite against fungi & bacteria
B: In cup plate assay showing activity against *Staphylococcus epidermidis*

B) Screening of organic acid and Amine producing organisms: For this serial dilutions of soil samples are prepared and spread on the surface of the medium so that after incubation well isolated colonies which do not touch the neighbouring colonies. The medium used contains sugars and pH indicating dye like neutral red or bromothymol blue, which indicates trace quantities of acid or amine produced by organism. In another method instead of pH indicating dye, calcium carbonate may be added. The production of acid or amine by colony is indicated by zone of dissolved $CaCo_3$.





C) Screening of organisms producing Vitamins, Amino acids and Growth Factors extracellularly: For isolation of such organisms medium must be totally lacking the metabolite under consideration. Again microbial source is diluted and plated to provide well isolated colonies. To this medium test organisms are also spreaded. The test organisms must posses a definite growth requirement for particular metabolite. Such metabolites will be indicated by zone of growth or increased growth.

2. Secondary screening:

Primary screening is usually followed by secondary screening to test the capabilities of organisms which are obtained in primary screening. Secondary screening allows the further sorting of those microorganisms that have real value for industrial purposes and discarding valueless microorganisms. Thus the secondary screening allows determination of the most efficient and suitable organism to be used for commercial production of the fermentation product from different potential strain as detected and isolated by primary screening.

Secondary screening is conducted on agar plates, in flasks or small fermentors containing liquid media. Agar plates are not as sensitive as liquid culture but have advantage in secondary screening because more information is obtained, also agar plates take relatively little space in incubator & do not require amount of handling & workup effort associated with liquid culture. Agar culture provides only limited indication of actual product yield among various isolates. To obtain this information we must employ liquid culture because it provides a much better picture of nutritional, physical & production responses of an organism to actual fermentation production conditions.

There are two types of secondary screening techniques:

a) Qualitative secondary screening

b) Quantitative secondary screening

The qualitative secondary screening tells us the spectrum or range of microorganisms which are sensitive to newly discovered antibiotic but quantitative secondary screening tells us yield of antibiotic which can be expected when microorganisms is grown in various different media.

Secondary screening should yield the type of information that is needed in order to evaluate the potential microorganisms for industrial use. E.g.

1. Secondary screening determines whether the microorganisms are producing new fermentation product or not. This is determined by Paper Chromatography, TLC, HOLC, GLC etc.
2. It gives an idea about the economic position of the fermentation process involving the use of a newly discovered culture. Thus one may have a comparative study of this process with processes that are already known, so far as the economic status picture is concerned.
3. It helps in providing information regarding the product yield potentials of different isolates. Thus this is useful in selecting efficient cultures for the fermentation processes.
4. It should determine what types of microorganisms are involved & whether they are classified at least to families or genera. This information is important because it allows comparison of the newly isolated organism with already present in literature. Classification of organism also allows whether the organism posses the pathogenicity to animal, Plants & Humans or not.
5. It provides information pertaining to the effect of different components of a medium. This is valuable in designing the medium that may be attractive so far as economic consideration.
6. It should determine whether microorganisms are actually producing new chemical compound, which is not previously described.
7. It should reveal pH, aeration and other nutritional requirements to particular microorganism.
8. It determines the chemical stability of the product.

9. Information about the solubility of the product in various organic solvents is made available. (Useful in product recovery operation and purification).
10. Chemical, physical and biological properties of a product are also determined during secondary screening. Moreover, it reveals whether a product produced in the culture broth occurs in more than one chemical form.
11. It reveals whether the culture is homofermentative or heterofermentative.
12. Determination of the structure of product is done. The product may have a simple, complex or even a macromolecular structure.
13. With certain types of products (e.g. antibiotics) determination of the toxicity for animals, plants or man are made if they are to be used for therapeutic purpose.
14. It should reveal whether product resulting from fermentation occur in culture broth in more than one form.
15. It should reveal whether the microorganisms are able to alter or destroy their own fermentation product. It is determined because after accumulation of high level of product in culture broth, some organisms produce adaptive enzymes, which destroy the usefulness of product. Thus microorganism might produce a recemase enzyme that change L-configuration of an Amino acid to D & L isomers. Microorganisms that produce the adaptive enzyme, decarboxylase can remove carbon dioxide from amino acid, leaving behind an organic amine.
16. It gives information about number of products in single fermentation.
17. It determines genetic stability of the Microbial strain.

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- **Preservation methods for industrially important microorganisms STOCK CULTURE AND ITS MAINTENANCE**

INTRODUCTION:

Several methods have been devised for preserving microbial cultures. None of them can be said to apply exclusively to industrial microorganisms. Furthermore, no one method is suitable for preserving all organisms. The method most suited to any particular organism must therefore be determined by experimentation unless the information is already available. Methods employed in the preservation of microorganisms all involve some limitation on the rate of

metabolism of the organism. A low rate of spontaneous mutation exists during the growth of microorganisms, about one in every 10^9 division. Lowering the metabolic rate of the organism will further reduce the chances of occurrence of mutations. Many methods of preservation for microorganisms have been developed. Here, it is to be noted that there exist different types of microorganisms (bacteria, viruses, algae, protozoa, yeasts and mold). Therefore, there are two criteria for selecting a method of preservation for a given culture. They are:

- The period of preservation desired, and
- The nature of a culture to be preserved.

Definition: - Stock cultures are those cultures of microorganisms that are stored or maintained for future use in such a fashion that their growth and productive capacities remains unaltered.

There are two types of stock cultures: (i) working stocks and (ii) Primary stocks. The working stock cultures are those which are used frequently and they must be maintained in a vigorous and uncontaminated condition. These cultures are maintained as agar slants, agar stabs, spore preparations or broth cultures and they are held under refrigeration. They must be checked constantly for possible changes in growth characteristics, nutrition, productive capacity and contamination.

Primary stocks are cultures that are held in reserve for practical or new fermentations, for comparative purposes, for biological assays or for possible later screening programs. These cultures are not maintained in a state of high physiological activity and they are delved into only rarely. Transfers from these cultures are made only when a new working-stock is required, or when the primary stock culture must be sub-cultured to avoid death of the cells. Thus, primary stock cultures are stored in such a manner as to require the least possible numbers of transfers over a period of time.

There are three basic aims in maintaining and preserving the microorganisms. They are: - i. To keep culture alive ii. Uncontaminated and iii. As healthy as possible, both physically and physiologically.

Following are the methods of preservations of microorganisms—

1. Serial subculture

2. Microbial Preservation Methods Based on the Reduction of the Temperature of Growth (Freezing)/ By using low temperature

A) Preservation on agar with ordinary refrigeration (4 – 10°C)

- Aerobic microorganisms

  - Agar slants
  - Petri dishes

- Anaerobic microorganisms

  - Agar stabs overlaid with petroleum jelly
  - Overlaying cultures with mineral oil

B) Preservation in Deep Freezers at about -20°C, or between -60°C and -80°C

  - Preservation on glass beads
  - Storage of agar cores with microbial growth

C) Storage in low temperature liquid or vapor phase nitrogen (-156°C to -196°C)

  - Liquid nitrogen storage (Cryogenic storage)

3. Microbial Preservation Methods Based on Dehydration / Removal of water

  - Drying on sterile silica gel
  - Preservation on sterile filter paper
  - Preservation in sterile dry soil /Soil culture /Soil stock
  - Freeze-drying (drying with freezing)/ lyophilization

1. Serial subculture: -

This is the simplest and most common method of maintaining microbial cultures. Microbes are grown on agar slants and are transferred to fresh media before they exhaust all the nutrients or dry out. An exception to this is aerobic Streptomyces spp. Where drying up of the medium has been found successful, provided the initial growth showed the production of serial hyphae. The drying of medium appeared to encourage good sporulation and the preserved specimen became simply a dried out strand of agar coated with spores, which remained viable for a few years at room temperature. For some microbial cultures no other methods have been found satisfactory, but for the majority of species other methods are available.

2. Microbial Preservation Methods Based on the Reduction of the Temperature of Growth (Freezing)

Organisms growing on suitable agar at normal growth temperatures attain the stationary phase and begin to die because of the release of toxic materials and the exhaustion of the nutrients. Agar-grown organisms are therefore **refrigerated** as soon as adequate growth is attained as to preserve them. Freezing is rapidly gaining acceptance for preserving organisms because of its dual use for working and primary stock maintenance as well as its storage effectiveness for up to three years. It is useful for a wide range of organisms, and survival rates have been shown to be as good as freeze-drying in many organisms.

Advantages of the freezing methods

- the methods are simple to use and require a minimum of equipment
- they save space as many hundreds of cultures can be stored in a small space;
- beads thaw rapidly and hence the method saves time,
- differently bead colors can represent different bacteria and so recognizing them is easy;
- the methods can be adapted for both aerobic and anaerobic organisms;
- the methods are suitable for situations or countries where power outages occur, as the freezer can remain cold for some time during power failures.

Following are the methods used in freezing

A) Preservation on agar with ordinary refrigeration (4 – 10°C)

- Aerobic microorganisms

- Agar slants
- Petri dishes

- Anaerobic microorganisms

- Agar stabs overlaid with petroleum jelly
- Overlaying cultures with mineral oil

B) Preservation in Deep Freezers at about -20°C, or between -60°C and -80°C

- Preservation on glass beads
- Storage of agar cores with microbial growth

C) Storage in low temperature liquid or vapor phase nitrogen (-156°C to -196°C)

- Liquid nitrogen storage

A) Preservation on agar with ordinary refrigeration (4 – 10°C)

Culture grown on agar slope may be stored in a refrigerator (4 - 10^0 C) and sub cultured at approximately 6 months to 1year. They are transferred more frequently. These frequent culture transfers and the many cell generations accompanying these transfers allow the possible occurrence of and selection for undesired genetic changes in the organisms. Also the potential for contamination is markedly increased with frequent transfer of the cultures.

- Aerobic microorganisms

- Agar slants
- Petri dishes

- Anaerobic microorganisms

  - Agar stabs overlaid with petroleum jelly
  - Overlaying cultures with mineral oil

- ***Aerobic microorganisms***

  - ***Agar slants*****:** Aerobic organisms may be grown on **agar slants** and refrigerated at 4 – 10°C as soon as they have shown growth.

  - ***Petri dishes*****:** Aerobic organisms may also be stored on Petri dishes. The plates may be sealed with electrical tapes to prevent the plates from drying out on account of evaporation and refrigerated at 4 – 10°C as soon as they have shown growth. Electrical tapes of different colors may be used to identify special attributes or groups among the cultures.

- ***Anaerobic microorganisms***:

  - **Agar stabs overlaid with petroleum jelly:** Anaerobic organisms may be stored on *agar stabs* which are then sealed with sterile molten petroleum jelly.
  - **Overlaying cultures with mineral oil**

Agar slant and stab cultures of many microorganisms will survive several years at room temperature if the growth is submerged under sterile mineral oil. The oil overlay provides dissolved oxygen, prevents drying of the agar and apparently decreases the metabolic activity of the cells to an almost negligible rate. However, genetic changes do occur in cultures stored in this manner. The steps involved in this method are-

- Inoculation of agar slant /stab with the culture to be maintained.
- Inoculated agar slant/stab is subjected to incubation until good growth appears.
- Using sterile technique, a healthy agar slant/stab culture is covered with sterile mineral oil to a depth of about 1 cm above the top of the agar slant. If a short slant is used, less oil is required.

