FOOD & INDUSTRIAL MICROBIOLOGY

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(MSC MICROBIOLOGY)

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Lesson No. 1

MICROORGANISMS ASSOCIATED WITH FOODS

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1.0 OBJECTIVE

This lesson is intended to know the association of different types of microorganisms with the foods and also about the sources of microbial contamination of foods. It also elaborates how the human beings serve as a potential source of microbial contamination of various food materials.

1.1 INTRODUCTION

Food serves as an interacting medium between various living species because it is a source of nutrients for humans, animals as well as microorganisms. Human food is basically a plant or animal origin. The food fit for consumption by human is also a medium for the growth and activity of microorganisms. So human food is always associated with a variety of microorganisms, as plant and animal material harbour various microorganisms. The significance of microorganisms in foods depends upon the number and types of microorganisms in foods, type of food, type of treatment

given to food, processing and storage treatments of food, the mode in which food is eaten and the individuals that consume the food. Several hundreds of microorganisms associate with food products in one way or other.

Microorganisms may have one or more functions in a food and those that cause foodborne illness are more concern than the others. The forms that spoil the foods by association produce undesirable changes in the odour, color, taste, texture of foods. On the other hand, the useful organisms produce desirable changes in foods like conversion of milk to cheese and sugar to alcohol.

1.2 MICROORGANISMS ASSOCIATED WITH FOODS :

The microorganisms that associate with various foods are of importance in food microbiology include bacteria, yeasts, molds and few protozoa. Bacteria, yeasts and molds attack virtually all the constituents of foods. Depending on nature and availability of enzymes, some ferment sugars and hydrolyze starch and cellulose while others hydrolyze fats and produce rancidity. Some microorganisms digest proteins and produce putrid and ammonia like odours. Some participate in the formation of acid and make the food sour, while others produce gas and make the food foamy, still others produce pigments that contribute to the discoloration of food. The growth and activity of the microorganisms depend on the nature of the food and its composition. The factors that govern the microbial activity in a food include both intrinsic and extrinsic factors. The intrinsic factors include pH, water activity, oxidation-reduction potential, nutrient content and presence of inhibitors in the food. The extrinsic factors include temperature, relative humidity and the atmosphere surrounding the food. These factors operate individually as well as in combination and affect the growth and activity of microorganisms in foods.

1.2.1 Bacteria:

All bacteria that associate with foods are unicellular, small in size, morphologically cocci or bacilli or spiral and multiply best between 16° – 38°C temperature. Some important genera of bacteria that associate with various foods include *Acinetobacter, Aeromonas, Alcaligenes, Alteromonas, Bacillus, Brochothrix, Campylobacter, Citrobacter, Clostridium, Corynebacterium, Enterococcus, Enterobacter, Erwinia, Escherichia, Flavobacterium, Hafnia, Lactococcus, Lactobacillus, Leuconostoc, Listeria, Micrococcus, Moraxella, Pediococcus, Proteus, Pseudomonas, Psychrobacter, Salmonella, Serratia, Shigella, Staphylococcus, Vagococcus, Vibrio, Yersinia* etc.

Importance of Bacteria in Foods: Bacteria that play significant roles in foods are often grouped on the basis of their activity in foods without regard to their systematic classification. The activity of fermenting sugars to lactic acid by lactic acid bacteria viz., *Leuconostoc, Lactobacillus, Streptococcus* and *Pediococcus* is desirable in a variety of foods such as sauerkraut and other pickled vegetables and dairy products for the production of flavour. Acetic acid bacteria oxidize ethanol to acetic acid. Species of genera *Acetobacter* and *Gluconobacter* are the most common. *Acetobacter* species are capable of oxidizing acetic acid to carbon dioxide. They are useful in vinegar manufacture but are undesirable in alcoholic beverages. Butyric acid bacteria are mostly the spore forming anaerobes of the genus *Clostridium*. They produce butyric acid by fermenting sugars. Propionic acid bacteria which produce propionic acid belong to the genus *Propionibacterium*.

Proteolytic bacteria include a heterogenous group of bacteria which produce extracellular proteases. Most species of this group belong to *Clostridium, Bacillus, Pseudomonas* and *Proteus*. Lipolytic bacteria are also a heterogenous group of bacteria which produce lipases and the important lipolytic bacteria include *Pseudomonas, Alcaligenes*, *Staphylococcus, Serratia* and *Micrococcus*. *Bacillus subtilis* and *Clostridium butyricum* are the examples of both saccharolytic and amylolytic bacteria that hydrolyze di- and polysaccharides, and starch, respectively. Species of *Bacillus*, *Achromobacter*, *Aeromonas*, *Arthrobacter* and *Flavobacterium* are pectinolytic and produce pectinases that are responsible for softening of plant tissues or loss of gelling power in various plant foods.

Gas forming bacteria include species of *Leuconostoc*, *Lactobacillus* and *Propionibacterium* which produce carbon dioxide. Species of *Escherichia*, *Enterobacter*, *Proteus*, *Bacillus* and *Clostridium* produce both carbon dioxide and hydrogen. *Clostridium* species ferment carbohydrates with the production of butyric and other acids and gases namely carbon dioxide and hydrogen. They are found commonly in soils and involve in the gaseous spoilage of canned vegetables and putrefaction of foods. Off-flavour forming bacteria include those of genus *Streptomyces* which produce undesirable flavours and musty or earthy of odour and taste. The pigmented bacteria produce colours during their growth in foods. Examples include the species belonging to genera *Flavobacterium* (yellow to orange), *Serratia* (red), *Halococcus* (red to orange) and *Halobacterium* (pink, red and orange). *Flavobacterium* species cause discolouration on the surface of meat and spoilage of shellfish, poultry, eggs, butter and milk.

Alcaligenes viscolactis, *Enterobacter aerogenes*, *Klebsiella oxytoca* and some species of *Streptococcus* and *Lactobacillus* are the examples of slime or rope forming bacteria that cause the ropiness or slime in milk. Coliform bacteria are aerobic and facultatively anaerobic non-spore forming bacteria which ferment lactose with gas formation. They cause spoilage of a variety of foods producing off-flavours and sliminess. The important species include *Escherichia coli* and *Enterobacter aerogenes*.

1.2.2 Yeasts:

These are the unicellular fungi of larger size than bacteria. Morphologically yeast cells are oval or elongate or elliptical or spherical in shape with about 20 µm length and 6-7 µm in diameter. Most common yeasts require comparatively more moisture than molds. Yeasts also grow in the presence of relatively high concentrations of solutes as salt, sugar or alcohol. The optimum temperature of 25° to 30°C and pH of 4.0 to 4.5 are required for the better growth. They grow best under aerobic conditions, however the fermentative types can grow under anaerobic conditions.

Yeasts are grouped into oxidative or fermentative types according to their function or activity. The oxidative yeasts oxidize organic acids and alcohol, whereas, the fermentative yeasts produce alcohol and carbon dioxide. Yeasts are employed in the manufacture of foods such as bread, beer, wine and vinegar and for surface ripening of cheese. On the other hand, yeasts can also cause spoilage of fruit juices, syrups, honey, jellies, meat, wine, beer, molasses etc. The important yeasts that associate with foods include the species of *Brettanomyces, Candida, Debaryomyces, Hanseniaspora, Kluyveromyces, Pichia, Rhodotorula, Saccharomyces, Schizosaccharomyces, Torulopsis, Trichosporon, Zygosaccharomyces* etc.

Importance of Yeasts in Foods: *Saccharomyces* species are the most widely used yeasts. The leading species *S.cerevisiae* is used in the manufacture of many foods, with special strains used for the leavening of bread and for the production of ale, wine, alcohol, glycerol and invertase. *S.cerevisiae var ellipsoideus* is a high alcohol yielding variety used in the production of industrial alcohol, wine and distilled liquors. *S.uvarum* is a bottom yeast used in beer manufacture. *S.fragilis* and *S.lactis* have the ability to ferment lactose and hence are important in milk products. *Zygosaccharomyces* species are osmophilic and are involved in the spoilage of honey, syrups and molasses. They also find use in the fermentation of soya sauce and some wines. *Candida* species spoil foods that are high in acid and salt content. *C.lipolytica* is known to spoil butter. *Brettanomyces* species are involved in the fermentation of Belgian and English beers and in French wines. *Rhodotorula* species cause discolouration of foods by forming coloured spots on meat and sauerkraut.

1.2.3 Molds :

The molds are larger and more complex in nature than yeasts. They are the multicellular filamentous fungi growing as a network of mycelia with a fuzzy or cottony appearance. They produce fruiting bodies and the mold spores are referred to as conidia. Most of the molds are mesophilic with optimum temperature of 25° – 30°C and few are thermophilic. A number of molds are psychrotrophic and can grow well at refrigerator temperature while some molds can grow slowly at temperature below freezing. They tolerate low water activity. They can grow over a range of pH between 2.0 and 8.5 of the substrate but prefer acidic pH. Molds are aerobic in nature and possess a variety of hydrolytic enzymes.

The growth of molds in foods can be inhibited by mycostatic substances like sorbic acid, propionates and acetates. Some molds produce toxins called mycotoxins. But, some molds can be used in the manufacture of soya sauce and for ripening of cheeses. The mold genera that associate with foods include *Alternaria, Aspergillus, Aureobasidium, Botrytis, Byssochlamys, Cladosporium, Colletotrichum, Fusarium, Geotrichum, Monilia, Mucor, Penicillium, Rhizopus, Trichothecium, Xeromyces* etc.

Improtance of Molds in Foods: *Mucor* species, though involve in the spoilage of some foods, they find use in the manufacture of certain foods such as Oriental foods and ripening of cheese. *Aspergillus* is a widespread genus. The widespread *A.niger* species is used in the manufacture of citric and gluconic acids and also a variety of enzyme preparations. *Penicillium* is an another widespread genus and some of the species cause spoilage of foods while certain others are useful. *P.expansum* causes soft rots of fruits. *P.digitatum* and *P.italicum* cause rotting of citrus fruits. Other molds belonging to genera such as *Trichoderma*, *Botrytis*, *Cladosporium*, *Endomyces*, *Fusarium* and *Sclerotinia* cause spoilage of foods.

1.3 SOURCES OF MICROBIAL CONTAMINATION :

The microbial flora of a food consists of the microorganisms associated with the raw material, those acquired during handling and processing and those surviving any preservation treatment and

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storage. These microorganisms contaminate the food at some stage of production, harvesting, handling, processing, storage, distribution and/or preparation for consumption. Most foods are subjected to many potential sources of microbial contamination like soil, water, air, plants, feed or fertilizer, animals, humans, sewage, processing equipment, ingredients and packaging materials (Fig. 9.1). The microorganisms can also be exchanged between these sources. For example, animals contaminate the soil with faecal material which may be washed into rivers by rain. When this rain water is used for irrigation, it may contaminate the plants used for food.