- Finally, oiled cultures can be stored at room temperature. But better viability is obtained when stored at lower temperatures (15 °C).
- **Note:** The depth of oil of 1 cm is fairly critical, as the oxygen transmission by layers of mineral oil in excess of 1 cm becomes less favorable. If less oil is used, strands of mycelium may be exposed which allows the cultures to dry out. If the bottles or screw capped tubes are used, the rubber liners should be removed from the caps as the oil tends to dissolve the rubber and this can be toxic to the culture.
- Practically all bacterial species or strains tested live longer under oil than in the control tubes without oil. Some bacterial species have been preserved satisfactorily for 15-20 years.

B) Preservation in Deep Freezers at about -20°C, or between -60°C and -80°C

- **Preservation on glass beads**
- **Storage of agar cores with microbial growth**

The regular home freezer attains a temperature of about -20 °C. Laboratory deep freezers used for molecular biology work range in temperature between -60 °C and -80 °C. It is possible to store microorganisms in either type of deep freezers in the form of agar plugs or on sterile glass beads coated with the organism to be stored.

- ***Preservation on glass beads***

The bacteria to be preserved are placed in broth containing cryoprotective compounds such as glycerol, raffinose, lactose, or trehalose. Sterile glass beads are placed in the glass vials containing the bacterial cultures. The vials are gently shaken before being put in the deep freezers.

- ***Storage of agar cores with microbial growth***

Bacteria, but especially molds, yeasts, and actinomycetes may be stored as agar plugs made from plates of the confluent growths bacteria or of hyphae of filamentous organisms. It consists of placing agar plugs of confluent growth of bacteria and yeasts and hyphae of molds or actinomycete in glass vials containing a suitable cryoprotectant and freezing the vials in deep freezers as above. To initiate growth a plug is placed in warm broth and plated out.

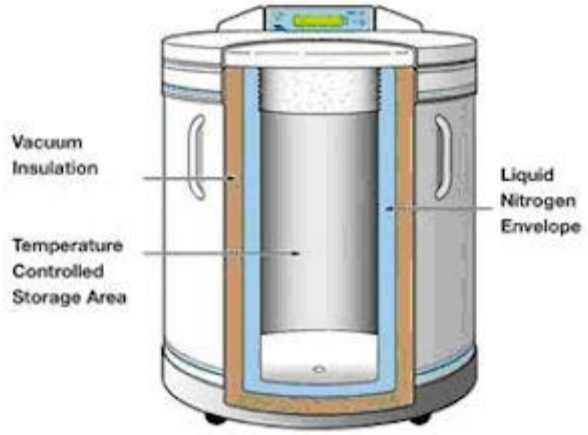
C) Storage in low temperature liquid or vapor phase nitrogen (-156°C to -196°C)

The liquid or vapor phase of nitrogen at -156°C to -196°C is widely used for preserving microorganisms and cultured cells. Fungi, bacteriophages, viruses, algae, protozoa, bacteria, yeasts, animal and plant cells, and tissue cultures have all been successfully preserved in it.

❖ Liquid nitrogen storage (Cryogenic Storage):

This method is also called as Cryogenic storage. It is like lyophilization, a satisfactory method for the long-term preservation of microorganisms. It has also been successful with many specimens which cannot be preserved by lyophilization. The maintenance of microbes is done by suspended metabolism. Life is regarded as "Stand still" at –130°C and below, so at the temperature of liquid nitrogen (-196°C), provided the cultures survive the treatment, the period of preservation should be indefinite. Thus, long-term preservation without any change in the cultural characteristics is now attainable. Major steps involved in the methods are:

- The culture to be maintained is suspended (thick suspension) as a cell or spore suspension in 10% glycerol.
- This thick suspension in glycerol is distributed into ampoules (resistant to cold-shock).
- Ampoules filled with a culture suspension are frozen (at the rate of about 1°C per minute to –20 to –35°C) and are sealed.
- The frozen ampoules are then clipped on metal (aluminium) canes, one above the other and six to each cane. The canes in turn are packed in metal boxes or canisters (aluminium), which holds about 20 canes. These are perforated to allow the free running of the liquid nitrogen.
- The cultures are revived by removing form the container rapidly thawing and culturing them in the usual manner.



Advantages of Liquid Nitrogen Storage:

i. It is effective method of preservation.

ii. No subculturing is required.

iii. The cultural characteristics remain unchanged.

iv. The ampoules are not open to contamination or infection by mites, since they are sealed.

v. The living material of a type, which would not normally grow in a culture and would not be preserved in a culture collection, can be retained in a viable state.

3. Microbial Preservation Methods Based on Dehydration

Just as reduction in temperature limits the metabolism of the organism, dehydration removes water a necessity for the metabolism of the organism. Several methods may used to achieve desiccation as a basis for preserving microorganisms.

- **Drying on sterile silica gel**
- **Preservation on sterile filter paper**
- **Preservation in sterile dry soil /Soil culture /Soil stock**
- **Lyophilization or freeze-drying or drying with freezing**

❖ Drying on sterile silica gel

Many organisms including actinomycetes and fungi are dried by this method. Screw-cap tubes half-filled silica gel are sterilized in an oven. On cooling a skim-milk suspension of spores and the cells of the fungus or actinomycetes is placed over the silica gel and cooled. They are dried at 25°C, cooled and stored in closed containers containing desiccants.

❖ Preservation on sterile filter paper

Spore-forming microorganisms such as fungi, actinomycetes, or *Bacillus* spp may be preserved on sterile filter paper by placing drops of broth containing the spores on sterile filter paper in a Petri dish and drying in a low temperature oven or in a dessicator. Alternatively, sterile filter paper may be soaked in the broth culture of the organism to be dried, placed in a tube, which is then evacuated and sealed. After drying the filter paper may be placed in sterile screw caps bottles and stored either at room temperature or in the refrigerator.

❖ Preservation in sterile dry soil /Soil culture /Soil stock

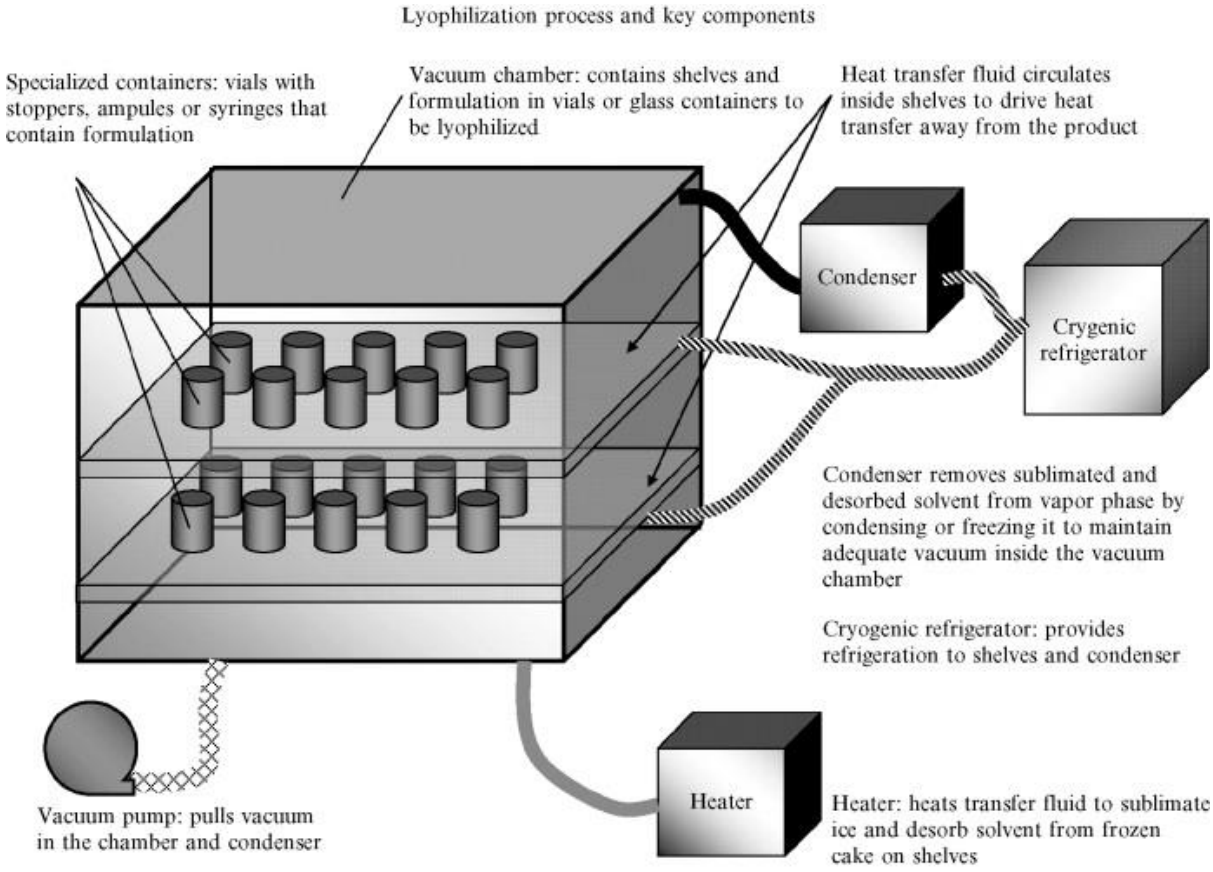
Sterile soil has found wide use for the stock culture maintenance of microorganisms that form spores. This method is particularly applied for the preservation of sporing microbes specially fungi. In fact, microorganisms that do not form spores also will survive in sterile soil, but they may die out unexpectedly after a period of time. Soil stocks are prepared as follows:

- A mixture of soil (20 %), sand (78%) and calcium carbonate (2%) is prepared and distributed into tubes (a few grams per tube).
- They are sterilized for 8-15 hours at 130°C and then cooled.
- A small volume of thick suspension of spores or of an actively growing culture is then added to the sterile soil and incubated till good growth.
- The inoculated tubes are kept in desiccators under vacuum. The reason behind this is to evaporate the excess water. Then the tubes are sealed.
- Soil stocks, thus prepared are stored at room temperatures with cotton plugs or screw caps protected from dust. These cultures can be stored in refrigerators at about 5-8 °C temperature.

❖ Lyophilization or freeze-drying or drying with freezing

Lyophilization is the most satisfactory method of long-term preservation of microorganisms. It is universally used for the preservation of bacteria, viruses, fungi, sera, toxins, enzymes and other biological materials. Lyophilization is the most popular form of suspended metabolism. It consists of drying of cultures or a spore suspension from the frozen state under reduced pressure. This can be accomplished in several ways. The major steps involve in this techniques are:

- A thick cell or spore suspension is prepared in a suitable protective medium (10% skimmed milk or bovine serum, 5% inositol in distilled water).
- Using sterile techniques, this thick suspension is distributed in small quantities into glass ampoules.
- These ampoules are subjected to deep-freezing by keeping the cultures at lower temperature (-20°C).
- Then the chilled ampoules are connected with a high vacuum system usually incorporating a desiccant (e.g. phosphorous pentoxide, silica gel or a freezing trap), and immersed into a freezing mixture of dry ice and alcohol (-78°C).
- The vacuum pump is turned on and the ampoules are evacuated till drying is complete.
- Freeze dried ampoules are then immediately sealed off and stored under refrigeration. If properly prepared and stored, most lyophilized cultures will remain viable for long periods (> 20 yrs.), without the occurrence of genetic changes.
- When needed, the cultures are recovered from the ampoules by suspending the lyophilized cells in a minimal amount of growth medium and then incubating.



Advantages of lyophilization:

i. As the ampoules are sealed there is no risk of contamination of infection with mites.
ii. The prepared ampoules are easily stored, they are not readily broken and most species remain viable for many years.
iii. There is less opportunity for the cultures to undergo changes in characteristics (i.e. they remain unchanged during storage period).
iv. Owing to the small size of glass ampoules, hundreds of lyophilized cultures can be stored in a small storage space. In addition to this, the ampoules size makes them ideal for postage.
v. Lyophilization cuts down the number of transfers.
vi.

- **Strain Development Methods / Strategy / increasing product yield**

Increasing product yield

Several options are open to an industrial microbiology organization seeking to maximize its profits. The organization may undertake -

- more aggressive marketing tactics, including more attractive packaging while leaving its technical procedures unchanged.
- It may use its human resources more efficiently and hence reduce costs, or it may adopt a more efficient extraction system for obtaining the material from the fermentation broth.
- The operations in the fermentor may also be improved by its use of a more productive medium, better environmental conditions, better engineering control of the fermentor processes,
- It may genetically improve the productivity of the microbial strain it is using.

  Of all the above options, strain improvement appears to be the one single factor with the greatest potential for contributing to greater profitability.

An ideal industrial strain should have the following qualities:

a) The organism should be able to use wide range of low cost and easily available substrate throughout the year.
b) The organism should produce large biomass and high amount of industrial product.
c) The organism should be non-pathogenic and non-allergic.
d) Organism should not degrade with passage of time i.e. it should be genetically stable.
e) It should be easy to perform genetic manipulation on it.

Unfortunately the organism isolated from the environment for industrial use does not fulfill all these requirements.

The first task for an industrial microbiologist is to find a suitable microorganism for use in the desired process. Approaches are available ranging from

A) Isolating / Finding microorganisms from the environment / Nature.

B) Genetic Manipulation of Microorganisms by using sophisticated molecular techniques to modify an existing microorganism.

A) Isolating / Finding microorganisms from the environment / Nature

Until recently, the major sources of microbial cultures for use in industrial microbiology were natural materials such as soil samples, waters, and spoiled bread and fruit. Cultures from all areas of the world were examined in an attempt to identify strains with desirable characteristics. Interest in "hunting" for new microorganisms continues unabated. Because only a minor portion of the microbial species in most environments has been isolated or cultured. There is a continuing effort throughout the world to find new microorganisms, even using environments that have been examined for decades. In spite of these long-term efforts, few microorganisms have been cultured and studied; most microbes that can be observed in nature have not been cultured or identified, although molecular techniques are making it possible to obtain information on these uncultured microorganisms. With increased interest in microbial diversity and microbial ecology, and especially in microorganisms from extreme environments, microbiologists are rapidly expanding the pool of known microorganisms with characteristics desirable for use in industrial microbiology and biotechnology.

They are also identifying microorganisms involved in mutualistic and protocooperative relationships with other microorganisms and with higher plants and animals. There is continuing interest in bioprospecting in all areas of the world, and major companies have been organized to continue to explore microbial diversity and identify microorganisms with new capabilities.

B) Genetic Manipulation of Microorganisms by using sophisticated molecular techniques to modify an existing microorganism.

Genetic manipulations are used to produce microorganisms with new and desirable characteristics. The classical methods of microbial genetics play a vital role in the development of cultures for industrial microbiology. Following are the methods used to modify microbial genetics –

1. Transformation, Conjugation and Transduction
2. Mutation
3. Protoplast Fusion
4. Insertion of Short DNA Sequences
5. Transfer of Genetic Information between Different Organisms / Genetic engineering / Recombinant DNA technology
6. Modification of Gene Expression
7. Natural Genetic Engineering

1. Transformation, Conjugation and Transduction

Transformation is a change in genetic property of a microorganism which is brought about when foreign DNA is absorbed by, and integrates with the genome of, the donor cell. Transformation has been used to introduce streptomycin production into *Streptomyces olivaceus* with DNA from *Streptomycin grisesus.* Oxytetracycline producing ability was transformed into irradiated wild-type *S. rimosus*, using DNA from a wild-type strain. An inactive strain of *Bacillus* was transformed to one producing the antibiotic bacitracin with the same method.

Conjugation involves cell to cell contact or through sex pili (*singular*, pilus) and the transfer of plasmids. Conjugation involves a donor cell which contains a particular type of conjugative plasmid, and a recipient cell which does not. The donor strain's plasmid must possess a sex factor as a prerequisite for conjugation; only donor cells produce pili. The sex factor may on occasion transfer part of the hosts' DNA. Mycelial 'conjugation' takes place among actinomycetes with DNA transfer as in the case of eubacteria. Plasmids play an important role in the formation of some industrial products, including many antibiotics.

Transduction is the transfer of bacterial DNA from one bacterial cell to another by means of a bacteriophage. It is now possible by methods to excise genes responsible for producing certain enzymes and attach them on the special mutant viral particles, which do not cause the lysis of their hosts. Several

hundreds of virus particles carrying the attached gene may therefore be present in one single bacterial cell following viral replication in it. The result is that the enzyme specified by the attached gene may be produced up to 1,000-fold.

2. Mutation

Once a promising culture is found, a variety of techniques can be used for culture improvement, including physical and chemical mutagens. As an example, the first cultures of *Penicillium notatum*, which could be grown only under static conditions, yielded low concentrations of penicillin. In 1943, a strain of *Penicillium chrysogenum* was isolated— strain NRRL 1951—which was further improved through mutation. Today most penicillin is produced with *Penicillium chrysogenum*, grown in aerobic stirred fermentors, which gives 55-fold higher penicillin yields than the original static cultures.

Physical agents such as ionizing radiations and ultraviolet light are used to induce mutations. ***Ionizing radiations*** include X-rays, gamma rays, alpha-particles and fast neutrons. The mutagenic range of ultraviolet light lies between wave length 200 and 300 nm. The main effect of ultraviolet light on DNA is the formation of covalent bonds between adjacent pyrimidine (thymine and cytosine) bases. Thymine is mainly affected, and hence the major effect of UV light is thymine dimerization, although it can also cause thymine-cytosin and cytosin-cytosin dimers. Dimerization causes a distortion of the DNA double strand and the ultimate effect is to inhibit transcription and finally the organism dies.

Chemical mutagens may be divided into three groups:

(i) Those that act on DNA of resting or non-dividing organisms; e.g. nitrous acid, Alkylating agents and nitrosoguanidine. These chemicals work by causing chemical modifications of purine and pyrimidine bases that alter their hydrogen bonding properties. For example, nitrous acid converts cytosine to uracil which then forms hydrogen bonds with adenine rather than guanine.

(ii) DNA base analogues which may be incorporated into DNA during replication e. g. 2-amino purine, 5-bromouracil (5BU). These are compounds

which because they are similar to base nucleotides in composition may be incorporated into a dividing DNA in place of the natural base.

(iii) Those that cause frame-shift mutations. E. g. Acridines. Frameshift or intercalating agents are planar three-ringed molecules that are about the same size as a nucleotide base pair. During DNA replication, these compounds can insert or intercalate between adjacent base pairs thus pushing the nucleotides far enough apart that an extra nucleotide is often added to the growing chain during DNA replication. A mutation of this sort changes all the amino acids downstream and is very likely to create a nonfunctional product.

3. Protoplast Fusion

Protoplast fusion is now widely used with yeasts and molds. Most of these microorganisms are asexual or of a single mating type, which decreases the chance of random mutations that could lead to strain degeneration. To carry out genetic studies with these microorganisms, protoplasts are prepared by growing the cells in an isotonic solution while treating them with enzymes, including cellulase and beta-galacturonidase. The protoplasts are then regenerated using osmotic stabilizers such as sucrose. If fusion occurs to form hybrids, desired recombinants are identified by means of selective plating techniques. After regeneration of the cell wall, the new protoplasm fusion product can be used in further studies.

A major advantage of the protoplast fusion technique is that protoplasts of different microbial species can be fused, even if they are not closely linked taxonomically. For example, protoplasts of *Penicillium roquefortii* have been fused with those of *P. chrysogenum.* Even yeast protoplasts and erythrocytes can be fused.

4. Insertion of Short DNA Sequences (Site directed mutagenesis)

Short lengths of chemically synthesized DNA sequences can be inserted into recipient microorganisms by the process of **site directed mutagenesis.** This can create small genetic alterations leading to a change of one or several amino acids in the target protein. Such minor amino acid changes have been found to lead, in many cases, to unexpected changes in protein characteristics, and have resulted in new products such as more environmentally resistant

enzymes and enzymes that can catalyze desired reactions. These approaches are part of the field of **protein engineering. E**nzymes and bioactive peptides with markedly different characteristics (stability, kinetics, and activities) can be created.

The most common method involves use of a chemically synthesized oligonucleotide mutant which can hybridize with the DNA target molecule; the resulting mismatch-carrying DNA duplex may then be transfected into a bacterial cell line and the mutant strands recovered. The DNA of the specific gene to be mutated is isolated, and the sequence of bases in the gene determined. Certain pre-determined bases are replaced and the 'new' gene is reinserted into the organism.

5. Transfer of Genetic Information between Different Organisms (Genetic engineering, Recombinant DNA technology, Molecular cloning or Gene cloning)

New alternatives have arisen through the transfer of nucleic acids between different organisms, which are part of the rapidly developing field of **combinatorial biology**. This involves the transfer of genes for the synthesis of a specific product from one organism into another, giving the recipient varied capabilities such as an increased capacity to carry out hydrocarbon degradation. An important early example of this approach was the creation of the "superbug," patented by A. M. Chakarabarty in 1974, which had an increased capability of hydrocarbon degradation.

The genes for antibiotic production can be transferred to a microorganism that produces another antibiotic, or even to a non-antibiotic-producing microorganism. For example, the genes for synthesis of bialophos (an antibiotic herbicide) were transferred from *Streptomyces hygroscopicus* to *S. lividans.* Other examples are the expression in *E. coli*, of the enzyme creatininase from *Pseudomonas putida* and the production of pediocin, a bacteriocin, in yeast used in wine fermentation for the purpose of controlling bacterial contaminants.

DNA expression in different organisms can improve production efficiency and minimize the purification steps required before the product is ready for use. For example, recombinant baculoviruses can be replicated in insect larvae to achieve rapid large scale production of a desired virus or protein.