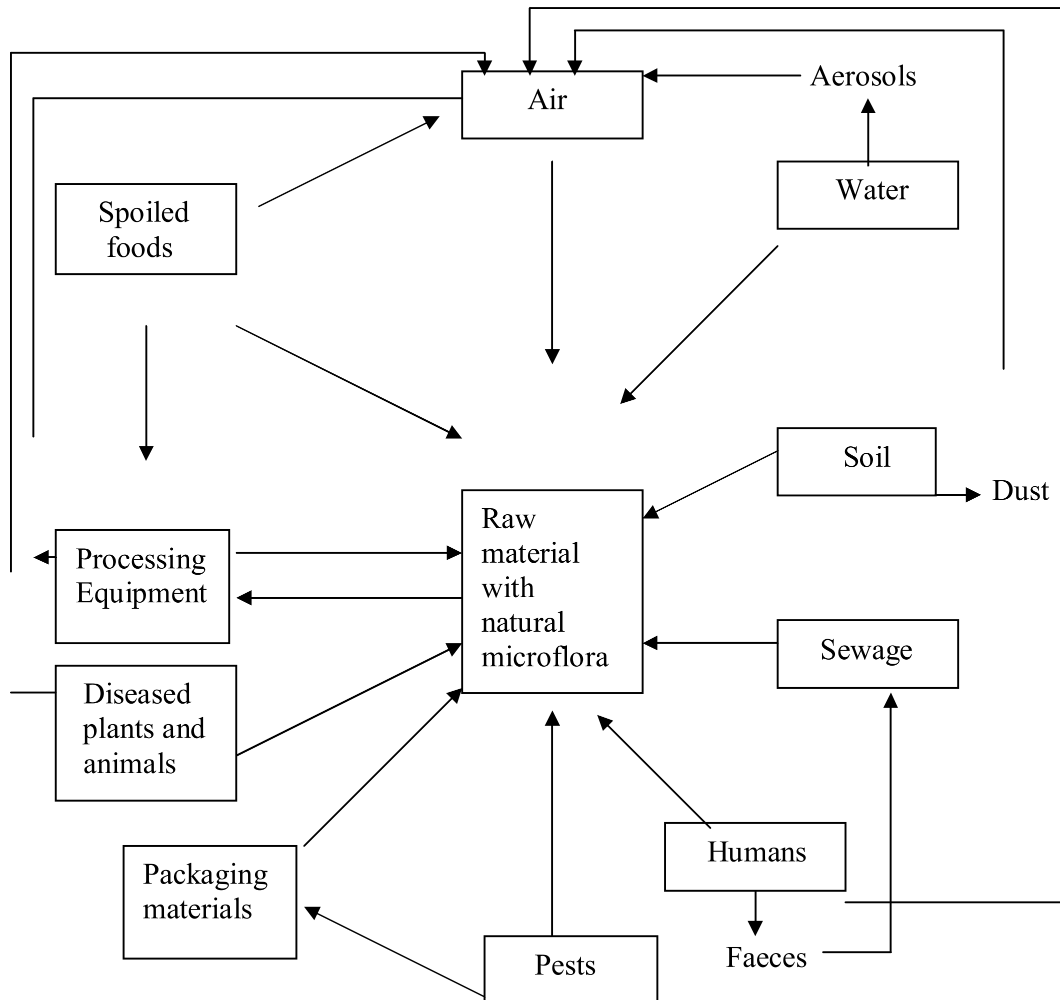

Fig. 9.1 Sources of contamination of foods

1.3.1 Soil :

It is the natural habitat for many types of microorganisms. The microbial density in the soil is greater near the surface and decreases with depth. The types and number of microorganisms vary

with soil type as well as the environmental conditions. In most soils, bacteria outnumber the other microorganisms and the most common bacteria in soil and found in food include *Acinetobacter, Alcaligenes, Bacillus, Campylobacter, Citrobacter, Clostridium, Corynebacterium, Flavobacterium, Micrococcus, Pseudomonas,* and *Streptomyces*. The fungal flora is primarily of different molds. Yeasts are found in soil. The microorganisms in the soil can contaminate tubers or root crops by direct contact. Dirt that is blown by the wind and splashed by rain fall can contaminate crops such as strawberries, beans, cabbage or peas that grow near the ground level. The microbial numbers and types on crops are influenced by the degree of contamination of the soil in which they are grown. Mechanical harvesting has increased the amount of soil contamination as well as breakage of fruits and vegetables.

1.3.2 Water :

A potential source of microbial contamination of food. Rain contains microorganisms that are washed from the air. As the water lands on the ground, it is further contaminated by soil microorganisms. In the ocean, organisms are in greater abundance near the shore than in regions distant from land. The dumping of wastes such as sewage and the runoff from animal feedlots results in considerable microbial contamination of waterways with enteric types of bacteria. Water contacts food during production, harvesting and processing. If the water used for irrigation of various crops is contaminated, the fruits and vegetables can be potential health hazards. Seafoods are harvested from water. The microorganisms in the water contaminate the surface gills and intestinal tract of fish and shellfish.

The occurrence of fecal coliforms in fish is a reflection of the pollution level of their water environment. When bivalve mollusks feed, they filter large quantities of water and concentrate bacteria and viruses that are present in water environment. If the drinking water of animals is contaminated by potential pathogens, they can be a health hazard to humans who handle the animals and can cause contamination of the carcass during slaughter. During harvesting, water may be used for hydrocooling of vegetables. Since many vegetables and fruits are eaten raw, the use of untreated water to wash these foods can serve as a vehicle of transmission of pathogenic organisms. Ice is used to cool and maintain coolness in a variety of fresh foods. As the ice melts, the organisms associated with it are passed on to the foods. The use of water in a food processing plant can be a source of microorganisms for contaminating food. In the food canning operations, water is used to cool the hot cans of food after heat processing. Due to heat and expansion of metal, seals on the cans are under stress and leakage can occur. *Leuconostoc mesenteriodes* spoilage of canned tomato juice is due to contaminated cooling water. The microorganisms that contaminate the water include the genera namely *Pseudomonas, Flavobacterium, Cytophaga, Acinetobacter, Moraxella, Aeromonas, Corynebacterium, Streptococcus, Klebsiella, Alcaligenes, Bacillus* and *Micrococcus.*

1.3.3 Air :

The main concern of today's air pollution is with chemicals such as CO, hydrocarbons, soot and fly ash. But nature is the major contributor of not only the chemical pollutants but also of biological agents such as plant cells, animal hair, pollen, algae, protozoa, bacteria, yeasts, mold spores and viruses. The air near the earth is more contaminated than at higher altitudes. Air over land

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is more contaminated than air over the ocean. The upper atmosphere over the ocean is more contaminated than air near the ocean surface. The clouds often contain high levels of bacteria and fungal spores. The atmosphere is more contaminated in the summer than in the winter. Until the food is sealed in a package, it is subjected to airborne contamination. Actually, there is no natural or normal microflora. It is contaminated from various sources. Contamination of the air is caused by gusts of wind picking up the microorganisms or spores. The types of organisms in air are often associated with the type of activity in the area. For example, downwind from a sewage treatment plant consists *Klebsiella, Bacillus, Flavobacterium, Streptococcus* and *Micrococcus.* Humans also produce aerosols during talking, coughing and sneezing. In an enclosed space, the microbial load of the air is proportional to the number of persons, their activity and rate of air circulation. In a processing room humans can be a significant source of microorganisms.

1.3.4 Plants and plant products :

Plants are contaminated by microorganisms from several sources like dirt, water, air, fertilizer, animals and humans. Many or most soil organisms and water organisms contaminate plants. However, only a relatively small number of microorganisms find the plant environment suitable to their well-being. The microbial flora on plant surfaces vary with the kind of plant. Some organisms persist on plant products due to their capacity to adhere to plant surfaces, so that they are not easily washed away as they are able to obtain nutrients from plants. Not only the surface but also the interior tissue of plants can be contaminated. Vegetables can harbour fecal streptococci within unopened pods, heads, and other structures. The microorganisms that commonly associate with plants include *Corynebacterium, Pseudomonas, Xanthomonas* of bacteria, *Saccharomyces* and *Hansenula* of yeasts, and *Torulopsis, Candida, Rhodotorula* and *Kloeckera* of imperfect fungi.

1.3.5 Animals :

The animals possesses a normal or natural microflora that is established very early in life. Besides this microflora, they tend to harbour organisms that can cause food spoilage or food-borne disease. Organisms are found in the animal's gastrointestinal tract, nasal passages, cutaneous lesions and on the skin, feet, hair or feathers on the outer surface. These organisms are readily transferred to the edible portions of the animal during processing. The digestive tract of animals contains numerous microorganisms and is a source of contaminants for soil, water, humans and food. The species of microorganisms present in the digestive system are influenced by the environment present in the various portion of the gastrointestinal tract, the type and age of the animal, the diet and the husbandry. Coliforms, enterococci, lactobacilli and bacteriodes are the usual dominant types of organisms in the fecal material of animals. The intestinal tract of animals as well as humans is the natural habitat for pathogenic *Salmonella*. Mastitis condition of milking animals may result in yielding contaminated milk, probably with coagulase positive staphylococci, coliforms and streptococci. Most of the contaminants on egg shells are of intestinal origin, indicating that the hen is a prime source of bacterial contamination of eggs.

1.3.6 Humans:

The skin, in humans, is the most available part of the body for microbial colonization after birth. This colonization is followed by microbial invasion of the nose, oral cavity, throat and the respiratory, digestive and urogenital tracts. Besides the microbial population on or in a human, clothing can be contaminated by external sources or by the human, and then serve to pass the microorganisms on to the human or to food products. Humans are a source of airborne microorganisms as well as an important source of food contamination through handling of food. The considerable number of microorganisms present on hands are passed on to food products when they are handled. Beards, sideburns, mustaches may also contribute to contamination in a food handling area.

In shellfish industry, the hand shucking of oysters and clams, shelling of shrimp and picking crab meat from the shell are associated with increased bacterial counts. Slicing, weighing and hand packaging of meat products, and unpacking, trimming, sorting and repacking of fresh fruits and vegetables increase the contamination through human handling of the product. An important cause of microbial contamination of foods is the carelessness of humans with respect to improper cleaning and sanitizing the equipment, failing to wash one’s hands, handling the food with infection, poor personal hygiene, lack of care in handling the food, failing to keep foods at proper temperature etc.

1.3.7 Sewage :

Animal manure or human wastes are used as fertilizer on crops. These biologically produced substances contain microorganisms including human pathogens. When added to soil, these pathogens may survive for periods sufficient to contaminate the harvested crop. The septic tanks in rural areas are, quite often, not properly installed and not properly operated and so that raw sewage leaks into the soil.

1.3.8 Equipment :

Machines were developed during the course of Industrial Revolution to do most of the human work. This has been resulted in less contact of persons with food and more contact is between machines and food. Small equipment like knives, cutting boards and bins are used in food processing or handling establishments. Metal processing equipment usually does not support growth of microbes. It has no natural or normal microbial flora. Yet, food processing equipment is one of the major sources of contamination of foods. Equipment that is cleaned or sanitized may have food deposits or films that provide micro environments acceptable for survival and growth of microorganisms. The potentiality for microbial buildup is further enhanced by improper cleaning. The pitted surfaces or poorly soldered joints are the places in which foods can lodge on the equipment. During the course of daily operation, bacterial growth will occur in these food films and deposits. This then serves as a source of contamination when food contacts these surfaces. The genera of microorganisms that found on food utensils mainly depend upon the types of foods handled. Utensils that are stored in the open areas where dust can fall, have airborne bacteria, yeasts and molds.

1.3.9 Animal feeds :

A variety of genera of bacteria, yeasts and molds may be found in animal feeds. The types of organisms in a feed depend on the source of feeds, containers in which they are stored etc. Animal feeds are of great importance in the spread of food-poisoning *Salmonella*. Organisms from this source have been shown to be rapidly disseminated throughout processing plants where feeds are handled.

1.3.10 Ingredients :

The quality of a processed food is influenced by the quality of the ingredients. Although ingredients may constitute a small part of the total food, they add a substantial number of microorganisms. Spices are often the source of high microbial numbers, because spices are the parts of the plants such as dried seeds, buds, fruits, flower parts, barks or roots, usually of tropical region. These seasonings may contain over 10^8 aerobic bacteria per gram weight. The flour material used for food preparations may also harbour various microorganisms. The types of microorganisms in an ingredient or product are often more important than the total numbers that are present. Since ingredients are an important source of microorganisms, food processors must establish acceptable microbiological limits for these substances.

1.3.11 Product-to-product :

If the foods are in contact with each other, the transfer of microorganisms from one food to another food occur. But more often, transfer occurs through contamination of wash water, equipment and handling. The cross contamination of food was found to be one of the 10 main factors that contribute to foodborne illness. People handling both cooked and raw foods can transfer microorganisms form the raw to the cooked product. If this was taken as such with out any further treatment, it is a potential health hazard.

1.3.12 Packaging :

There are many studies concerning the effect of different types of packaging materials on the shelf life of food. The boxes or the cardboard material to prepare packaging boxes that used to pack the food material may be contaminated by dust, rodents and birds. The reuse of packaging containers can cause problem in food industry. Usually, packages are meant as a protective covering to limit or prevent microbial contamination but cannot prevent the microbial growth. So, it is so important that preparation of food for packaging is designed to limit or prevent microbial contamination prior to packaging. Also, the package must be durable enough to maintain its integrity during storage and distribution.

1.4 SUMMARY :

Foods, by their very nature, need to be nutritious and metabolizable and it should be expected that they will offer suitable substrates for the growth and metabolism of microorganism. The foods that will be consumed are rarely if ever sterile, they carry microbial associations whose composition depends upon which organisms gain access and how they grow, survive and interact in the food over time. The microorganisms present will originate from the natural microflora of the raw material and those organisms introduced in the course of harvesting or slaughter, processing, storage and distribution. The numerical balance between the various types will be determined by the properties of the food, its storage environment, properties of the organisms themselves and the effects of processing. In most cases this microflora has no discernible effect and the food is consumed without objection and with no adverse consequences. In some instances though, microorganisms manifest their presence in one of several ways viz., they can cause spoilage, they can cause foodborne illness, and they can transform a food's properties in a beneficial way i.e. food fermentation. The growth and activity of the microorganisms in a food depends on the intrinsic factors as well as extrinsic factors.

1.5 MODEL QUESTIONS

1.Give a general account on the association of microorganisms in foods.
2.Write an essay on different potential sources of microbial contamination of food materials.
3. Short questions:
   i. Bacterial association of foods
   ii. Soil source of microbial contamination of foods.
   iii. Equipment and humans as sources of microbial contamination of foods.
   iv. Product-to-product source of microbial contamination in foods.
   v. Association of molds and yeasts with foods.
   vi. Importance of bacteria and yeasts in foods.

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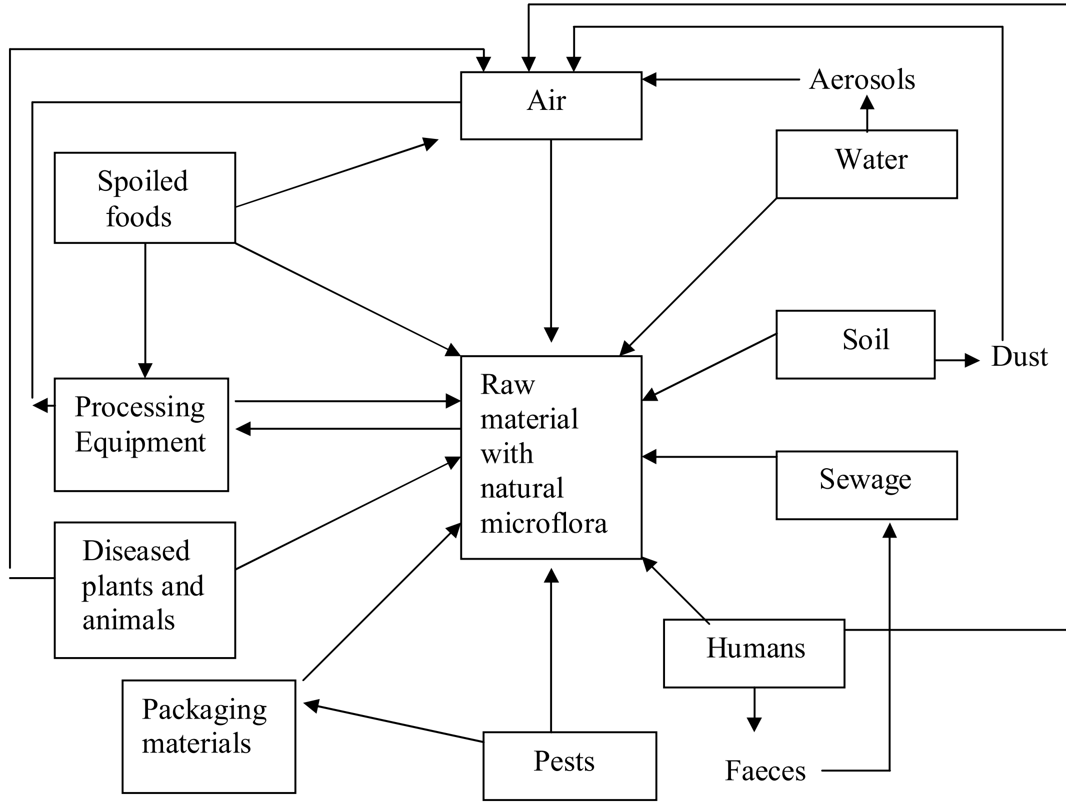
Air
Aerosols
Water
Spoiled foods
Soil
Dust
Processing Equipment
Raw material with natural microflora
Sewage
Diseased plants and animals
Humans
Packaging materials
Pests
Faeces

M.Sc. MICROBIOLOGY (Final)

Lesson No. 2

ENUMERATION OF MICROORGANISMS IN FOODS

2.0 Objective
2.1 Introduction
2.2 Traditional methods
2.2.1 Direct Microscopic Count
2.2.2 Standard Plate Count
2.2.3 Membrane filtration technique
2.2.4 Most Probable Number technique
2.2.5 Dye reduction tests
2.3 Rapid methods
2.3.1 ATP Photometry
2.3.2 Direct epifluorescent filter technique
2.3.3 Electrical impedance method
2.4 Summary
2.5 Model questions
2.6 References

2.0 OBJECTIVE

The main aim of this lesson plan is to make the reader to understand the various methods or analyses that are routinely and normally employed for assessing the microbiological quality of foods and levels of contamination in the processing environment.

2.1 INTRODUCTION

The microbiological examination of a food may be necessary for one or more number of reasons. A determination of the microbiological quality of a food or food constituent may be required in order to estimate its shelf-life or its suitability for human consumption. Although knowledge of the total viable count may be desirable, it is often more useful to obtain an estimate of the numbers of a particular component of the total flora such as molds in cereal, psychrotrophic bacteria in a product to be stored at low temperature, anaerobes in a vacuum-packed food, or yeasts in a fruit beverage. Methods used to assess the numbers of organisms or the presence of various types of organisms in processed foods, raw materials and the processing environment are used extensively in routine quality control laboratories throughout the food industry, laboratories that carry out contract work for the food industry and public health laboratories.

Microbiological analysis can be carried out on raw materials and final products to find :

- The total number of organisms present
- The presence or absence of organisms
- The levels of indicators
- The levels of specific pathogens
- The presence or absence of specific pathogens

In the processing environment to look at:

- The microbiological quality of air
- Levels of microbial contamination on surfaces
- The microbiological quality of the water used

The tests that are actually carried out will vary according to the nature of the raw materials, the process and the criteria used to assess quality.

2.2 TRADITIONAL METHODS:

These methods are also commonly referred as conventional methods. Some of these methods are relatively older but are still found be useful and efficient for enumeration purpose. These methods include the microscopic techniques, cultural techniques, membrane filter technique and dye reduction tests.

2.2.1 Direct Microscopic Count (DMC):

This is the simplest method and consists of making smears of food specimens or cultures onto a microscope slide, staining with an appropriate dye, and viewing and counting cells with the aid of a microscope. A small sample of food (0.01 ml liquid food or food homogenized in diluents) is spread as evenly as possible in a 1 cm^2 area etched on a microscope slide. The slide is dried, stained with a suitable stain, e.g. methylene blue, and examined with the oil immersion lens on the microscope. The numbers of organisms in 25 random microscope fields can then be counted and a calculation, based on the field diameter, made to give the number of organisms/g or ml original sample. The use of direct microscopic counts is more or less limited to liquid or semi-solid foods with no cell debris. DMC is most widely used in dairy industry for assessing the microbial quality of raw milk and other dairy products, and the specific method employed is the Breed count method. This method is also used as a quick check of yoghurt starters and yoghurt quality for the relative numbers of lactobacilli and streptococci.

Among the advantages of DMC are: it is rapid and simple; cell morphology can be assessed; and it lends itself to fluorescent probes for improved efficiency. Although the technique is quick to carry out with results available in just a few minutes and requires little in the way of consumables, it does suffer from a number of drawbacks. Among its disadvantages are : it is a microscopic method and therefore fatiguing to the analyst; both viable and nonviable cells are enumerated; food particles are not always distinguishable from microorganisms; microbial cells are not uniformly distributed

relative to single cells and clumps; some cells do not take the stain well and may not be counted; and DMC counts are invariably higher than counts made by SPC. In spite of its drawbacks, it remains the fastest way to make an assessment of microbial cells in a food product.

The Howard mold count is a direct microscopic method developed by B.J.Howard in 1911 for the purpose of determining the quality of tomato products, e.g. tomato sauce, in relation to mould content of the raw materials. The technique involves counting the numbers of hyphal fragments in a known volume of tomato product. There is also a similar method for quantifying *Geotrichum candidum* in canned beverages and fruits.

2.2.2 Standard Plate Count (SPC):

The SPC is by far the most widely used method for determining the numbers of viable cells or colony-forming units (cfu) in a food product. The use of plate counts is based on the fact that living bacterial cells or clumps of cells will grow and increase in numbers in or on the surface of a suitable agar medium to give visible colonies that can be counted. In this method, portions of food samples are blended or homogenized, serially diluted in an appropriate diluent, plated in or onto a suitable agar medium, incubated at an appropriate temperature for a given time, after which all visible colonies are counted by use of a Quebec or electronic counter. When total viable counts are reported for a product, the counts should be viewed as a function of at least some of the following factors:

- Sampling methods employed
- Distribution of the organisms in the food sample
- Nature of the food flora
- Nature of the food material
- The pre-examination history of the food product
- Nutritional adequacy of the plating medium employed
- Incubation temperature and time used
- pH, a_w and oxidation-reduction potential (Eh) of the plating medium
- Type of diluent used
- Relative number of organisms in food sample
- Existence of other competing or antagonistic organisms

The first step or stage in carrying out a traditional plate count on a food involves producing a homogenate of the sample and a series of dilutions. Producing a homogenate of the food sample gives an evenly dispersed microbial population in a liquid that can be easily pipetted, plated out or spread. Production of the food homogenate and dilution series is followed by the inoculation of agar plates with samples from each dilution, incubating the plates and counting the number of colonies produced. A simple calculation can then be used to determine the number of colony forming units (cfus) in the original sample. Diluting the sample and plating out each dilution is essential to ensure a countable plate with between 30 and 300 colonies. The number of dilutions used in practice is determined by the criterion applied to assess whether the sample is acceptable, and previous experience of examining a particular raw material or product.

The information obtained from a plate count depends on – the choice of diluent used to prepare the food homogenate and the dilution series; the medium used; the plating method; incubation conditions; and method used to homogenize the sample. The two most commonly used plating techniques are the pour plate technique and the spread plate technique. The plating methods, particularly those associated with assessing the total number of bacteria in foods, tend to be expensive in terms of consumables used, e.g. a standard plate count using the spread plate method may use six dilutions and, if samples are plated in duplicate, consume 12 agar plates for each sample analysed. Several attempts have been made to reduce the quantity of agar plates used, e.g. Miles and Misra, spiral plating and the Colworth droplet method, but because of problems involved with ease of counting, sensitivity and the presence of food particles, none have gained wide acceptance for analyzing foods. If large numbers of samples are being examined, counting time can be reduced by employing devices such as laser counters that will count the number of colonies on a plate automatically. However, there is a relatively large capital outlay involved in purchasing the necessary equipment.

The most common plating technique to determine the bacterial content of foods is the Standard Plate Count (SPC), also called as the Aerobic Plate Count (APC), in which a general purpose medium, normally tryptone dextrose yeast extract agar is used. After inoculation, plates are incubated aerobically at 30°C for 48 hours. The technique gives a valuable indication of the quality of foods in terms of the numbers of aerobic psychrotrophic and mesophilic organisms present. In the dairy industry, plate counts carried out on milk samples are sometimes described as Total Bacterial Counts (TBCs). SPCs can be used to

- check the microbiological quality of raw materials and final products
- check the conditions of hygiene during the manufacturing process
- determine whether a food has been subjected to temperature abuse during production, transport and storage
- estimate the potential storage life of a product
- comply with established criteria for a product
- determine levels of contamination in the processing environment

Plate counts can also assess the numbers of pathogens, indicators and specific spoilage organisms in foods. For some foods, quality is determined by counting the numbers of molds present. However, total viable counts for molds have doubtful validity as a single sporing head, of say *Penicillium* spp, present in a food can release many hundreds of spores into the homogenizing diluent, each of which will give rise to a colony and be counted as a separate organism.

2.2.3 Membrane Filtration Technique:

Membrane filtration involves passing a known volume of liquid through a cellulose acetate membrane with a pore size of 0.45 µm that will retain the bacteria but allow water of diluent to pass are used. Bacteria, yeasts and molds are removed from the liquid and precipitated on the membrane surface. When the membrane is transferred to an agar plate or an absorbent pad saturated with the

culture medium of choice and incubated, nutrients diffuse through the membrane so that organisms can grow on the membrane surface giving visible colonies that can be counted. Differential media can be used to detect specific organisms or groups of organisms in the filtered liquid.

Alternatively, a DMC can be made. In this case, the organisms collected on the membrane are viewed and counted microscopically following appropriate staining, washing, and treatment of the membrane to render it transparent. These methods are especially suited for samples that contain low numbers of bacteria. The overall efficiency of membrane filter methods for determining microbial numbers by the DMC has been improved by the introduction of fluorescent dyes. Membrane filtration as a technique for assessing microbial numbers is limited to clear liquids that do not contain debris or other materials that will block the filter, e.g. it will not work for food homogenates, milk or fruit juices containing fruit debris.