Transgenic plants may be used to manufacture large quantities of a variety of metabolic products. A most imaginative way of incorporating new DNA into a plant is to simply shoot it in using DNA coated microprojectiles and a gene gun.

A wide range of genetic information also can be inserted into microorganisms using vectors and recombinant DNA techniques. Vectors include artificial chromosomes such as those for yeasts (YACs), bacteria (BACs), P1 bacteriophage-derived chromosomes (PACs), and mammalian artificial chromosomes (MACs). YACs are especially valuable because large DNA sequences (over 100 kb) can be maintained in the YAC as a separate chromosome in yeast cells.

A good example of vector use is provided by the virus that causes foot and mouth disease of cattle and other livestock. Genetic information for a foot-and-mouth disease virus antigen can be incorporated into *E. coli,* followed by the expression of this genetic information and synthesis of the gene product for use in vaccine production.

6. Modification of Gene Expression (Metabolic engineering, metabolic control engineering)

In addition to inserting new genes in organisms, it also is possible to modify gene regulation by changing gene transcription, fusing proteins, creating hybrid promoters, and removing feedback regulation controls. These approaches make it possible to overproduce a wide variety of products. As a further example, genes for the synthesis of the antibiotic actinorhodin have been transferred into strains producing another antibiotic, resulting in the production of two antibiotics by the same cell.

This approach of modifying gene expression also can be used to intentionally alter metabolic pathways by inactivation or deregulation of specific genes, which is the field of **pathway architecture.** Alternative routes can be used to add three functional groups to a molecule. Some of these pathways may be more efficient than the others. Understanding pathway architecture makes it possible to design a pathway that will be most efficient by avoiding slower or energetically more costly routes. This approach has been used to improve penicillin production by ***m*etabolic *p*athway *e*ngineering (MPE).**

An interesting recent development in modifying gene expression, which illustrates **metabolic control engineering,** is that of altering controls for the synthesis of lycopene, an important antioxidant normally present at high levels in tomatoes and tomato products. In this case, an engineered regulatory circuit was designed to control lycopene synthesis in response to the internal metabolic state of *E. coli.* An artificially engineered region that controls two key lycopene synthesis enzymes is stimulated by excess glycolytic activity and influences acetyl phosphate levels, thus allowing a significant increase in lycopene production while reducing negative impacts of metabolic imbalances.

Other examples include the increased synthesis of antibiotics and cellulases, modification of gene expression, DNA amplification, greater protein synthesis, and interactive enzyme overproduction or removal of feedback inhibition. Recombinant plasminogen, for example, may comprise 20 to 40% of the soluble protein in a modified strain, a tenfold increase in concentration over that in the original strain.

7. Natural Genetic Engineering

The newest approach for creating new metabolic capabilities in a given microorganism is the area of **natural genetic engineering,** which employs **forced evolution** and **adaptive mutations.** This is the process of using specific environmental stresses to "force" microorganisms to mutate and adapt, thus creating microorganisms with new biological capabilities. The mechanisms of these adaptive mutational processes include DNA rearrangements in which transposable elements and various types of recombination play critical roles. Such "environmentally directed" mutations have the potential of producing microbes with new degradative or biosynthetic capabilities.

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- **Inoculum development**

An inoculum can be defined as the population of microorganisms or cells that is introduced in the fermentation medium or any other suitable medium. It is prepared and optimized before the fermentation process commences. The inoculum needs to be optimized for better performance, which can be done on the basis of various parameters, including mutation (DNA recombination, radiation, and chemical addition).

In medical terminology, an inoculum can be defined as a population of bacteria or viruses, which is introduced in the body to cause or to increase immunity to a specific disease or condition, or into the medium for different medical microbiological procedures.

Preparation of the Inoculums

Microbial inoculums have to be prepared from the preservation culture so that it can be used for the fermentation process. The aim of inoculum preparation is to select microorganisms with high productivity and to minimize low productive, mutant strains. The process involves several steps:

First generation culture is prepared from the preservation culture on agar slants which is then sub-cultured to prepare "working culture". At this stage the microorganisms start growing. In small scale fermentation processes working culture is used as inoculum, but for large scale fermentation inoculum preparation involves additional steps.

Second, sterile saline water or liquid nutrient medium containing glass beads is added to the agar slants and shaken so that microbial suspension is prepared. This suspension is transferred to a flatbed bottle which contains sterile agar medium. The microorganisms are allowed to grow by incubating the bottle.

Third, the microbial cells from the flat bed bottles are transferred to a shaker flask containing sterile liquid nutrient medium and is placed on a rotary shaker bed in an incubator. Microorganisms grow at a rapid rate due to aeration.

Fourth, microbial cells from the shaker flask can be used as seed culture which are then added to small fermenters and allowed to grow for 1-2 days. This simulates conditions that exist in the larger fermenters to be used for production of metabolites. Finally, the microorganisms are transferred to the main fermentation vessel containing essential media and nutrients.

During the first stage of inoculum development, the inoculum is taken from the working stock culture to initiate the growth in a suitable liquid medium. Growth at this stage is influenced by the adaptation of inoculum to the new environment, which further influences the final product.

Bacterial vegetative cells and spores are suspended in sterile tap water or sterile saline, which is then added to the broth. In case of nonsporulating fungi

and actinomycetes, hyphae are used for inoculum, and then transferred to the final solution.

Inoculum development is done in sequential steps to increase the volume of the initial inoculum to the desired level. At each step, the inoculum used is 0.5–5% of the medium volume, which constitutes a 20–200-fold increase in the inoculum volume at each step from its original size. An active growth stage is required in the final production stage of fermentation processes.

Many fermentation processes have several stages of development and scale-up of inocular microbial growth. The objective is usually to achieve a high level of viable biomass in a suitable physiological form and a growth stage for use as an inoculum for the next stage where small cell protein (SCP) for enzymes, proteins, or metabolite production, or viable biomass is the required end product.

In microbiological techniques, inoculation of samples and microbiological cultures in the relevant media is crucial to obtain a proper yield for diagnosis and antimicrobial sensitivity, both for bacterial and for fungal cultures. Correct preparation of inoculum for cultures is integral to many microbiological procedures. One main component is standardization of size of cultures, which has implications mostly in antimicrobial susceptibility testing.

Counting methods of bacteria also require standardization of bacterial inoculum for the same. In case of viral cultures, inoculation requires meticulous preparation of viral stock to ensure maximum viral yield. Animal cells from tissues are cultivated followed by expansion of the cells for mammalian cell-culture techniques for industrial and medical use.

Criteria for inoculum Preparation for Fermentation Process

1. **Physiology and Morphology**

Optimized fermentation is often associated with particular physiological and morphological forms. For instance, in streptomycin-producing strains, mycelia fragmentation, loss of conidia formation, and progressive change to noncardial-type morphologies are associated with culture degeneration and antibiotic producing capacity. The production of some microbial products is associated with spore formation, whereas the synthesis of other products is inhibited by spore formation. Spore formation, in turn, can be regulated by

media design. In general, low levels of complex nitrogen induce spore formation.

Certain medium-related conditions have been shown to affect the morphology. These include pH, viscosity, divalent cations, chelating agents, anionic polymers, surface-active agents, and the presence of solids in medium.

2. **Healthy and Active inoculum**

In order to achieve exponential phase in less time, microbes should have a short lag period, that is, they take less time to adapt to the environmental conditions. This is possible only with healthy and actively growing microbial growth. Healthy and active growth can be obtained by providing microbes with proper medium, necessary conditions, and a good monitoring system. The initial size of the inoculum influences the duration of the lag period, which is represented by the mathematical equation.

$$\lambda_n = -\frac{1}{\mu}\log\left(\sum_{i=1}^{n}\alpha_i\right)$$

Where n is the size of inoculum. It has been demonstrated that the expected log value with initial cell number n is always greater than the population with double (2n) initial cell number.

3. **Optimum Size**

Starting from a slant or stock, the inoculum is usually built up in two or three stages in the laboratory followed by one or more stages in conventional fermentors. During this process, the cells may undergo 20–50 generations or more. At each step, the inoculum is used at 0.5–5% of the medium volume, which allows a 200-fold increase in inoculum volume at every step. Mostly, the inoculum used for the production stage is approximately 5% of the medium volume, or it can be adjusted to obtain maximum fermentor productivity. Low inoculum levels result in long fermentation cycles, lowered productivity, and increased likelihood of contamination.

Taking into consideration another perspective of inoculum size, at high cell densities, a phenomenon called inoculum effect has been observed with some organisms, which is attributed to several mechanisms. Prominent among

the proposed mechanisms is quorum sensing. Studies have demonstrated that inoculum size has a significant influence on the growth of culture under extreme conditions. Studies have also demonstrated that, for identical combination of sodium chloride and pH, a difference was observed between medium- and low-level inoculua, with medium inoculum exhibiting more growth at more extreme conditions, indicating that inoculum size plays a very important role in the growth and, eventually, the yield.

4. Contamination

The risk of contamination is always present in inoculum development. Contamination can result in lower productivity by killing the microbes used for inoculum preparation, or by competition by the contaminating organisms. Therefore, every effort must be made to detect and prevent contamination. This can be done by various sterilization methods and offline and online monitoring.

5. Retaining Productivity

The culture should retain its essential product-forming capabilities. A culture usually loses its productivity due to depletion of media, degeneration of culture, accumulation of toxins, contamination, etc. A common example is antibiotic production by fermentation where the reversion of high-yielding strains is rare. The retention of productivity depends on the likelihood of stability of culture, which, in turn, depends on the medium conditions. If the product is growth associated, medium conditions have to satisfy cell growth and product formation in an optimal manner. In case of enzymes and antibiotics, the optimum rate of product formation is often observed during the post exponential phase. Thus, the main objective of controlled media and other environmental conditions is to extend the exponential phase as long as possible, that is, to retain productivity for as long as possible.

6. Culture Medium

Culture media are designed for rapid microbial growth, and little or no product accumulation will normally occur. Requirements chiefly depend on the type of microorganism being used in the fermentation process. However, the basic essentials for organisms remain the same, that is, source of energy, water, carbon source, nitrogen source, vitamins, and minerals.

The culture medium should allow high yield of the desired product at a rapid rate, cause suppression of undesired products, should be easily sterilized,

yield consistent products with minimum batch variation, be economical, readily available, and compatible with the fermentation process, and have minimum environmental hazards during the entire fermentation process. Important components of the medium are carbon sources, nitrogen sources, minerals, growth factors, chelating agents, buffers, antifoaming agents, air, steam, and fermentation vessels. Defined or formulated media have very little batch variation, but are expensive. The media should support the metabolic process of the organism to facilitate the biosynthesis of the desired products. Media are designed based on the following equation, using the minimum required components and producing the maximum yield:

Carbon and energy source + Nitrogen source + Nutrients →

Products + carbon dioxide + water + heat + biomass

7. Preparation of the Inoculum

The microbial inoculum has to be prepared from the preservation culture so that it can be used for the fermentation. The process involves multiple steps to ensure maximum yield. First-generation culture is prepared from the preservation culture on agar slants, which is than subcultured to prepare working culture. At this stage, microorganisms start growing. In small fermentation processes, working culture is used as an inoculum, but for large-scale fermentation, inoculum preparation involves additional steps. Sterile saline water or liquid nutrient medium containing glass beads is added to the agar slant and shaken so that a microbial suspension is obtained. This suspension is transferred to a flat-bed bottle, which contains sterile agar medium. The microorganisms are allowed to grow by incubating the bottle. Then, the microbial cells from flat-bed bottles are transferred to a shake flask containing sterile liquid nutrient medium, which is placed on a rotary shaker bed in an incubator. The aeration helps microorganisms to grow at a rapid rate. The purpose of this step is to increase the microbial biomass, which influences the final yield of the fermentation process because yield is defined as a ratio of biomass to mass of substrate.