The technique has an important advantage in being able to detect low numbers of organisms in large volumes of liquids, so that it is particularly useful for testing treated water supplies for the presence of coliforms. The standard technique for testing water supplies in quality control laboratories in the water industry involves filtering 100 ml samples of water, transferring the membrane to a pad soaked in membrane enriched teepol broth and counting typical coliform colonies after incubation at $37^{o}C$ for 48 hours. The method will detect one coliform cfu in the 100 ml sample filtered.

2.2.4 Most Probable Number (MPN) Technique:

Most probable number or multiple tube techniques are used to estimate the number of cfus in food samples if the criterion for a particular organism requires detection of low numbers. This method was introduced by McCrady in 1915. This is not a precise method of analysis. The MPN technique is a statistical method based on the probability of transferring a cfu from an original dilution using multiple samples and is employed in conjunction with a statistical table (MPN table) that given MPN values for various combinations of positive tubes. MPN tables are frequently published with 95% confidence limits for each of the table values.

The technique is particularly useful for indicators, e.g. *E.coli*. In this method, dilutions of food samples are prepared following the 10-fold serial dilution technique. Three serial aliquots or dilutions are then planted into 9 or 15 tubes of appropriate medium for the 3- or 5-tube method, respectively. The typical MPN technique for food analysis involves replicate tubes in groups of three containing a liquid medium (normally a selective and differential medium designed to detect a specific organism or group of organisms) inoculated with 1 ml samples from a dilution series. The decision as to whether a viable organism has been transferred to a tube is based on growth to give visible turbidity often in conjunction with another characteristic of the organism, e.g. the ability to produce gas and / or acid from a sugar.

Among the advantages MPN technique offers are: relatively simple; results from one laboratory are more likely to agree with those from another laboratory; specific groups of organisms can be determined by use of appropriate selective and differential media; a method of choice for determining fecal coliform densities. Among the drawbacks to its use is the large volume of

glassware required, the lack of opportunity to observe the colonial morphology of the organisms, and its lack of precision.

2.2.5 Dye Reduction Tests:

Dye reduction tests have a long history of use in the dairy industry for assessing the overall microbial quality of raw milk. Two redox dyes namely methylene blue and resazurin are commonly used to estimate the number of viable organisms in suitable products and to test the quality of milk. To conduct a dye-reduction test, properly prepared supernatants of foods are added to standard solutions of either dye for reduction from blue to white for methylene blue and from slate blue to pink or white for resazurin. The test is based on the assumption that the microflora present in the milk will metabolize carbohydrate to produce reducing substances that in turn reduce the dye. If tubes containing known amounts of milk or other sample are mixed with a standard amount of dye and incubated at a predetermined temperature, then the rate at which the dye is reduced reflects the numbers of bacteria present and therefore gives some indication of quality. The system will work well for samples in which the dominant flora is primarily mesophilic, fermentative and active in reducing the dye, e.g. samples containing streptococci and coliforms.

Dye reduction tests are operated very successfully to assess milk quality when milk was stored and distributed at ambient temperatures. However, in modern distribution and storage systems in which milk is refrigerated to chill temperatures, the dominant microflora is psychrotrophic. The psychrotrophic bacteria do not actively reduce redox dyes, making the tests much less reliable, to that the methylene blue dye reduction test previously used as the statutory test to assess pasteurized milk quality in England and Wales has been replaced by plate counts. The resazurin test is still applied as a quick test to assess the quality of bulk tanker milk before it is accepted at the dairy. Among their advantages are: they are simple, rapid, and inexpensive; and only viable cells actively reduce the dyes. The disadvantages are: not all organisms reduce the dyes equally; and they are not applicable to food specimens that contain reductive enzymes unless special steps are employed; difficulty in recognition of colour changes against a homogenized food background.

2.3 RAPID METHODS:

Although traditional techniques for the microbiological analysis of foods have been used for several decades and have given excellent service and continue to do so, they do suffer from a number of disadvantages. The most obvious of these is the time it takes to get results. The term rapid method can be applied to any technique involved in the microbiological analysis of foods that reduces the time taken to get results in comparison with the traditional technique. Some of the rapid techniques available have gained acceptance in the food industry as reliable methods for the routine assessment of the numbers of organisms in foods.

2.3.1 ATP Photometry:

The cells of living organisms contain adenosine triphosphate (ATP), which is used by the cell as an energy source to drive various cellular activities, i.e. synthesis of new cell materials, uptake of

materials from the environment, movement and light production. A substrate (luciferin) and an enzyme (luciferase) can be extracted from light-producing organisms. The tails of fireflies are used as a commercial source of the purified enzyme and substrate. When purified enzyme and substrate are mixed together with ATP in the laboratory, light is emitted. The amount of light produced is proportional to the concentration of ATP (1 photon for each ATP molecule) so that the reaction can be used to assay the amount of ATP in living cells. Light emitted can be measured using a photometer. Microbial cells of a particular type appear to contain more or less the same amount of ATP. Bacterial cells contain about 1 fg of ATP and yeast cells contain about 100 fg of ATP.

The basic sequence for the analysis of food using ATP photometry is:

- break down of non-microbial cells in the food (somatic cells) to release their ATP
- removal of non-microbial ATP using the enzyme ATPase
- release of bacterial ATP from bacterial cells
- assay of the amount of bacterial ATP by the addition of firefly luciferin or luciferase
- recording of the amount of light emitted using the ATP photometer
- using the number of relative light units directly to assess quality or use a correlation curve to convert relative light units to cfus/ml.

ATP photometry has been used successfully to assess the quality of fresh meat and milk, measure the activity of starter cultures and test UHT milk for sterility. It has also been found to be particularly useful for the rapid monitoring of surface contamination of processing equipment in the food industry. The problem areas can be quickly defined and the effectiveness of cleaning procedures assessed more or less immediately.

2.3.2 Direct Epifluorescent Filter Technique (DEFT):

This is viewed as an improved modification of the basic membrane filter technique. The direct epifluorescent filter technique uses a combination of direct microscopy and membrane filtration to assess the numbers of organisms in food samples. This technique employs fluorescent dyes and fluorescent microscopy. Foods normally need some form of pretreatment to enable samples to pass through the membrane filter. DEFT was originally developed to monitor the microbiological quality of farm milk arriving at the dairy. In order to pass the milk through the filter membrane, it needs to be pretreated using a surfactant to emulsify fat globules and a proteolytic enzyme (trypsin) to remove somatic cells. Stomached suspensions of solid foods require pre-filtering to remove food debris. The technique involves the following basic stages:

- a known volume of liquid sample of diluted food homogenate is filtered through a polycarbonate membrane filter.

- Acridine orange, a fluorescent dye, is poured through the filter to stain the organisms left on the surface.

- The filter is mounted under a coverglass and viewed using epifluorescent microscopy. Fluorescent cells or groups of cells can then be counted and numbers of organisms / ml original sample calculated on the basis of the volume of liquid passed through the filter, the filter area, the area of the microscope field and the number of fields counted.

In epifluorescent microscopy, the mercury vapour lamp emits light at the correct wavelength to cause cells stained with acridine orange to fluoresce. As no incubation for growth is needed, the technique is faster than membrane filtration, taking only about 45 minutes to complete. The advantages over a conventional DMC are:

- Concentration of organisms on the filter before counting means that lower numbers of organisms can be detected.
- Organisms fluorescing against a dark background are easier to see than cells stained and viewed by conventional microscopy. This makes the cells easier to count and the likelihood of counting debris is reduced.
- Counting can be automated.

2.3.3 Electrical impedance methods:

Impedance is the resistance to the flow of an alternating current through a conducting material, e.g. a microbiological culture medium. Impedance has two components: Capacitance, which is associated with the accumulation of charged ions at the interface between the electrodes and the culture medium, and Conductance, which is the reciprocal of resistance to current flow determined by the concentration of charged ions in the culture medium. Culture media with high conductance have low resistance and vice versa. Changes in impedance in a culture medium are associated with the conversion of uncharged or weakly charged substrates into smaller more mobile and / of highly charged molecules by the metabolic activities of organisms. For example, amino acids are deaminated with the release of ammonium ions. This causes a decrease in the impedance of the medium. Both the mobility of molecules and their charge are responsible for impedance changes. Bacterial growth normally leads to an increase in conductivity i.e. decrease in impedance.

Equipment is available, e.g. Bactometer, that is capable of monitoring the changes in impedance that take place as an organism grows in a culture medium. The Bactometer consists of the Bactometer Processing Unit (BPU), which monitors impedance change and also serves as an incubator, linked to a computer with software designed to analyse, record and display the information produced by the BPU. Samples, e.g. a food homogenized in a suitable growth medium, are pipetted into wells in a Bactometer module. Each well is supplied with two stainless steel electrodes. When the module is plugged into the Bactometer processing unit, a small alternating current is passed between the electrodes and any change in impedance monitored.

However, a significant change in impedance can only be detected when the number of organisms reaches $10^6 – 10^7$ cells / ml medium in the wells. The time taken to reach this threshold is called the detection time (DT) and depends on the number of organisms in the original sample. The lower the number of organisms the longer the detection time and vice versa. The factors that influence the length of detection time include incubation temperature, medium used, types of organisms present and the length of their lag phase. An advantage of this technique is that it measures changes

associated with metabolic activity, which means that each individual cell from a colony forming unit is likely to influence impedance. Using appropriate media, electrical impedance methods can be used as an alternative to standard plate counts and coliform counts for sterility testing, and for the enumeration, and detection of molds, yeasts and staphylococci in foods.

2.4 Summary

The examination of foods for the presence, types, and numbers of microorganisms and / or their products is basic to food microbiology. In spite of the importance of this, none of the methods in common use permits the determination of exact numbers of microorganisms in a food product. Although some methods of analysis are better than others, every method has certain inherent limitations associated with its use. There are several methods or techniques for enumeration of microorganisms in foods. These methods can be broadly categorized into two groups namely traditional methods and rapid methods. Each technique will have their own merits and demerits. The choice of method used can be governed by several factors and the relative merits of different methods is a topic of constant investigation and debate.

2.5 Model questions

01. Describe the various methods used for the enumeration of microorganisms in foods.
02. Write an essay on the traditional methods of enumeration of microorganims.
03. Give an account on rapid methods employed in enumeration of microorganisms in foods.
04. Short answers:
    a) ATP photometry
    b) Standard plate count
    c) Membrane filter technique
    d) MPN technique
    e) Dye reduction tests
    f) Electrical impedance method
    g) Direct epifluorescent filter technique
    h) DMC method

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Lesson No. 3

FOOD SPOILAGE

3.0 Objective

3.1 Introduction

3.2 Causes of food spoilage

- 3.2.1 Activity of Microorganisms
- 3.2.2 Action of Native Enzymes
- 3.2.3 Insects, Parasites and Rodents
- 3.2.4 Chemical reactions in foods
- 3.2.5 Environmental factors

3.3 Microbial spoilage of Vegetables and Fruits

3.4 Microbial spoilage of Cereals and Cereal products

3.5 Microbial spoilage of Poultry and Eggs

3.6 Microbial spoilage of Meat

3.7 Microbial spoilage of Canned foods

- 3.7.1 Underprocessing
- 3.7.2 Survival of thermophilic bacteria
- 3.7.3 Leaker spoilage

3.8 Summary

3.9 Model questions

3.10 References

3.0 OBJECTIVE

From the health point of view, it is very important for any one to know about the spoilage of various types of foods by different pathogenic microorganisms. After going through this topic, one can have, at least, a considerable level of understanding about the fitness of the foods for consumption.

3.1 INTRODUCTION

The spoiled food may be defined as "food that has been damaged or injured so as to make it undesirable for human consumption". So, a spoiled food is simply a food that is unacceptable to a consumer for reasons of smell, taste, appearance, texture or the presence of foreign bodies. Basing on their ease of spoilage, foods are classified into three groups viz.,

(i) Stable or non-perishable foods – foods which do not spoil normally unless handled carelessly. Eg: Sugar, Flour, Dry beans, Grains

(ii) Semi-perishable foods – foods which remain unspoiled for relatively long period under proper handling or storing conditions. Eg: Potatoes, Onions, Waxed fruits, Nutmeats, thick skinned fruits and vegetables.

(iii) Perishable foods – foods that spoil readily unless special preservative methods are adopted. Most of our daily foods belongs to this group.
Eg: Meat, Fish, Poultry, Milk, Vegetables, Fruits, Eggs.

Foods are mostly subjected to physical, chemical, and biological changes which may cause the deterioration in the quality and ultimately the spoilage of food. Such changes may not always be microbiological in origin, but by and large most food spoilage is a result of microbial activity. Microbiological food spoilage can manifest itself in several different ways, some of which often occur in combination. Visible microbial growth may be apparent in the form of surface colonies, degradation of structural components of the food that cause loss of texture, but the most common manifestation will be chemical products of microbial metabolism i.e., gas, pigments, polysaccharides, off odours and flavours.