Microbial cells from the shaker flask can be used as seed culture, which are then added to a smaller fermentor and allowed to grow for 1–2 days. This simulates conditions that exist in the larger fermentor meant to be used for the

production of metabolites. Finally, the microorganisms are transferred to the main fermentation vessels containing essential media and nutrients.

8. **Monitoring inoculum Development**

Standardization of culture conditions and the monitoring system is required for determining the optimum transfer time, to maintain proper physiological conditions, and the optimized production process. Biomass is a key factor in the fermentation process, directly influencing the performance of the fermentation system as well as the quality and yield of the product. Biomass levels can be measured by monitoring parameters such as packed cell volume, dry weight, wet weight, turbidity, respiration, residual nutrient concentration, and morphology.

A new generation of highly specific biosensors has been developed by interfacing the immobilized enzymes with electrochemical sensors, that is, glucose and sensitive alcohol electrodes. For example, a glucose sensor determines concentrations based on the glucose oxidase enzyme. Control of particular parameters involves a sensor, which can measure the property, and a controller, which compares the measurements with a predetermined set point and activates equipments to adjust the property back toward the set-point. The adjustment usually involves the modification of a valve opening or pump settings. Sensor may be online, that is, connected to the fermentor installation or in contact with the process stream, or off-line, where a sample is aseptically removed for analysis.

Commonly online sensors are used for the physical measurement of temperature, pressure, impeller rpm (rotation per minute), liquid and gas flow rate, and for the physiochemical measurement of pH and gas concentrations in the liquid and gas phases. Currently, exhaust-gas analysis provides the greatest online insight into the state of fermentation. Mass spectrometers operate unattended for long periods and can measure carbon dioxide, oxygen, nitrogen, and other gases to a high degree of accuracy.

Off-line analysis is still commonplace for the measurement of biomass, substrate, metabolites, enzymes, and cell constituents. Computers can be used in fermentation processes to log data coming from sensors. They can analyze or process the data, present the analysis on display devices, and store it or use it for process control by activation switches, valves, and pumps.

Apart from maintaining the records for quality assurance and inspection by regulatory agencies, significant process improvements are often achieved by the proper analysis of process records. Thus, a fully computerized integrated fermentation system requires detailed process models, which can detect and report changes in the culture conditions that may influence cell physiology and productivity.

9. The Development of inocula for Bacterial Processes

- *The main objective of inoculum development for traditional bacterial fermentations is to decrease lag phase.*
- *A long lag phase is not only is wastage of time but also medium is consumed in maintaining a viable culture prior to growth.*
- *The length of the lag phase is affected by the size of the inoculum and its physiological condition.*
- *Bacterial inocula should be transferred, when the cells are still metabolically active.*
- *The age of the inoculum is particularly important in the growth of sporulating bacteria, for sporulation is induced at the end of the logarithmic phase and the use of an inoculum containing a high percentage of spores would result in a long lag phase in a successive fermentation.*
- *5% inoculum of thermophilic Bacillus in logarithm phase is used for the commercial production of proteases.*
- *A two-stage inoculum development programme is used for the production of proteases by Bacillus subtilis. Inoculum for a seed fermenter was grown for 1 to 2 days on a solid or liquid medium and then transferred to a seed vessel where the organism was allowed to grow for a further ten generations before transfer to the production stage.*
- *The lag phase in plant fermenters could be almost completely eliminated by using inoculum medium of the same composition as used in the production fermenter and employing large inocula of actively growing seed cultures in the production of bacterial enzymes.*

- *The inoculum development programme for a pilot-plant scale process for the production of vitamin B12 from **Pseudomonas denitrificans** is shown below.*

STOCK CULTURE

Lyophilised with skim milk

↓

MAINTENANCE CULTURE

Agar slope incubated 4 days at 28°

↓

SEED CULTURE - FIRST STAGE

2 dm^3 flask containing 0.6 dm^3 medium inoculated with culture from one slope; incubated with shaking for 48h at 28°

↓

SEED CULTURE - SECOND STAGE

40 - 80 dm^3 fermenter containing 25 - 50 dm^3 medium inoculated with 1 - 1.2% first stage seed culture. Incubated 25 - 30h at 32°

↓

PRODUCTION CULTURE

- 500 dm^3 fermenter with 300 dm^3 medium inoculated with 5% second stage seed culture. Incubated at 32° for 140 - 160 h
- The acetic-acid bacteria used in the vinegar process are extremely sensitive to oxygen starvation therefore it is essential to use an inoculum in an active physiological state.
- The cells at the end of fermentation are used as inoculum for the next batch by removing approximately 60% of the culture and restoring the original level with fresh medium.
- In this process there are enough chances of strain degeneration and contaminant accumulation.

- However, strain stability is a major concern in inoculum development for fermentations employing recombinant bacteria.
- Plasmid stability and productivity is improved if stationary, rather than exponential phase, cells were used as inoculum due to loss of plasmid in fermentation.
- High quality inoculum of ***Lactococcus lactis*** 10^{-1} on a laboratory scale is obtained using electrodialysis which reduced the lactate in the inoculum and reduced the length of the lag phase in the production fermentation.

10. Development of Inocula for Anaerobic Bacterial Processes

- Clostridial Acetone-Butanol fermentation is anaerobic process.
- Though the process was outcompeted by the petrochemical industry but there is still considerable interest in reestablishing the fermentation.
- The inoculum development programme described by McNeil and Kristiansen (1986) is given as below

Heat-shocked spore suspension inoculated

into 150 cm^3 of potato glucose medium

↓

Stage 1 culture used as inoculum for 500 cm^3molasses medium

↓

Stage 2 culture used as inoculum for 9 dm^3 molasses medium

↓

Stage 3 culture used as inoculum for 90,000 dm^3 molasses medium

- The stock culture is heat shocked to stimulate spore germination and to eliminate the weaker spores.
- The production stage is inoculated with a very low volume.

- The use of such small inocula necessitates the achievement of as near perfect conditions as possible to prevent contamination and to avoid an abnormally long lag phase.

11. Measurement of Bacteria and inoculum

Preparation Measuring or counting bacteria is an integral part of inoculum preparation. For viable count, many methods are used.

i. **Pour Plate Method**
ii. **Surface Viable Count by Spreading Method**
iii. **Surface Viable Count by Miles and Misra Method**
iv. **McFarland standard**: For aerobic bacteria, CLSI recommends 0.5 McFarland standard. The 0.5 standard is prepared by adding 0.5 ml of 1.175% barium chloride to 99.5 ml of 1% H2SO4. Density equivalent to a bacterial suspension of 1.5 x 10^8 CFU ml is obtained.

12. The Development of inocula for fungal Processes

Fungus to be stored as spore suspension in sterile distilled water at room temperature. The isolates are subcultured on relevant media, such as potato dextrose agar, to ensure purity and sterility. The stock conidial suspension prepared is of concentration 10^6 spores ml^{-1}. The stock solution is further diluted to obtain a final inoculum concentration of 0.4 x 10^4 to 5 x 10^4 CFU ml^{-1}. This constitutes the finally prepared inoculums.

For yeast, inoculum preparation involves making suspension of colonies grown on Sabourauds dextrose agar (SDA). The inoculum is prepared in 5 ml of 0.85% sterile normal saline to achieve 0.5 McFarland turbidity to yield a yeast inoculum size of 1x10^6 to 5x10^6 cells ml^{-1}. This inoculum size is able to produce a semi-confluent growth in order to make the reading interpretable.

For filamentous fungi, standardization of inoculum size may be done using a hemocytometer or a spectrophotometer. Three- to 5-day-old cultures grown on SDA or potato dextrose agar are considered for this purpose. The colonies are covered with 5 ml of sterile distilled water and agitated by using a sterile loop or by being shaken vigorously for 15s with a vortex mixer. The

suspension of fungus obtained is transferred to a sterile tube. For obtaining the spore-rich inoculum, the suspension should be filtered by a Millipore membrane of 11μm pore diameter for adjusting the size of inoculum, followed by microscopic enumeration by a hemocytometer. The inoculum size should be adjusted to between 10^6 and 5 x 10^6 CFU ml^{-1}. Alternatively, the suspension adjusted could be quantified by plating on an SDA plate. The dilution prepared could be 1:1000 and different volumes of aliquots of 100, 50, and 25 μl are spread on SDA. The plates should be incubated at 35 and 30 ºC, and observed daily for growth. The colonies are to be counted, the moment any growth appears. Inoculum size standardized by either method shows a high percent agreement of 97.2%. Thus, either method can be used for the standardization of the same. However, counting by hemocytometer is independent of the variety of the strain and the size of spores. On the other hand, counting by spectrophotometer requires that each species to be standardized separately.

Besides standardization of the size of inoculum, the nature of inoculum is also important. This has been seen in case of dermatophytes. When unfiltered inoculum containing microconidia and hyphae is used compared with filtered inoculum containing only microconidia, MIC for most antimicrobials is found to be significantly higher ($P < 0.01$) for unfiltered inoculum. Ease of reading as well as reproducibility of test is seen with filtered inoculum because of homogeneous growth on microplates.

Inoculum for susceptibility testing is prepared by transferring isolates to potato–dextrose agar and incubating at 28 ºC for 7 days to produce conidia. The colonies are covered with sterile saline (0.9%) followed by gently probing the surface with the tip of a Pasteur pipette. The suspension obtained is taken in two aliquots. The contents of one aliquot are filtered with Whatman paper (pore size, 8 μm) to obtain a filtered extract containing only conidia. In the other tube, both conidia and hyphae are allowed to settle. Both inoculum so obtained may be quantified by plating 0.01 ml of each type on SDA.

13. The Development of inocula for viral Processes

The inoculation of specimen for viral cultures in cell lines requires proper preparation of the inoculum to achieve maximum viral yield. Samples for viral cultures include mostly swabs, aspirates, feces, sterile fluids, and tissue. Sterile fluids such as cerebrospinal fluid are inoculated directly into the cell cultures. For other samples, some form of pretreatment is usually required, which

includes vortexing to break the virus-containing cells and the adding of antibiotic solution (usually eight drops) to decrease undesirable microbial contamination. Proper treatment helps to reduce the toxicity caused by undesirable substances present in the primary inoculum.

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- **Fermentation media**

- Thorough analysis is essential to establish a suitable medium for an individual fermentation process.
- All most all microbes need water, energy sources, sources of carbon and nitrogen, certain mineral elements and perhaps vitamins plus oxygen if microbes are aerobic.
- It is easy to devise a medium containing pure compounds on a small scale but this medium may be unsuitable for use in a large scale fermentation processes.