3.2 CAUSES OF FOOD SPOILAGE

The major causes of food spoilage include (i) Activity of microorganisms, (ii) Action of native enzymes, (iii) Insects, rodents and parasites, (iv) Chemical constituents of the food components, (v) Environmental factors such as temperature, moisture, air and light, and (vi) Time. All these factors may operate simultaneously affecting the quality of food. So, it is highly impossible to completely eliminate these factors and secure total prevention of food spoilage. Various preservation methods may minimize the effect of these factors and enhance the storage stability and maintain the quality of foods for prolonged periods.

3.2.1 Activity of microorganisms :

Microorganisms capable of spoiling food are available commonly in soil, water and air, on the skins of cattle, fruits and vegetables, on the feathers of poultry, on the hulls of grains, and shells of nuts, on the clothing and skin of handling personnel, on processing equipment and within the intestines and body cavities of animal and human body. However, they are not found within the healthy living tissues of plants and animals. Microorganisms invade the plants and animal tissues through a break in skin or by penetration through skin.

Most raw foods contain a variety of bacteria, yeasts and molds and their numbers increase due to contamination and also introduce new kinds. Microorganisms may be symbiotic, antagonistic or synergistic. The synergistic forms bring bout changes by growing together which cannot be brought when grown alone. Eg: *Pseudomonas syncyanea* alone in milk causes a light brownish tinge and growth of *Streptococcus lactis* alone causes no change in colour. When both the organisms grown they cause bright blue colour.

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Microorganisms, in most cases, produce a metabiotic effect i.e., one organism grows and makes the favourable conditions for the growth of the second organism. Most natural fermentations in raw foods are primarily due to metabiosis which involves the succession of microorganisms. Eg: Raw milk supports acid fermentation by the growth of *Streptococcus lactis* and coliform bacteria until the formed acid inhibits their growth. In the next stage of fermentation, acid tolerant lactobacilli grow and produce more acid. Then a film of yeasts and molds grow over the top and reduce the acidity facilitating the growth of proteolytic bacteria at a subsequent stage.

3.2.2 Action of Native Enzymes :

The activity of the endogenous enzymes in plant and animal foods is often intensified after harvest or slaughter due to lack of control mechanisms in the harvested plant food or slaughtered animal. For example, pepsin in a living animal helps in the digestion of protein but does not affect the intestine itself but when the animal is dead, pepsin does contribute to proteolysis of the organs containing it. Similarly, uncontrolled ripening of vegetables and fruits results in their spoilage. However, the enzymatic changes in foods are desirable to some extent as in the case of natural tenderization of meat during aging and ripening of fruits, but continued enzymatic activity beyond an optimum point becomes food deterioration due to rotting.

3.2.3 Insects, Parasites and Rodents :

Insects destroy cereal grains, fruits and vegetables by not only consuming the food but by contaminating the food. They also facilitate microbial attack on foods. The insect eggs which are resistant to controlling treatments may persist in stored plant products and are of great concern in spoilage. The parasites enter the human body mostly through the improperly cooked poultry and hogs. Rodents apart from consuming considerable quantity of food also contaminate the food through their droppings, urine and filth.

3.2.4 Chemical reactions in foods :

The quality of foods deteriorate due to chemical reactions of the constituents of food. The unsaturated fatty acid components undergo oxidation due to exposure to atmospheric air giving rise to oxidative rancidity in fat rich foods. Free fatty acids may also be released due to hydrolytic reactions causing odour as well as undesirable changes in the texture of food.

3.2.5 Environmental factors :

The most important environmental factors which affect the quality of food include temperature, moisture and humidity, air and light.

Temperature – Much of the food is handled at a temperature range of about 10-40°C. Excessive heat denatures proteins, destroys vitamins, enhances the rates of chemical as well as enzymatic reactions and dries out food by removing moisture. On the other hand, excessive cold also spoils

foods. Freezing disrupts the texture of fruits and vegetables and also crack the skin rendering the food susceptible to microbial attack. Uncontrolled freezing also spoils liquid foods like milk.

Moisture and humidity – Moisture content in the food and humidity of the surrounding atmosphere also have an important role in the deterioration of the quality of food. Surface moisture causes surface defects such as mottling, crystallization and stickiness. Condensation of moisture on the surface of the food facilitates the growth and activity of microorganisms. Even in moisture proof packages, fruits and vegetables give off moisture from respiration and transpiration sufficient to support the growth of the microorganisms.

Air – Oxygen and air can have detrimental effects on vitamins A and C, food colours, flavours and other food constituents. The presence of air facilitate several oxidation reactions.

Light – The light destroys riboflavin, vitamins A and C and also promotes light induced oxidation reactions affecting flavour and colour of foods. Light also causes destruction of proteins as in the case of milk.

Time – The quality of food remains at its peak for some time soon after its harvest or slaughter and thereafter as time progresses, deterioration in the quality of food also progresses.

3.3 MICROBIAL SPOILAGE OF VEGETABLES AND FRUITS :

The deterioration of raw vegetables and fruits may result from physical factors, action of their own enzymes, microbial action, or combinations of these agencies. As estimated, about 20% of all fruits and vegetables harvested for human consumption are lost through microbial spoilage by one or more of 250 market diseases caused by various microorganisms. Members of Bacteria, Yeasts and Molds are the primary causative agents of microbial spoilage of vegetables and fruits. In general, molds and bacteria are by far the most important agents of food spoilage than yeasts. The type of spoilage commonly varies with the kind of fruit / vegetable and also to some extent with the variety.

Microbial spoilage of vegetables and fruits may be due to (i) plant pathogens acting on the stems, leaves, flowers or roots of the plant, on the fruits or other special parts used as foods and (ii) saprophytic organisms which may be secondary invaders after the action of a plant pathogen or may enter a healthy fruit or vegetable as in the case of various rots or grow on its surface. A saprophyte may succeed a pathogen or a succession of saprophytes may be involved in the spoilage. The enzyme production by microorganisms, though not multiplying, over an extended storage time decompose the food and cause spoilage. However, usually the organisms that cause spoilage are those that can multiply and become dominant. Though each fruit / vegetable has certain types of decomposition and kinds of microorganisms predominant in its spoilage, some general types of microbial spoilage are found more often. Bacterial soft rot is widespread for the most part among the vegetables which are not very acid, and among fruits is limited to those which are not highly acid. Fungal spoilage of vegetables often results in water-soaked, mushy areas, while fungal rots of fleshy fruits such as apples and peaches frequently show brown or cream coloured areas in which mold mycelia are growing in the tissue below the skin and aerial hyphae and spores may appear later.

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Most commonly occurring spoilage types include

No.	Type of Spoilage	Causative organism
1.	Bacterial soft rot	*Erwinia carotovora, Pseudomonas marginalis, Clostridium* spp., *Bacillus* spp.
2.	Gray mold rot	*Botrytis cinerea*
3.	Rhizopus soft rot	*Rhizopus stolonifer*
4.	Anthracnose	*Colletotrichum lindemuthianum, Colletotrichum coccodes*
5.	Alternaria rot	*Alternaria tenuis*
6.	Blue mold rot	*Penicillium digitatum*
7.	Downy mildew	*Phytophthora* spp., *Bremia* spp.
8.	Watery soft rot	*Sclerotinia sclerotiorum*
9.	Stem-end rots	Species of *Diplodia, Alternaria, Phomopsis, Fusarium*
10.	Black mold rot	*Aspergillus niger*
11.	Black rot	*Alternaria* and *Physalospora* spp.
12.	Pink mold rot	*Trichothecium roseum*
13.	Fusarium rot	*Fusarium* spp.
14.	Green mold rot	*Cladosporium* and *Trichoderma* species
15.	Brown rot	*Sclerotinia*
16.	Sliminess or souring	Saprophytic bacteria

Since vegetables are harvested from or near the soil, they are subjected to a heterogenous flora of soil, as well as airborne microorganisms. In general, the pH of vegetables is near neutral so that the bacteria as well as fungi cause deterioration. Fruits like strawberries, cucumbers, peppers, melons may be in direct contact with the surface of the soil. Leaves, stems and flowers as in lettuce, the greens, cabbage, asparagus are especially exposed to contamination by plant pathogens or damage by birds or insects. The location of plant part used is also important from spoilage point of view. The underground parts like roots, tubers, or bulbs as in radishes, beets, carrots and potatoes are in direct contact with moist soil and become infected from that source. The character of spoilage will also depend on the product attacked and the attacking organism.

On average, the composition of vegetables is about 88% water, 8.6% carbohydrates, 1.9% proteins, 0.3% fat and 0.84% ash. The percentage of vitamins, nucleic acids and others is generally less than 1%. Nutritionally, vegetables are capable of supporting the growth of molds, yeasts and bacteria and so can be spoiled by these organisms. The high water content and the low carbohydrate and fat

contents favour the growth of spoilage bacteria. Bacteria are the common agents of microbial spoilage of vegetables as the pH range of most vegetables is within the growth range of large number of bacteria. Aerobic and facultatively anaerobic types are more important than obligate anaerobes due to relatively high oxidation-reduction potential of vegetables.

The composition, on average, of fruits is 85% water, 13% carbohydrate, 0.9% protein, 0.5% fat and 0.5% ash. The pH of the most fruits is below that of needed by bacteria. So, bacteria are of no importance in initiation of fruit spoilage. Yeasts by their faster growth rate, attack sugars and bring out fermentation with alcohol and CO_2 production. The yeast growth is preceded by mold growth which utilizes alcohol as energy source and later destroy the remaining parts of the fruits such as structural polysaccharides and rinds.

3.3 MICROBIAL SPOILAGE OF CEREALS AND CEREAL PRODUCTS :

The microbial flora of wheat, rye, corn and related products may be derived from soil, storage environments and from during the processing of these commodities. In proper storage conditions, by their low water activity, the cereals and related products are usually resistant to microbial attack. If the water activity favours the microbial growth, usually bacteria of the genus *Bacillus* and molds of several genera are the only ones to develop. Many aerobic spore-formers capable of producing amylase enables them to utilize the starch in flours. Mold growth occurs at less moisture condition, whereas high moisture allows the growth of bacteria and yeasts.

Cereal grains and meals are not generally processed greatly to reduce their natural microbial flora. So, they likely contain molds, yeasts and bacteria that can readily grow when enough moisture is added. The major factors involved in the spoilage of stored grains by molds include microbial content, moisture levels above 12-13%, physical damage and temperature. A wet mash of cereal grains or meals will undergo an acid fermentation by lactic acid bacteria and coliform bacteria that normally present on plant surfaces. This may be followed by an alcoholic fermentation by yeasts as soon as enough acidity is developed. Finally, molds grow on top surface. Of the numerous and different molds involve in cereal spoilage, most common ones are the species of *Aspergillus*, *Penicillium* and *Fusarium*. These genera are capable of producing mycotoxins. Grains spoiled by molds are a potential animal or human health hazard as well as a great economic loss.

A moisture content of flour of less than 13% prevent the growth of all microorganisms. Slight moistening (15%) of wheat flour brings about spoilage by molds and 17% moistening may favour the bacterial growth. The type of spoilage in a flour paste is difficult to predict as the microbial content varies with different lots of flour. If acid-forming bacteria are present, acid fermentation begins followed by alcoholic fermentation by yeasts, then acetic acid by *Acetobacter* species. This succession of changes would be more likely in freshly milled flour than in flour stored for a long period with a consequent reduction in kinds and numbers of microorganisms. In the absence of lactics and coliforms, micrococci have been found to acidify the paste. And *Bacillus* spp. may grow which produce lactic acid, gas, alcohol, acetoin and small amounts of esters and other aromatic compounds.

3.5 MICROBIAL SPOILAGE OF POULTRY AND EGGS :

Poultry spoilage occurs mostly on the surface as the inner portions of poultry tissue are generally sterile. Fresh poultry stored in humid conditions is susceptible to aerobic bacterial growth. As the poultry is chilled and held at cold storage, psychrotrophic microorganisms predominate and cause deterioration. The principal spoilage organisms include the species of *Pseudomonas*, *Aeromonas*, *Moraxella*, *Alcaligenes*, *Flavobacterium* and *Micrococcus*. Yeasts are not the important organisms on normally processed poultry. When antibiotics are used on poultry, yeasts become the important spoilage microorganisms. The main defects of poultry spoilage are off-odour and slime formation. The chemical compounds found on spoiled chicken due to microbial spoilage are H_2S, methyl mercaptan, dimethyl sulphide, acetone, toluene, ethyl acetate, heptadiene, methanol and ethanol. The presence of these compound types is influenced by the storage temperature.