Following are the criteria imperative to consider while designing a medium for large scale production

- The medium should support the maximum production of yield of product per gram of substrate used.
- It should promote maximum accumulation of the product.
- The maximum rate of product formation should be achieved.
- There should be minimum production of unwanted products.
- Constituents of the medium should be available throughout the year at cheaper rate and nearby area.
- There should not be any undesirable changes in the consistency of the medium during preparation of media and after sterilization.
- There should not be any difficulty in the operations like aeration, agitation during the production process and downstream operations like detection, isolation, extraction, purification and waste treatment.

CARBON SOURCES

- The rate at which the formation of the product takes place is influenced by the rate at which the carbon source is metabolized.

- Many time, sugars which metabolize rapidly results in fast growth of the organism and less productivity of secondary metabolites.

Culture Medium

Media requirements depend on the type of microorganism being used in the fermentation process, but the basic requirements remain the same source of energy, water, carbon source, nitrogen source, vitamins, and minerals. Designing the media for small scale laboratory purpose is relatively easy, but media for industrial purpose are difficult to prepare.

The culture medium should:

- Allow high yield of the desired product and at fast rate,
- Allow low yield of undesired products,
- Be sterilized easily,
- Yield consistent products i.e., minimum batch variation,
- Be cheap and readily available,
- Be compatible with the fermentation process,
- Not pose environmental problems before, during, or after the fermentation process.

The culture medium will affect the design of the fermenter. For example, hydrocarbons in the media require high oxygen content so an air-lift fermenter should be used. Natural media ingredients are cheap but they have high batch variation. On the other hand pure ingredients (also called defined media or formulated media) have very little batch variation but are expensive. The media should support the metabolic process of the microorganisms and allow bio-synthesis of the desired products.

Carbon & Energy source + Nitrogen source + Nutrients Product(s) + Carbon Dioxide + Water + Heat + Biomass

Media are designed based on the above equation using minimum components required to produce maximum product yield.

Important components of the medium are carbon sources, nitrogen sources, minerals, growth factors, chelating agents, buffers, antifoaming agents, air, steam, and fermentations vessels.

Carbon Sources: Product formation is directly dependent on the rate at which the carbon source is metabolized; also the main product of fermentation determines the type of carbon source to be used. Carbon sources include carbohydrates, oils and fats, and hydrocarbons.

Carbohydrates: These are the most commonly used carbon sources in the fermentation process. Starch is easily available carbohydrate obtained from maize, cereals, and potatoes. It is widely used in alcohol fermentation. Grains like maize are used directly in the form of ground powder as carbohydrate. Malt and beer made from barley grains contain high concentrations of different carbohydrates like starch, sucrose, cellulose and other sugars. Sucrose is obtained from sugar cane and molasses. Molasses is one of the cheapest sources of carbohydrate. It contains high sugar concentration and other components like nitrogenous substances and vitamins and is used in alcohol, SCP (Single-cell Protein), amino acid, and organic acid fermentations.

Extraction and purification of the products is expensive. Sulfite waste liquor is the by-product of the paper industry; it contains carbohydrates and is used in yeast cultivation. Whey is the byproduct of dairy industry. It is used in alcohol, SCP, gum, vitamins, and lactic acid fermentation.

Oils and Fats: Vegetable oils are used as a carbon source. Oils provide more energy per weight compared to sugars. They also have anti-foaming properties but are generally used as additives rather than as the sole carbon source. Examples are olive oil, cotton seed oil, soya bean oil, linseed oil, and lard (animal fat).

Hydrocarbons: C12-C18 alkanes can be used as carbon sources. They are cheap, and have more carbon and energy content per weight than sugars. They can be used in organic acids, amino acids, antibiotics, enzymes, and proteins fermentation.

Nitrogen Sources: Ammonia, ammonium salts, and urea are the most commonly used nitrogen sources in the fermentation process. Ammonia also serves the purpose of pH control. Other substances used as nitrogen sources are corn-steep liquor, soya meal, peanut meal, cotton seed meal, amino acids, and proteins.

Minerals: Calcium, chlorine, magnesium, phosphorous, potassium and sulfur are the essential minerals for all media. Other minerals like copper, cobalt, iron,

manganese, molybdenum, and zinc are needed in trace amounts and are generally present as impurities in other components. The specific concentration on these elements depends on the micro-organism being used.

Growth Factors: Vitamins, amino acids, and fatty acids are used as growth factors in the fermentation process to complement the cell components of the microorganisms.

Chelating Agents: Chelating agents prevent formation of insoluble metal precipitates. They form complexes with the metal ions present in the medium and can be utilized by the microorganisms. Chelating agents are not required in large scale fermentation processes since some of the other ingredients like yeast extract will perform the function of forming complexes with the metal ions.

One example of a chelating agent is EDTA (ethylene diamine tetra acetic acid). EDTA is a versatile, being able to form six bonds with a metal ion. It is frequently used in soaps and detergents because it forms a complex with calcium and magnesium ions. These ions are in hard water and interfere with the cleaning action of soaps and detergents. Other chelating agents are citric acid and pyrophosphates.

Buffers: Buffers are used to maintain the pH of the medium as microbial growth is affected by the pH changes. Optimum pH for most microorganisms is 7.0. Commonly used buffers are calcium carbonate, ammonia, and sodium hydroxide.

Antifoaming Agents: Microbial process produces a large amount of foam in the fermentation vessel. This is due to microbial proteins or other components of the media. Foaming causes removal of cells from the media and their autolysis, thus, releasing more microbial foam-producing proteins, and aggravating the problem. Foam will reduce the working volume in the fermentation vessel, decrease rate of heat transfer, and deposit cells on the top of the fermenter. The air filter exits become wet allowing growth of contaminating microorganisms. Antifoaming agents are also called surfactants, i.e. they reduce the surface tension in the foam and destabilize the foam producing proteins.

Commonly used antifoaming agents are stearyl alcohol, cotton seed oil, linseed oil, olive oil, castor oil, soy bean oil, cod liver oil, silicones, and sulphonates.

Air: Air is required for aeration and is supplied to the fermenter by means of pumps or compressors. It is sterilized by passing through filters before being introduced. The amount of air required and the extent of purity depends on the fermentation process being carried out.

Steam: Steam is used to sterilize fermenters and other equipment and to control temperature. Continuous dry steam supply is required for the fermentation process and care should be taken to prevent condensation.

For secondary metabolites such as antibiotics or enzymes, the production media are so designed that a key nutrient becomes limiting at a critical stage to initiate secondary metabolism in the organism. The nutrient to be made limiting depends on the process, for example, glucose for penicillin production and phosphate for several antibiotics produced by Streptomyces.

Media may be manipulated to affect cell permeability. Biotin limitation in glutamic acid fermentation inhibits the biosynthesis of oleic acid by glutamic acid-producing bacteria and modifies the cell permeability so that glutamic acid is secreted into the medium. Antibiotics, such as penicillin, inhibit the formation of peptidoglycan cross-links, result in the formation of swollen cells, and also leak glutamic acid into the medium. Some surface-active agents have shown to increase the rate of secretion of microbial extracellular enzymes. Antifoaming agents are added at the appropriate stage of fermentation to minimize foaming; silicones, vegetable oils, etc. are used for this purpose.

The pH is controlled by using buffers such as inorganic phosphate, calcium carbonate, hydroxide ions, liquid or gaseous ammonia, sulfuric acid, or hydrochloric acid. The pH can also be indirectly controlled by balancing carbohydrate and nitrogen sources. Carbohydrates contribute to the lower pH by the formation of organic acids whereas nitrate assimilation results in alkaline drift by the liberation of free acids.

- **Fermentation media – Raw materials used**

The media used in industrial fermentations 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 undefinable. Most are derived from natural plant and animal materials, often byproducts of other industries, with varied and variable composition. The effects of such batch to batch variations must be determined. 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 final 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.
4. Formulation, mixing, complexing and viscosity characteristics that may influence agitation, aeration and foaming during fermentation and downstream processing stages.
5. The concentration of target product attained its rate of formation and yield per gram of substrate utilized.
6. The levels and range of impurities, and the potential for generating further undesired products during the process.
7. Overall health and safety implications.
8. Levels of impurities which affect purification steps in downstream processing and waste treatment.
9. Physical and chemical properties of the formulated medium can influence the sterilization operations employed.
10. Media characteristics can affect product recovery and purification, and the ease with which the cells are separated from the spent medium.

Following are some raw materials used in industrial fermentation –

A) Raw materials rich in Carbon and Energy sources

1. Molasses
2. Malt extract
3. Starch and Dextrin
4. Sulphite Waste Liquor

5. Cellulose
6. Whey
7. Alkanes and Alcohols
8. Fats and oils (Pharmamedia)

B) Raw materials rich in Nitrogen Sources

1. Corn Steep Liquor
2. Yeast Extract
3. Peptones
4. Soya Bean Meal
5. Distillers Solubles

A) Raw materials rich in Carbon and Energy sources

1. Molasses
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5. Cellulose
6. Whey
7. Alkanes and Alcohols
8. Fats and oils

1. Molasses

Molasses is a source of sugar, and is used in many fermentation industries including the production of potable and industrial alcohol, acetone, citric acid, glycerol, and yeasts. It is a by-product of the sugar industry. There are two types of molasses depending on whether the sugar is produced from the tropical crop,

Cane molasses: sugar is produced from crop, sugar cane (*Saccharum officinarum*)

Beet molasses: sugar is produced from crop, beet (*Beta alba*).

Blackstrap molasses: -

In the fourth and final stage of *crystallization,* sugar crystals begin to form with increasing heat and under vacuum, yielding thick brown syrup which contains the crystals, which is centrifuged to remove the sugar crystals and the remaining liquid is known as molasses. The first sugar so collected is 'A' and the liquid is 'A' molasses. 'A' molasses is further boiled to extract sugar crystals to yield 'B' sugar and 'B' molasses. Two or more boiling may be required before it is no longer profitable to attempt further extractions. This final molasses is known as **'blackstrap molasses'.**

Cane and beet molasses differ slightly in composition. Beet molasses is alkaline while cane molasses is acid.

High test molasses **(inverted molasses):**

It is brown thick syrup liquid used in the distilling industry and containing about 75% total sugars (sucrose and reducing sugars) and about 18% moisture. Strictly speaking, it is not molasses at all but invert sugar, (i. e. reducing sugars resulting from sucrose hydrolysis). It is produced by the hydrolysis of the concentrated juice with acid.

Table: - Average composition of beet and cane molasses

Composition	***Beet Molasses*** *% (W/W)*	***Cane Molasses*** *% (W/W)*
Water	16.5	20.0
Sugars:	53.0	64.0
Sucrose	51.0	32.0
Fructose	1.0	15.0
Glucose	--	14.0
Raffinose	1.0	--
Non-sugar (nitrogeneous Materials, acids, gums, etc.)	19.0	10.0
Ash	11.5	8.0

2. Malt extract

Aqueous extracts of malted barley can be concentrated to form syrups that are particularly useful carbon sources for the cultivation of filamentous fungi, yeasts and actinomycetes. Extract preparation is essentially the same as for malt wort production in beer brewing. The composition of malt extracts varies to some extent, but they usually contain approximately 90% carbohydrate, on a dry weight basis.

Table: - Average composition of Malt extract

Composition	*% (W/W)*
Glucose and Fructose	*20*
Maltose	*55*
Maltotriose	*10*
Dextrins	*5 to 20*
Proteins, peptides, amino acids & Vitamins	*5*

3. Starch and Dextrin

These polysaccharides are not as readily utilized as monosaccharides and disaccharides, but can be directly metabolized by amylase-producing microorganisms, particularly filamentous fungi. Their extracellular enzymes hydrolyse the substrate to a mixture of glucose, maltose or maltotriose to produce a sugar spectrum similar to that found in many malt extracts. Maize starch is most widely used, but it may also be obtained from other cereal and root crops. To allow use in a wider range of fermentations, the starch is usually converted into sugar syrup, containing mostly glucose. It is first gelatinized and then hydrolysed by dilute acids or amylolytic enzymes, often microbial glucoamylases.

4. Sulphite Waste Liquor

It is the aqueous effluent resulting from the sulfite process for manufacturing cellulose or pulp from wood. Depending on the type, most woods contain about 50% cellulose, about 25% lignins and about 25% of hemicelluloses. During the sulfite process, hemicelluloses hydrolyze and dissolve to yield the hexose sugars, glucose, mannose, galactose, fructose and the pentose sugars, xylose, and arabinsoe.

The acid reagent breaks the chemical bonds between lignin and cellulose; subsequently they dissolve the lignin. Depending on the severity of the treatment some of the cellulose will continue to exist as fibers and can be recovered as pulp. The presence of calcium ions provides a buffer and helps neutralize the strong lignin sulfonic acid. The degradation of cellulose yields glucose. Portions of the various sugars are converted to sugar sulfonic acids, which are not fermentable. Variable but sometimes large amounts of acetic, formic and glactronic acids are also produced.

Sulfite liquor of various compositions is produced, depending on the severity of the treatment and the type of wood. Hardwoods not only yield a higher amount of sugar (up to 3% dry weight of liquor) but the sugars are largely pentose, in the form of xylose. Hardwood hydrolyzates also contain a higher amount of acetic acid. Soft woods yield a product with about 75% hexose, mainly mannose.

Sulfite liquor is used as a medium for the growth of microorganisms after being suitably neutralized with $CaCO_3$ and enriched with ammonium salts or urea, and other nutrients. It has been used for the manufacture of yeasts and alcohol.

5. Cellulose

Cellulose is predominantly found as lignocellulose in plant cell walls, which is composed of three polymers: cellulose, hemicellulose and lignin. Lignocellulose is available from agricultural, forestry, industrial and domestic wastes. Relatively few microorganisms can utilize it directly, as it is difficult to hydrolyse. The cellulose component is in part crystalline, encrusted with lignin, and provides little surface area for enzyme attack.

At present it is mainly used in solid-substrate fermentations to produce various mushrooms. However, it is potentially a very valuable renewable source of fermentable sugars once hydrolyzed, particularly in the bioconversion to ethanol for fuel use.

6. Whey

Whey is an aqueous byproduct of the dairy industry. The annual worldwide production is over 80 million tonnes, containing over 1 million tonnes of lactose and 0.2 million tonnes of milk protein. This material is expensive to store and transport. Therefore, lactose concentrates are often prepared for later fermentation by evaporation of the whey, following removal of milk proteins for use as food supplements. This disaccharide was formerly used extensively in penicillin fermentations and it is still employed for producing ethanol, single cell protein, lactic acid, xanthan gum, vitamin B_{12} and gibberellic acid.

7. Alkanes and Alcohols

n-Alkanes of chain length C_{10}–C_{20} are readily metabolized by certain microorganisms. Mixtures, rather than a single compound, are usually most suitable for microbial fermentations. However, their industrial use is dependent upon the prevailing price of petroleum. Methane is utilized as a carbon source by a few microorganisms, but its conversion product methanol is often preferred for industrial fermentations as it presents fewer technical problems.

Methanol has high per cent carbon content and is relatively cheap, although only a limited number of organisms will metabolize it. Ethanol is less toxic than methanol and is used as a sole or cosubstrate by many microorganisms, but it is too expensive for general use as a carbon source. However, its biotransformation to acetic acid by acetic acid bacteria remains a major fermentation process.

8. Fats and oils

Plant oils primarily from cotton seed, linseed, maize, olive, palm, rape seed and soya are used as the primary or supplementary carbon source,

especially in antibiotic production. Plant oils are mostly composed of oleic and linoleic acids, but linseed and soya oil also have a substantial amount of linolenic acid. The oils contain more energy per unit weight than carbohydrates.

Pharmamedia (proflo): -

Also known as proflo, this is a yellow fine powder made from cotton-seed embryo. It is used in the manufacture of tetracycline and some semi-synthetic penicillins. It is rich in protein, (56% w/v) and contains 24% carbohydrate, 5% oil, and 4% ash, the last of which is rich in calcium, iron, chloride, phosphorous, and sulfate.

B) Raw materials rich in Nitrogen Sources

1. Corn Steep Liquor
2. Yeast Extract
3. Peptones
4. Soya Bean Meal
5. Distillers Solubles

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. Ammonia can also be used to adjust the pH of the fermentation. 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.

1. Corn Steep Liquor

This is a by-product of starch manufacture from maize. Sulfur dioxide is added to the water in which maize is steeped. The lowered pH inhibits most other organisms, but encourages the development of naturally occurring lactic acid bacteria especially homofermentative thermophilic *Lactobacillus* spp. which raise the temperature to 38-55°C. Under these conditions, much of the

protein present in maize is converted to peptides which along with sugars leach out of the maize and provide nourishment for the lactic acid bacteria. Lactic fermentation stops when the SO_2 concentration reaches about 0.04% and the concentration of lactic acid between 1.0 and 1.5%. At this time the pH is about 4. Acid conditions soften the kernels and the resulting maize grains mill better while the gel-forming property of the starch is not hindered.

The supernatant drained from the maize steep is corn steep liquor. Before use, the liquor is usually filtered and concentrated by heat to about 50% solid concentration. The heating process kills the bacteria. Corn steep liquor is rich in carbohydrates, nitrogen, vitamins, and minerals. Its composition is highly variable and would depend on the maize variety, conditions of steeping, extent of boiling etc. As corn steep liquor is highly acidic, it must be neutralized (usually with $CaCO_3$) before use. It is used mainly in the production of antibiotics. E.g. Penicillin.

The composition of a typical sample of corn steep liquor is given in the table.

Table: Approximate composition of corn steep liquor (%)

Components	**Percentage**
Lactose	3.0 - 4.0
Glucose	0. - 0.5
Non-reducing carbohydrates (mainly starch)	1.5
Acetic acid	0.05
Glucose lactic acid	0.5
Phenylethylamine	0.05
Amino aids (peptides, mines)	0.5
Total solids	80-90
Total nitrogen	0.15 - 0.2

2. Yeast Extract

Yeast extracts may be produced from waste baker's and brewer's yeast, or other strains of *S. cerevisiae*. Those extracts used in the formulation of fermentation media are normally salt-free concentrates of soluble components

of hydrolyzed yeast cells. Yeast cell hydrolysis is often achieved by autolysis, using the cell's endogenous enzymes, usually without the need for additional hydrolytic enzymes. Autolysis is performed at 50–55°C for several hours before the temperature is raised to 75°C to inactivate the enzymes. Finally, the cells are disrupted by plasmolysis or mechanical disruption. Cell wall materials and other debris are removed by filtration or centrifugation and the resultant extract is rapidly concentrated.

Extracts are available as liquids containing 50–65% solids, viscous pastes or dry powders. They contain amino acids, peptides, water soluble vitamins and some glucose.

Table: Composition of yeast extract

Components	**Quantity**
Total proteins, peptides & amino acids (%, w/v)	73–75
Free amino acids	35–40
Peptides less than	10–15
Other material	20–30
Vitamins (µg/g)	
Thiamin	30
Riboflavin	120
Niacin	700
Pyridoxine	20
Folic acid	30
Calcium pantothenate	300
Biotin	2.5

3. Peptones

Peptones are usually too expensive for large-scale industrial fermentations. They are prepared by acid or enzyme hydrolysis of high protein materials: meat, casein, gelatin, keratin, peanuts, soy meal, cotton seeds, etc. Their amino acid compositions vary depending upon the original protein source. Peptones from plant sources invariably contain relatively large quantities of carbohydrates.

4. Soya Bean Meal

Residues remaining after soya beans have been processed to extract the bulk of their oil are composed of 50% protein, 8% non-protein nitrogenous compounds, 30% carbohydrates and 1% oil. This residual soya meal is often used in antibiotic fermentations because the components are only slowly metabolized, thereby eliminating the possibility of repression of product formation.

Components	**Percentage**
Protein	50
Non protein Nitrogenous compounds	8
Carbohydrates	30
Oil	1

5. Distillers Solubles

This is a by-product of the distillation of alcohol from fermented grain. It is prepared by filtering away the solids from the material left after distilling fermented cereals (maize or barley) for whiskey or grain alcohol. The filtrate is then concentrated to about one-third solid content to give syrup which is then drum-dried to give distillers soluble. It is rich in nitrogen, minerals, and growth factors.

Table: Composition of maize distillers soluble

Components	***%***
Protein	27
Lipid	9
Fibre	5
Carbohydrate	43
Ash (mainly K, Na, Mg, CO3, and P)	11
Moisture	5

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- **Sterilization in fermentation process**

Most industrial fermentations are carried out as pure cultures in which only selected strains are allowed to grow. If foreign microorganisms exist in the medium or any parts of the equipment, the production organisms have to compete with the contaminants for the limited nutrients. The foreign microorganisms can produce harmful products which can limit the growth of the production organisms.

Therefore, before starting fermentation, the medium and all fermentation equipment have to be free from any living organisms; in other words, they have to be completely sterilized. Furthermore, the aseptic condition has to be maintained. Sterilization of fermentation media or equipment can be accomplished by destroying all living organisms by means of heat (moist or dry), chemical agents, radiation (ultraviolet or X-rays), and mechanical means (some or ultrasonic vibrations). Another approach is to remove the living organisms by means of filtration or high-speed centrifugation.

Heat is the most widely used means of sterilization, which can be employed for both liquid medium and heatable solid objects. It can be applied as dry or moist heat (steam). The moist heat is more effective than the dry heat, because the intrinsic heat resistance of vegetative bacterial cells is greatly increased in a completely dry state. As a result the death rate is much lower for the dry cells than for moist ones. The heat conduction in dry air is also less rapid than in steam. Therefore, dry heat is used only for the sterilization of glassware or heatable solid materials.

Chemical agents can be used to kill microorganisms as the result of their oxidizing or alkylating abilities. However, they cannot be used for the sterilization of medium because the residual chemical can inhibit the fermentation organisms.

Media and vessel sterilization

Sterilization of the medium in a fermentor can be carried out in batch mode by direct steam sparging, by electrical heaters, or by circulating constant pressure condensing steam through heating coil. The sterilization cycles are composed of heating, holding, and cooling.