Freshly laid eggs are generally sterile with an exception due to ovarian infections. Relatively short period after laying, numerous microorganisms may be found on the outside and inside of the egg. The egg contents are protected by the shell and associated membranes and chemical inhibitors in the egg albumen. The outer waxy shell membrane, the shell and inner shell membrane are individually effective to some degree in retarding the entry of microorganisms. Lysozyme, avidin, conalbumin and pH of 9.3 present in egg white contribute towards resistance of eggs for microbial spoilage. On the other hand, yolk material with 6.8 pH and nutrient contents serves as an excellent source of growth for most microorganisms.

The bacterial genera that predominate in spoilage of eggs include the species of *Pseudomonas*, *Acinetobacter*, *Proteus*, *Aeromonas*, *Alcaligenes*, *Escherichia*, *Micrococcus*, *Salmonella*, *Serratia*, *Enterobacter*, *Flavobacterium* and *Staphylococcus*. The mold genera include the members of *Mucor*, *Penicillium*, *Cladosporium, Alternaria* and *Aspergillus*. Of the yeasts, *Torula* genus is important in egg spoilage. In general more spoilage of eggs is caused by bacteria than by molds. The most common condition of bacterial spoilage of egg is referred to as `rotting'. Additionally, bacteria can also cause another condition known as `mustiness' by *Pseudomonas graveolens* and *Proteus* spp. And the species of *Pseudomonas* are the main spoilage organisms of `shell eggs' and `egg products'. Molds, though not a prominent causatives, involve in egg spoilage referred to as `pinspots'. The colour of these pinspots varies with the kind of mold species.

For the spoilage of an undamaged shell egg, the causal organism must (i) contaminate the shell, (ii) penetrate the pores of the moist shell to shell membranes, (iii) grow through shell membranes to reach white or yolk and (iv) grow in egg white to reach the yolk. The entry of microorganisms into whole eggs is favoured by high humidity. During the penetration of bacteria into eggs, the inner membrane of egg is the most important barrier followed by shell and outer membrane. Once inside the yolk, bacteria apparently grow in that nutrient medium producing by-products of protein and amino-acid metabolism such as H_2S and other foul smelling compounds. The effect of significant growth is to cause the yolk to become `runny' and `discoloured'.

The most common spoilage types in eggs include

S.No.	Type of Spoilage	Causative Organism
1.	Green rot	*Pseudomonas fluorescens*
2.	Colourless rot	Species of *Pseudomonas*, *Acinetobacter* and *Alcaligenes*
3.	Black rot	Species of *Proteus*, *Pseudomonas* and *Aeromonas*
4.	Pink rot	*Pseudomonas* spp.
5.	Red rot	*Serratia* spp.
6.	Custard rot	*Proteus vulgaris* and *Citrobacter* spp.
7.	Yellow rot	*Alcaligenes* spp.
8.	Yellow/blue/green pinspots	*Penicillium* spp.
9.	Dark green/black pinspots	*Cladosporium* spp.
10.	Pink pinspot	*Sporotrichum* spp.

3.6 MICROBIAL SPOILAGE OF MEAT :

Raw meat is subject to change by its own enzymes and by microbial action, and its fat may be oxidized chemically. In some instances, the autolytic changes may be difficult to distinguish from the microbial proteolytic actions. A great variety of kinds of organisms are involved in spoilage during slaughter, dressing and cutting that derive from exterior of the animal and its intestinal tract. More number of microorganisms are also added from knives, cloths, air, workers, carts, boxes, equipment etc.

The invasion of microorganisms into the tissues of animal on death is influenced by the factors such as (i) the load in the gut of the animal, (ii) the physiological condition of the animal immediately before slaughter, (iii) the method of killing and bleeding and (iv) the rate of cooling. Spoilage of meat is due to the growth and metabolism of large number of microorganisms on the surface or the interior. The most common indications of spoilage are 1) off-odour and slime due to the growth of aerobic bacteria on the cut surfaces of meat, 2) fungal growth favoured at water activities too low for bacterial growth, 3) bone-taint or deep spoilage due to anaerobic or facultative microorganisms and 4) discolouration due to alterations of myoglobin, the muscle pigment. The spoilage patterns of fresh and cured meat shows some differences. Fresh, chilled meat spoilage is evidenced by off-odour and slime due to *Pseudomonas*, *Acinetobacter* and *Alcaligenes*. Cured meats become sour due to the activity of *Micrococcus*, *Lactobacillus* and *Microbacterium.*

The rate of spoilage of fresh meat depends upon the numbers and types of microorganisms initially present, the storage temperature, pH and water activity of meat. The surfaces of meat cuts will support the growth of large numbers of bacteria, and ground meat offers not only ample and

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desirable surfaces, but a thorough inoculation of the meat during grinding. In the case of cured meat, the intrinsic factors such as pH, water activity and the presence of inhibitors play an important role in the microbial ecology of these products.

Some important microbial defects of meat are –

Meat Product	Defect	Organisms
Fresh Meat:		
Fresh, refrigerated (0-5°C)	Off-odour, slime, discolouration	*Pseudomonas, Aeromonas, Alcaligenes, Acinetobacter, Proteus, Flavobacterium, Saccharomyces* etc.
	Lipolysis, pungent odour	*Pseudomonas*, yeasts
	Moldy	*Penicillium*
	Black spot	*Cladosporium*
	White spot	*Sporotrichum*
	Gassy	*Clostridium perfringens*
Fresh (15-40°C)	Foul odour	*C.bifermentans, C.sporogenes*
	Acid,sweet,rancid	*Lactobacillus, Enterobacter, Microbacterium*
Vacuum packaged		
Cured Meat: Bacon	Cheesy,sour,rancid	*Micrococcus*
	Discolouration	Molds
	Slight souring	*Lactobacillus, Micrococcus, Vibrio*
	Putrefaction	*Clostridium sporogenes*
Brines	Turbid	*Debaryomyces*
Ham	Surface lime	*Micrococcus, Microbacterium*
	Gassy or puffy	*Clostridium*
	Green discolouration	*Lactobacillus, Streptococcus, Leuconostoc*
Sausages	Slime on surface	*Micrococcus*, Yeasts
	Gas production	*Lactobacillus*
Fermented sausage	Slime	Yeasts
	Spots	Molds
Canned : Semi-preserved	Souring, discolouration	*Steptococcus*
	Putrefaction, gas	*Bacillus, Clostridium*
Commercially sterile	Gas, putrefaction	*Bacillus, Clostridium*

The common types of meat spoilage can be classified on the basis of aerobic and/or anaerobic conditions.

Bacterial spoilages of meat under aerobic conditions include surface slime formation, changes in colour of meat pigments, changes in fats, phosphorescence, various surface colours due to pigmented bacteria and off-odours and off-tastes. Under aerobic conditions, yeasts may grow on the surface of meats and cause stickiness, whiskers, black spots, white spots, green patches, decomposition of fats and off-odours and off-tastes. The facultative and anaerobic bacteria are able to grow within the meat under anaerobic conditions and cause different types of spoilage like souring, taint and putrefaction.

3.7 MICROBIAL SPOILAGE OF CANNED FOODS :

The main objective of canning the foods is to destruct the microorganisms. However, the canned products also undergo microbial spoilage under certain conditions due to survival of organisms after the administration of heat treatment, leakage of the container after heat process, permitting the entry of microbes, under processing and inadequate cooling. Microorganisms that enter the cans through container leaks may be of various kinds and are not necessarily heat-resistant. The acidity of canned foods is important in determining the heat process required for sterilization and the type of spoilage to be expected, if inadequate process or leakage occurs.

Microorganisms that cause the spoilage of canned foods can be categorized as –

I) Mesophilic organisms : Putrefactive anaerobes, Butyric anaerobes, Aciduric flat sours, Lactobacilli, Yeasts and Molds.

II) Thermophilic organisms: Flat-sour spores, Thermophilic anaerobes producing sulphide and Thermophilic anaerobes not producing sulphide.

3.7.1 Under processing

In modern canning, under processing is rare. Even a mild heat treatment will kill vegetative cells of bacteria, yeasts and moulds so that spoilage of this type is caused by spore-forming anaerobes or facultative anaerobes. Low acid canned foods can be spoiled by mesophilic spore formers, eg., *Clostridium sporogenes*, a putrefactive anaerobe. The organisms produce spoilage symptoms that consist of a putrid off odour and gas (CO_2 and H_2) that cause the can to swell and eventually burst. Acid foods such as tomatoes can be spoiled by *Bacillus coagulans* that gives rise to flat sour spoilage. This type of spoilage is characterized by an increase in the acidity of the can content but no gas, so that the external appearance of the can is normal. Some fruits like pears can be spoiled by the ascomycete fungus, *Byssochlamys fulva*. Ascospores formed by the organism survive processing, germinate and the pectinolytic activity of the mycelium produced leads to maceration of the fruit. Again no gas is produced so that the can appears to be normal until opened by the consumer. *B.fulva* is relatively unusual for a mould fungus regarding its ability to grow under anaerobic conditions.

3.7.2 Survival of thermophilic bacteria

Low acid canned foods such as peas, can be spoiled by thermophilic bacteria like *Bacillus stearothermophilus* that can survive a normal heat process. The organism produces the typical flat sour spoilage with acidic can content due to lactic acid production but no gas. *B.stearothermophilus* will only grow at temperatures above 37°C, so that spoilage will only occur if cans are held above this level for long enough for spoilage to take place. This can happen if cans are stacked hot and allowed to cool naturally, so that spoilage can be prevented by rapidly cooling cans after processing. If cans are exported to countries with high ambient temperatures, storage temperatures can be sufficient to allow growth and spoilage. Under these circumstances the antibiotic nisin, that prevents spore out growth, can be added as a preservative.

3.7.3 Leaker spoilage

Leaker spoilage is by far the most common cause of canned food spoilage. Essentially what happens is that after heat processing, hot cans are cooled down and the negative pressure created inside the can sucks in cooling water through the double seam that holds the lid in place. If the water is contaminated, then organisms can enter the can and give rise to spoilage. Even good quality seams can allow the entrance of organisms through minute holes. Because contamination is post process, a wide variety of organisms can gain entrance, including those that are not heat resistant, so that the symptoms of spoilage are variable. Common symptoms are swollen cans due to gas formation and putrid odours. Microbiological examination of the can content often shows the presence of a mixed microflora characteristic of this type of spoilage. The level of contamination in the can cooling water is a critical factor in causing leaker spoilage.

Other factors that increase the number of leaker spoilages are –

- Underfilling
- Quality of the can seam
- Liquid in contact with the double seam
- Roughness of can handling
- Hygiene of the can transport system

3.8 SUMMARY

The human food supply consists basically of plants and animals or products derived from them, and it is understandable that our food supply can contain microorganisms in interaction with the food. In most cases microorganisms use our food supply as a source of nutrients for their growth and this results in deterioration of the food referred to be as food spoilage. Microorganisms can spoil a food by increasing their numbers, utilizing nutrients, producing enzymatic changes, and contributing off-flavours by means of breakdown of a product or synthesis of new compounds. When the microorganisms involved are pathogenic, their association with our food supply is critical from a public health point of view. A knowledge of the factors that favour or inhibit the growth of microorganisms is essential to an understanding of the principles of food spoilage.

A spoiled food is simply a food that is unacceptable to a consumer for reasons of smell, taste, appearance, texture or the presence of foreign bodies. The concept of a spoiled food is subjective and associated with individual taste. Personal preferences, ethnic origin and family background may play a role in an individual deciding whether a food is fit for consumption or not. The chemical and bacteriological changes associated with foods make them unacceptable to some consumers but a delicacy for others. For example, bananas that have become brown and sugary are considered overripe and therefore spoiled to many consumers but are perfectly acceptable to some. Various kinds of foods such as vegetables, fruits, cereals, cereal products, meat, poultry, eggs, etc., of human consumption can be subjected to spoilage by different microorganisms. Generally, the spoiled foods are considered as hazardous to health. However, some spoiled foods referred as fermented foods, for example yoghurt and cheese, are acceptable and safe to eat because of their more nutritious quality.

3.9 MODEL QUESTIONS

1. Write about the microbial spoilage of some important foods of human consumption.
2. Explain the causes of food spoilage of vegetables and fruits.
3. Give an account on the causes and spoilage types in meat and poultry & eggs.
4. Microbial spoilage of canned foods.
5. Microbial spoilage of cereal grains and related products.