Sterilization can be carried out in a continuous mode rather than in batches. Continuous sterilization offers several advantages:

i. It simplifies production planning, thus allowing maximum plant utilization and minimum delays.
ii. It provides reproducible conditions.
iii. It can be operated at a high temperature (140°C instead of 121°Cin batch sterilization); therefore, the sterilization time can be shortened (holding time of 1 to 2 minutes).
iv. It requires less steam by recovering heat from the sterilized medium. As a result, it also requires less cooling water.
v. It is easier to automate the process; thus, it is less labor intensive.

Air sterilization

For aerobic fermentations, air needs to be supplied continuously. Typical aeration rates for aerobic fermentation are 0.5 - 1.0 vvm (air volume per liquid volume per minute). This requires an enormous amount of air. Therefore, not only the medium but also the air must be free of microbial contaminants. All of the sterilization techniques discussed for medium can also be employed for air. However, sterilization of air by means of heat is economically impractical and is also ineffective due to the low heat-transfer efficiency of air compared with those of liquids. The most effective technique for air sterilization is filtration using fibrous or membrane filters.

These filters are designed to trap and contain microorganisms. Filters are made of glass fibre, mineral fibres, polytetrafluoroethylene (PTFE) or polyvinyl chloride (PVC), and must be steam sterilizable and easily changed.

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- **Contamination and its control**

The contamination of fermentors has a significant financial impact on manufacturers. The likely source of a contamination may depend on scale or the complexity of the fermentation process being operated. The prevention of contaminations can be achieved by good equipment design, the following of standard operating procedures and a detailed understanding of the various sterilization processes that ensure a sterile barrier is maintained around the

fermentor. Filtration can be used for the sterilization of liquids and gases entering the fermentor. Pre-filtration of air entering the fermentor can help protect against phage contaminations.

A more complex process which uses multiple feeds and a high sampling frequency correlate with an increased risk of contamination.

Contaminations in small-scale fermentors

Small-scale fermentors are typically designed for process flexibility rather than operating robustness. The ingress of contaminating microbes in these fermentors can occur by the following routes;

• Silicone tubing used to deliver feeds to the fermentor where insufficient care has been taken with tubing connections.

• Through bottom entry stirrer seal assemblies where a single-mechanical seal has been used and is not being continuously supplied with steam in order to prevent a hot spot.

• Improperly connected air vent filters.

• Improper cleaning of the sparger.

• Poor tightening of the fermentor top lid screws.

Contaminations in production-scale fermentors

Production fermentors are more heavily engineered and typically hard-piped with contaminations being more frequently attributable to tainted inocula or failures in the sterilization of liquids and gases entering the vessel.

Contaminated inocula

The isolation of pure cultures is critical to avoiding frequent contaminations. Methods for isolating pure cultures include dilution to extinction, pour plating and streak plating. Developing production strains requires considerable time, effort and expense. Once the development is complete the culture should be appropriately stored either at reduced temperature or by storage in a dehydrated form. The quality of these stock cultures should be monitored in order to ensure purity, viability and productivity by culturing in shake flasks and observing a characteristic growth profile. The preparation of an inoculum from the stored stock of cells requires manual

operations during which the risk of contamination occurring is increased. Good laboratory aseptic technique is required through the inoculum preparation process. Samples should be taken throughout this process even though the results are unlikely to be available before the inoculum reaches the production process.

Failures in sterilization processes

If it can be demonstrated that the inoculum used was indeed a pure culture then the next most likely source of contaminations are likely to be the inadequate sterilization of the fermentor, media, liquid feeds or sparged gases such as air. All equipment must be suitably maintained and calibrated to ensure sterilization can be achieved and aseptic conditions are maintained.

Phage contaminations

Phage contaminations are ubiquitous yet enigmatic. They can be liquid or air borne and the phages' small robust nature allows them to evade sterilization procedures. The impact of a phage contamination can vary from the slowing of growth to a dramatic total lysis of the producer organism in the fermentor.

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- **Inoculum media**

Inoculum preparation media are quite different from production media. These media are designed for rapid microbial growth, and little or no product accumulation will normally occur. Many production processes depend on inducible enzymes. In all such cases, the appropriate inducers must be included either in all the stages or at least in the final stages of inoculum development. This will ensure the presence of the concerned inducible enzymes at high levels for the production to start immediately after inoculation.

- **Buffers**

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.

- **Antifoam agents**

Foams are dispersions of gas in liquid. Aeration and agitation of a liquid medium can cause the production of foam. This is particularly true for the media containing high levels of proteins or peptides. If the foam is not controlled, it will rise in the head space of the tank and be forced from the tank along with the exit valve. This condition often causes contamination of the fermentation from organisms picked up by breaking of some of the foam which then drains back into the tank. Excessive foaming also causes other problems for fermentation.

Foams in industrial fermentations are controlled either by chemical or mechanical means. *Chemicals controlling foams* have been classified into **antifoams**, which are added in the medium to prevent foam formation, and **defoamers** which are added to knock down foams once these are formed. Some may not see much in the distinction and in this discussion the term antifoam will refer to both. The usual procedure for controlling foam is to add an antifoaming agent, although a supplementary impeller blade mounted high in the tank may at times be effective. An antifoam agent lowers surface tension and in the process decreases the stability of the foam bubbles so that they burst. The antifoam may be added at media makeup or may be added after sterilization or as called for during the fermentation process.

Basically, antifoams enter the lamella between the bubbles by spreading over or mixing with the positively absorbed surfactants monolayer and thus destroying the film elasticity. The result is that the film collapses. Ideally, therefore, the antifoam should be miscible with the foaming liquid. Antifoams

used in industrial fermentation should ideally have the following properties. They should:

i. be non-toxic to microorganisms and higher animals, especially if the fermentation product is for internal use.
ii. have no effect on taste and odor.
iii. be autoclavable.
iv. not be metabolized by the microorganisms
v. not impair oxygen transfer.
vi. be active in small concentrations, cheap, and persistent.

There are two types of antifoam agents:

(i) Inert Antifoam agents e.g. Silicone compounds are ideal inert antifoam agents but are too expensive.

(ii) Antifoam agents made from crude organic materials.

e.g. Animal and vegetable oils , lard oil, corn and soybean oil, long chain alcohols such as octadecanol. In addition mixtures of oils and alcohols are effective in controlling foam.

Sr. No.	Category	Example	Chemical nature
1.	Natural oils and Fats	Peanut oil, Soyabean oil, Lard oil, Corn oil	Esters of glycerol & Long chain mono basic acids
2.	Alcohols	Sorbitan Alcohol, Octadecanol	Alcohols with 8-10 carbon atoms
3.	Sorbitan derivatives	Sorbitan monolaureate	Derivatives of sorbitol produced by reacting it with H_2SO_4 or ethylene.
4.	Polyethers	P400, P1200, P2000	Polymers of ethylene oxide and propylene oxide
5.	Silicones	Antifoam A	Polymers of Polydimethyl-siloxane fluids

Antifoam agents are often difficult to sterilize, particularly if they are of an oily nature, because of poor heat penetration and transport through the oil. When antifoam is required in a tank, it is added either manually or electrically. Obviously, manually addition requires that someone continuously observe the tank so that the antifoam can be added as required. Electrical addition of antifoam is usually preferred. To accomplish this automatic addition, a sensing mechanism is employed to determine when the foam has risen into the head space of bioreactor. Such a device is provided with two electrodes mounted in the top of the fermentor. These electrodes are connected to a pump associated with a reservoir of sterile antifoam and as the foam rises in the reactor it touches the two electrodes in the process allowing current to flow between them so as to activate the pump for addition of antifoam. The foam then collapses away from the electrode thus breaking the electrical connection between them and stopping further addition of antifoam agent.

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- **Precursors**

Some fermentations must be supplemented with specific precursors, notably for secondary metabolite production. When required, they are often added in controlled quantities and in a relatively pure form. Examples include phenylacetic acid or phenylacetamide added as side-chain precursors in penicillin production. D-threonine is used as a precursor in l-isoleucine production by *Serratia marsescens*, and anthranillic acid additions are made to fermentations of the yeast *Hansenula anomala* during l-tryptophan production.

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- **Phage contamination and control**

Phages (Viruses) are non-cellular entities which consist basically of protein and either DNA or RNA and replicate only within specific living cells. They have no cellular metabolism of their own and their genomes direct the genetic apparatuses of their hosts once they are within them. Viruses are important in the industrial microbiology for at least two reasons:

(i) Those that are pathogenic to man and animals are used to make vaccines against disease caused by the viruses.
(ii) Viruses can cause economic losses by destroying microorganisms used in fermentations.

We will therefore look at those viruses which attack organisms of industrial importance, namely bacteria (including actinomycetes) and fungi. Such viruses are known as bacteriophages, actinophages, or mycophages depending on whether they attack bacteria, actinomycetes, or fungi.

Prevention of Phage Contamination

Sterilization

Phages are as ubiquitous as microorganisms in general and are present in the air, water, soil, etc. The first cardinal rule in avoiding phage contamination therefore is routine general cleanliness and asepsis. Pipes, fermentors, utensils, and media, should all be well sterilized. The culture should be protected from aerial phage contamination, an insidious situation, which unlike bacterial or fungal contamination cannot be observed on agar. Air filters should be replaced or sterilized regularly. Aerosol sterilization of the factory with chlorine compounds, and other disinfectants, as well as UV irradiation of fermentation halls should be done routinely.

Use of Phage Resistant Mutants

Phages may of course be introduced as direct contaminants or be lysogenic in the organism being used in the fermentation. Mutants as productive as the original parent but resistant to various contaminating phages should be developed. Such mutants should have no tendency to revert to the phage-sensitive type.

Inhibition of Phage Multiplication with Chemicals

Specific chemicals selectively active on phages and which spare bacteria may be used in the fermentation medium.

i. To prevent infection by phages requiring divalent cations (Mg^{2+}; Ca^{2+}) for adsorption to host cell or for DNA injection into the host cell, chelating agents have been used. These sequester the cations from the medium and hence the phage cannot adsorb onto its host. Examples of the chelating agents are 0.2-0.3% tripolyphosphate and 0.1-0.2% citrate.
ii. Non-ionic detergents e.g. tween 20, tween 60, polyethylene glycol monoester also inhibit the adsorption of some phages or the multiplication of the phages in the cell. The above two agents usually have no effect on the growth of many industrial organisms.
iii. The addition of Fe^{2+} suppresses cell lysis by phages.
iv. Certain antibiotics may be added to prevent growth of phages, but only the selective ones should be used. Chloramphenicol has been used. It has no direct action on the phage, but it inhibits protein replication in phage-infected cells, probably due to selective absorption of the antibiotic by phage-infected cells.

Use of Adequate Media Conditions and Other Practices

Fermentation conditions and practices which adversely affect phage should be selected. Media unfavorable to phages (high pH, low Ca^{2+}, citrates, salts with cations reacting with –SH groups) should be developed. Pasteurization of the final beer and high temperature of incubation consistent with production should be used; both of these adversely affect phage development.