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M.Sc. MICROBIOLOGY (Final)

Lesson No. 4

FOOD PRESERVATION

4.0 Objective
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4.2 Low Temperatures
4.2.1 Chilling
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4.0 OBJECTIVE

To understand the ways and means by which different foodstuffs are preserved from getting spoiled by the microorganisms.

4.1 INTRODUCTION

Food preservation can be defined as " the science that deals with the process of prevention of decay or spoilage of food, thus allowing it to be stored in a fit condition for future use". Most kinds of food are readily decomposed by microorganisms unless some special methods are used for their preservation to prevent the spoilage. The chief methods of food preservation are –

- Asepsis or keeping out the microorganisms
- Removal of microorganisms
- Maintenance of anaerobic conditions in a sealed and evacuated container
- Use of high temperatures
- Use of low temperatures
- Drying
- Use of chemical preservatives
- Irradiation
- Mechanical destruction of microorganisms
- Combination of two or more of the above methods

The main principles involved in accomplishing the preservation of foods by various methods are –

1) Prevention or delay of microbial decomposition by means of – (i) keeping out the microorganisms, (ii) removing the microorganisms through filtration, (iii) hindering the growth and activity of microorganisms by low temperature, drying, anaerobic conditions and chemicals, and (iv) killing the microorganisms by heat or radiation.
2) Prevention or delay of self decomposition of the food by – (i) destruction or inactivation of food enzymes through blanching and (ii) prevention and delay of purely chemical reactions.
3) Prevention of damage caused by insects, animals and mechanical means.

Many common methods of food preservation depend not on the destruction or removal of microorganisms but on delay in the initiation of growth and hindrance to growth once it has begun. Some of the preservation methods can be used only for temporary preservation and some methods can be used for permanent preservation. Of the various methods, the most widely and routinely used preservation methods are use of low temperature, high temperature, drying, irradiation and chemical preservatives.

4.2 LOW TEMPERATURES

The use of low temperatures to preserve foods is based upon the fact that the activities of food-borne microorganisms can be slowed down and / or stopped at temperatures above freezing and generally stopped at sub-freezing temperatures. This is mainly reasoned to that all metabolic reactions of microorganisms are enzyme catalyzed and rate of enzyme-catalyzed reactions dependant on temperature. As the temperature is lowered the rate of these reactions decreases. The useful life of many foods can be increased by storage at low temperatures. A drop of 10°C might stop the growth of some organisms and slow down the growth of others. A further decrease of 10°C temperature would stop the growth of more microorganisms and make still slower the growth of others.

Foods are stored for preservation at least at two distinct low temperature ranges viz., Chilling temperatures and Refrigerator temperatures.

Chilling temperatures (10-15°C) – between the usual refrigerator temperatures and room temperatures and very much suitable for storage of certain vegetables and fruits such as cucumbers, potatoes, limes and so on.

Refrigerator temperatures (0°-7°C) – suitable for the storage of a large number of perishable and semi-perishable foods.

4.2.1 Chilling or Cold storage :

Chilling storage of foods is carried at temperatures not far above freezing, and usually involves cooling by ice or by mechanical refrigeration. This may be used as the main preservative method for foods or for temporary preservation until some other preservative process is applied. Several factors like temperature, RH, ventilation, composition of storage atmosphere and irradiation are of considerable concern in chilling storage. Most perishable foods that include eggs, dairy products, meats, seafood, vegetables and fruits may be held in chilling storage for a limited time with little change from their original condition. During this storage, enzymatic and microbial changes in the foods may not be prevented totally but considerably slowed down.

Chilling storage brings out qualitative changes in spoilage characteristics, as low temperature exert a selective effect preventing the growth of mesophiles and leading to a microflora dominated by psychrotrophs. But the growth of these psychrotrophs in chilled foods occur relatively slowly, so that the onset of spoilage is delayed. Chilling storage will produce a phenomenon known as `Cold shock' which causes death and injury in a proportion of the population. The extent of this cold shock depends on several factors like type of organism, growth phase of organism, temperature differential and the rate of cooling, and the type of growth medium.

The principal mechanism of cold shock appears the damage membranes by causing phase changes in membrane lipids. These changes create hydrophilic pores through which cytoplasmic contents can leak out. Mesophiles that survive cooling, albeit in an injured state, can persist in the food for extended periods and may recover and resume growth when conditions become favourable. Thus chilling will prevent an increase in risk from mesophilic pathogens but will not assume its elimination. Some foods are not amenable to chill storage as they suffer from cold injury that results in tissue breakdown leading to visual defects and accelerated microbial deterioration.

4.2.2 Freezing:

Freezing is the most successful technique for long-term preservation of foods since the nutrient content of foods is largely retained and the product resembles the fresh material more closely than in appertized foods. With the development of mechanical refrigeration and the quick-freezing processes and also because of availability of home deep freezers, the frozen food industry has rapidly expanded. The foods, prior to actual freezing, should be prepared suitable by selecting, sorting, washing, blanching and packaging the material. Foods in any state of detectable spoilage should be rejected for freezing and they should be as fresh as possible. Under the normal conditions of storage of frozen foods, the microbial growth is prevented entirely and the action of food enzymes is greatly retarded. The lower the storage temperature the slower will be any chemical or enzymatic reactions,

but most of them will continue slowly at any temperature. Therefore, it is common practice to blanch the vegetables before freezing, to inactivate their enzymes to retain the quality of food. This blanching can be done by brief immersion of foods into hot water or using the steam. The extent of blanching will vary with the type of the food.

There are two basic ways to achieve the freezing of foods namely *Quick freezing* and *Slow freezing*. The rate of freezing of foods depends upon a number of factors such as the method employed, the temperature, circulation of air or refrigerant, size and shape of package, kind of food etc.

Quick or fast freezing is a process by which the temperature of foods is lowered to about –20°C within 30 minutes or less time. This can be accomplished by one of the three general methods –

1. by direct immersion of the food or packaged food in a refrigerant. The refrigerants used should be non-toxic, pure and free from taste, odour, colour or bleaching action.

2. by indirect contact with the refrigerant, where the food or package is in contact with the passage through with the refrigerant at –17.8 to – 45.6°C flows.

3. by air-blast freezing, where frigid air at –17.8 to –34.4°C is blown across the materials to be frozen.

Slow freezing is a process whereby the desired temperature is achieved within the time of 3 to 72 hours. The temperature employed is usually –23.3°C or lower but sometimes vary from –15 to –29°C. Freezing is carried out with natural air or at best with electrical fans.

The quick freezing method claims several advantages over the slow freezing method and some of the important differences between these two methods are –

Quick freezing	**Slow freezing**
Small ice crystals are formed	Large ice crystals are formed
Metabolism is blocked or suppressed	Breakdown of metabolic rapport
Brief exposure to adverse factors	Longer exposure to adverse factors
No adaptation to low temperature	Gradual adaptation to low temperature
Thermal shock effect is present	No thermal shock effect
No protective effect	Accumulation of solutes with beneficial effects
Microorganisms frozen in crystals	
Avoid internal metabolic imbalance	

Effects of freezing upon microorganisms include –

- A sudden mortality immediately on freezing which may vary with species
- The proportion of cells surviving immediately after freezing is nearly independent of the rate of freezing

- The cells that are still viable immediately after freezing die gradually when stored in the frozen state
- The decline in numbers of microorganisms is relatively rapid at temperature just below the freezing point, especially about –2°C, but less so at lower temperature usually slow below –20°C.

4.3 HIGH TEMPERATURES:

Preservation of food by the use of heat finds very wide applications when compared to other methods. The use of high temperatures to preserve food is based on their destructive effects on microorganisms. The killing of microorganisms by heat is supposed to be caused by the denaturation of the proteins and especially by the inactivation of enzymes required for metabolism. The heat treatment necessary to kill organisms or their spores varies with the kind of organism, its state, and the environment during heating. Depending on the heat treatment employed, only some of the vegetative cells, most or all of the cells, part of the bacterial spores, or all of them may be killed. The heat treatment selected will depend on the kinds of organisms to be killed, other preservative methods to be employed, and the effect of heat on the food. In connection to food preservation, there are two temperature categories in common use namely Pasteurization and Sterilization.

4.3.1 Pasteurization

It is comparatively a low order heat treatment involving the heat processing at a temperature below the boiling point of water and normally within the range 60-80°C. The two primary objectives are (i) destruction of majority but not all pathogenic microorganisms in liquids like milk and liquid egg, and (ii) extending the product shelf-life from microbial and enzymatic point of view. This second objective finds more relevance in pasteurization of beer, wine and fruit juices.

Pasteurization may be carried out in batch mode or continuous mode. The continuous pasteurization of bulk foods is carried out by passing them through plate heat exchangers in four stages viz., preheating, heating, holding and cooling. Pasteurization of milk, bulk liquid egg and ice cream mixes are used primarily as mechanisms for conferring safety on products. The choice of temperature and time of pasteurization depends on food type. Pasteurization of bulk liquid egg is carried out at 64.4°C temperature for not less than 2.5 minutes. Pasteurization of ice cream involves the heating to 65.6°C for at least 30 minutes or at 71.1°C fro 10 minutes or at 79.4°C for at least 15 seconds.

Most popularity for pasteurization was gained with respect to milk heating process. The milk pasteurization can be achieved at different temperatures for different time periods as

- 145°F or 63°C for 30 minutes (low temperature long time –LTLT method)
- 161°F or 72°C for 15 seconds (high temperature short time –HTST)
- 191°F or 89°C for 1.0 second
- 194°F or 90°C for 0.5 second
- 201°F or 94°C for 0.1 second
- 212°F or 100°C for 0.01 second

All these treatments are equivalent and are sufficient to destroy the most heat-resistant of the non-spore forming pathogenic organisms like *Mycobacterium tuberculosis*, *Coxiella burnetti* etc. Milk pasteurization temperatures are sufficient to destroy all yeasts, molds, Gram negative bacteria and many Gram positive bacteria. The two groups of organisms that survive milk pasteurization are thermodurics which can withstand and survive at relatively high temperatures but not require the high temperatures (*Streptococcus* spp. and *Lactobacillus* spp.), and thermophiles that can survive at high temperatures and also require high temperatures (*Bacillus* spp. and *Clostridium* spp.). The efficiency of milk pasteurization or the percentage of reduction of numbers of microorganisms in milk during pasteurization depends on (1) the temperature of pasteurization, (2) the holding time, (3) the total numbers of bacteria, and (4) the proportion of the total microbial load that are spore-formers or thermoduric organisms.

4.3.2 Sterilization

The main means of sterilization is the destruction of all viable microorganisms in a material. Because of the resistance of certain bacterial spores to heat, a treatment at 121°C (250°F) of wet heat for 15 minutes or its equivalent is necessary for sterilization. During sterilization, every particle of the food must receive the heat treatment. In the case of canned foods, sterilization of foods may require several hours depending upon the size of the can and type of food. A variety of foods including whole or sliced vegetables and fruits, meat and meat products, fish and fish products and soups are canned. Two general methods of sterilization are commonly used in this canning industry viz., in-pack sterilization (sterilization inside containers) and sterilization of the food before placing in the containers.

The in-pack sterilization involves several steps like

- Cleaning and grading the raw materials of food
- Blanching to inactivate native enzymes particularly in vegetables and fruits
- Filling or placing the cleaned raw food in a sealable container, with added brine in the case of vegetables, meat and fish or sugar syrup in the case of fruits
- Deaeration to prevent bulging of can during heating followed by closing and sealing the container
- Heating the container in a retort at specified temperature for a specific holding time
- Cooling partially under pressure in the retort followed by cooling in a cooling tank
- Labeling, storing and / or marketing

The sterilization process may be operated in batch or continuous made involving vertical or horizontal retorts, pressure cooker, rotary or reel cooker or hydrostat cooker or non-pressurized methods. All these procedures use temperatures above 100°C and hence the pressure developed is greater than atmospheric pressure.

Aseptic canning process involves – (1) sterilizing the food separately outside the containers, (2) placing the food in previously sterilized cans under aseptic conditions and (3) sealing the containers. The advantages in this method include :

- high temperature processing at temperatures up to 150°C using high speed heat exchangers cutting down the processing time appreciably,
- a better product quality as deterioration due to over heating is prevented. The food is passed continuously through a plate or tubular heat exchanger where it is brought to sterilizing temperature almost instantaneously. The holding period is about 1-2 seconds at this temperature and the technique is known as Ultra-high temperature (UHT) sterilization. The sterilized food is then quickly cooled by another heat exchanger and enters the aseptic canning line, which consists of a tunnel maintained under sterile conditions through which sterilized cans are conveyed, filled and sealed.

Hot packing or hot filling technique involves the filling of previously sterilized food, while still hot, into clean but not necessarily sterile containers under clean but not necessarily aseptic conditions. The heat of the food and the holding time before cooling ensures commercial sterility of the filled product. This hot packing technique is suitable for acid foods. Most fruit juices such as orange, grape, grapefruit, tomato and acid fruits and vegetables and pickled vegetables such as sauerkraut are hot packed or hot filled. For example, fruit juices are heated in the range of 77-100°C for 30-60 seconds, hot filled at about 90°C and held at this temperature for about 3 minutes with an inversion of the can before cooling.

4.4 DRYING :

Preservation of foods by drying is the oldest method and has been practiced for centuries. Drying refers to the removal of relatively small amounts of water from a material as vapour. Some foods like grains are sufficiently dry when harvested and may remain unspoiled under proper storage conditions. But most of the foods contain enough moisture to permit the activity of native enzymes and microorganisms for spoilage, and so drying is necessary to reduce the water activity and prevent microbial spoilage. Drying is usually accomplished by the removal of water, but any method that reduces the amount of available moisture or lowers the water activity in a food is a form of drying.

The primary aims or purpose of drying of foodstuffs are – (1) preservation of foods, (2) decreasing the weight and bulk of the food to economize shipping and canning costs and (3) production of convenience items such as instant coffee and instant mashed potatoes. The drying characteristics of foods are influenced by various factors such as drying temperature, the relative humidity of air, velocity of air and drying time. Both the drying temperature and drying time vary with type of food and method of drying. During the process of drying, several changes occur in foods affecting the texture, size and appearance. Foods are dried by different methods namely Sun drying, Drying by mechanical driers and Freeze-drying.

4.4.1 Sun-drying

Sun-dried food has had moisture removed by exposure to the sun rays without any artificially produced heat and without controlled temperature, RH or air velocities. Sun-drying in direct sunlight or diffused light (shade drying) is one of the earliest methods of food preservation and is still used for the production of dried fruits, and also for drying nuts. Sun-drying of fruits and vegetables is widely practiced in tropical and sub-tropical regions where there is plenty of sun-shine. Sun-dried fruit can be produced only in climatic areas with relatively high temperature, low humidity and freedom from rainfall during the drying season. Sun-drying is limited to certain fruits such as raisins, prunes, figs, apricots, dates, pears, peaches, nectarines and salted fish. These fruits or fish are spread out on trays and are turned on their sides during drying. A combination of sun-drying and shade-drying is customarily used order to obtain dried fruits of proper texture and colour.

4.4.2 Drying by mechanical driers

This sort of drying includes the methods of drying by heated air or by direct contact with a heated surface or by the application of energy from a radiating microwave or dielectric source. Most methods of artificial drying involve the passage of heated air with controlled RH over the food to be dried or the passage of the food through such air.

Hot air driers – In the hot air driers, the food is in contact with a moving stream of hot air. Heat is supplied to the product mainly by convection. For drying the solid foods, different types of driers like Kiln driers, Cabinet tray or Compartment driers, Pneumatic driers, Tunnel driers, Conveyor driers, Bin driers, Fluidized bed driers, Rotary driers and Spray driers are employed using hot air. Liquid foods, such as milk, juices ans soups may be evaporated by the use of comparatively low temperatures and low pressures in a vacuum pan. Liquid foods may also be drum dried by their passage over a heated drum with or without vacuum, or spray dried by spraying liquids into a current of dry, hot air.

Drying by contact with heated surface – In direct contact drying, the food is brought into contact with a heated surface and heat is supplied to the product mainly by conduction. Examples include drum driers, roller driers and vacuum band driers. In such systems the necessary sensible and latent heat of evaporation is supplied to the material by conduction. In order to achieve reasonable drying times and to dry to a low moisture content, the heated surface temperature needs to be appreciably higher than 100oC and therefore, as drying nears completion the material temperature rises quite high. Thus the danger of heat damage to sensitive food materials is comparatively high. To reduce this hazard, drying by contact is often carried out under reduced pressure so that lower surface as well as material temperature may be employed.

Drying by microwaves or dielectric source – Microwave and dielectric sources may be used for supplying heat by radiation to the food for drying purposes. Infrared radiant driers have been used for drying breadcrumbs, starches, custard powder, cake mixes, tea, almond and spices. Dielectric heating has been used for drying of biscuits.

4.4.3 Freeze Drying

This mode of drying is also referred to as Sublimation-drying or Lyophilization. Foodstuffs, pharmaceuticals and biological materials which are heat sensitive even to low or moderate temperatures are freeze-dried. Freeze drying produces the highest quality food product with no loss of flavour and aroma and minimizes thermally induced degradation reactions. The method involves freezing of the material by exposure to cold air followed by sublimation of ice in vacuum from the frozen state to produce a dried product. Freezing leaves a mass with high structural rigidity, preventing its collapse during sublimation of the ice and when water is added later, the rehydrated product retains almost the original structural form. However, the method is costly due to the low rate of drying and the necessity of high vacuum equipment to maintain low pressures.

In most of the conventional freeze-drying systems, the vapour pressure gradient, necessary for sublimation, is attained by maintaining the total pressure in the drying chamber of the order of 0.1 – 2.0 torr. A condensing system is provided to remove water vapour formed and a heating system to supply the necessary latent heat of sublimation to the frozen material. In practice, it is not feasible to freeze all the liquid present in the food. The optimum rate of freezing depends largely on the material and the rate is usually experimentally determined. Material such as fruit juice concentrate is first frozen on trays in the lower chamber of a freeze drier and the frozen material is dried in the upper chamber under high vacuum. The material dries directly by sublimation of ice without passing through the intermediate liquid stage.

Applications of this freeze-drying in food industry include freeze-drying of coffee, tea, meat and fish and some vegetable and fruit products. The main advantages of this method are the retention of volatile odour and flavour compounds to relatively high extent and the high rate and extent of reconstitution of characteristics. However, the disadvantages include that freezing damages the cell structure and the product becomes brittle. The high capital and operating costs involved in freeze-drying and stringent packaging requirements of the products make it very expensive.

4.5 RADIATION :

Radiation may be defined as the emission and propagation of energy through space or a medium. Radiation of wavelengths less than 200nm, such as X-rays, gamma rays, alpha particles, beta particles and cosmic rays are called ionizing rays and are of importance in food preservation. These radiations have sufficient energy to ionize molecules in their path. They destroy microorganisms in foodstuffs without raising the temperature and hence the irradiation process is termed as Cold sterilization.

Ionizing radiations penetrate the food to varying degrees depending on the nature of the food and the characteristics of radiations. Gamma radiations have greater penetrating power than beta particles but the latter have greater ability to ionize molecules. When ionizing radiations pass through foods, breaking of chemical bonds occur resulting in the formation of ion pairs and free radicals. The reactions of these species with other molecules provide the mechanism of alteration of food constituents, enzymes and microorganisms. Radiations produce several undesirable changes of colour, odour, taste and physical properties in foods. In most foods the levels of vitamins such as

thiamine, pyridoxine, B_{12}, C, D, E and K are decreased while those of riboflavin and niacin are not affected.

Ionizing radiations destroy all types of microorganisms. Gram negative bacteria are less resistant to radiation than gram positive bacteria and spore formers are more resistant than non-spore formers. The bactericidal efficiency of a given dose of irradiation depends on several factors such as (1) the kind and species of organism, (2) the number of microorganisms or spores initially present in the food, (3) the composition of food, (4) oxygen availability and (5) age of the microorganism.

Prior to the irradiation, foods must be properly processed. Foods for irradiation must be selected on the basis of their freshness and overall desirable quality. Foods must be cleaned to make them free from debris and dirt. Finally, they should be packaged in proper containers such as cans. Glass containers cannot be used because they undergo colour changes on irradiation. Sterilizing doses of irradiation are insufficient to destroy the natural enzymes of foods. Hence to avoid post irradiation enzymatic changes blanching of vegetables and mild heat treatment of meat are necessary prior to irradiation.

Gamma radiation from radioisotopes of cobalt-60 or cesium-137 and electron beams from linear accelerators are commonly used for irradiation of foods. The radiation dosage depends on the nature of the food, the resistance of microorganisms and the resistance of enzymes apart from the cost of the process. In general, the radiation treatment is given to foods in any one of the three types namely radappertization, radicidation and radurization.

4.5.1 Radappertization

This is equivalent to radiation sterilization and the typical levels of radiation used are 30-40 kGy. Radappertization can be achieved by application of the proper dose of radiation under proper conditions. In this, a safe shelf-stable product is produced. In general, the radiation resistance of microorganisms increase at lower temperature and decrease at higher temperature. The minimum radiation doses (MRD) differ among food products. Viruses are considerably more resistant to radiation than bacteria. Enzymes are also highly resistant to radiation, and a dose of from 2-6 Mrad destroy only up to 75% of the proteolytic activity of ground beef. However, when blanching at 65-70°C was combined with radiation doses of 4.5-5.2 at least 95% of beef proteolytic activity was destroyed.

4.5.2 Radicidation

This is equivalent to pasteurization of milk i.e. radiation pasteurization. Radicidation refers to the reduction of the number of viable non-spore forming pathogens other than viruses. The method is so significant in removal of salmonellae from meat and poultry. The typical levels of dose normally used is 2.5-10 kGy. A radiation dosage up to 7 kGy (0.7 Mrad) has been approved by WHO as being ‘unconditionally safe for human consumption’. Fresh poultry, cod and red fish, spices and condiments have been approved for radicidation in some countries.

4.5.3 Radurization

It is also considered as equivalent to pasteurization. It enhances the keeping quality of foods by reducing the number of spoilage microbes. The common dosage levels are in the range of 0.75-2.5 kGy for fresh meats, poultry, seafood, fruits, vegetables and cereal grains. The shelf-life of shrimp, crab, haddock, clams may be extended from 2-6 folds by radurization with doses from 100,000-400,000 rads. The radurization at doses of 2-3 kGy can extend the shelf-life of various fruits to at least 14 days.

4.6 CHEMICAL PRESERVATIVES :

The chemical agents added to foods or produced by the activities of microorganisms that extend the shelf-life of a food by killing or inhibiting spoilage organisms and / or killing or inhibiting the growth of pathogens are referred to as chemical preservatives. The preservatives are defined in the UK Food Regulations (1989) as substances that are capable of inhibiting, retarding or arresting the growth of microorganisms or any deterioration relating to the presence of microorganisms. Preservatives may be microbicidal and kill the target organisms or they may be microbistatic in which case they simply prevent them growing. This is very often a dose-dependent feature; higher levels of an antimicrobial proving lethal while the lower concentrations that are generally permitted in foods tend to be microbistatic. For this reason chemical preservatives are useful only in controlling low levels of contamination and are not a substitute for food hygiene practices. Some of the chemicals used as preservatives are –

- **Organic acids and their esters**: citric acid, tartaric acid, malic acid, lactic acid, acetic acid, propionic acid, sorbic acid, benzoic acid, para-hydroxybenzoate.
- **Mineral acids**: phosphoric acid.
- **Inorganic anions**: sulphite, nitrite
- **Carbon dioxide**
- **Sodium chloride**
- **Antibiotics**: nisin, pimaricin or natamycin
- **Smoke**

The most important and routinely used chemical preservatives are –

4.6.1 Benzoic acid

Occurs naturally in cherry bark, cranberries, tea etc., but prepared synthetically for food use. It is relatively a strong acid and so its microbial activity principally lies in undissociated form. The undissociated molecule of benzoate interfere with membrane energetics and inhibit the amino acid uptake. It is so effective only in acid foods. Benzoic acid and its salts are the most effective agents against yeast, bacteria in foods which are acidic in nature. Sodium salt of benzoic acid is extensively used in foods as antimicrobial agent. At the levels of 0.05-0.1%, benzoic acid is used in jams, jellies, carbonated beverages, fruit salads, fruit drinks, pickles etc.

4.6.2 Sorbic acid

It is an unsaturated fatty acid namely 2,4-hexadienoic acid. Sorbic acid is used as a direct antimicrobial additive in foods in the form of calcium or sodium or potassium salts. It is active against yeasts, molds, catalase positive bacteria and effective at low pH values with a maximal level of use at about pH 6.5. At a pH of above 4.0, sorbic acid is effective than sodium benzoate. Most importantly, sorbic acid targets the plasma membrane and also inhibits a number of key enzymes of intermediary metabolism. Used as a preservative in cheeses, cheese products, baked foods, beverages, syrups, fruit juices, jellies, jams, fruit cocktails, dried fruits and pickles.

4.6.3 Sulphur dioxide & sulphites

Application is known wayback from Egyptians and Romans. In aqueous solutions, sulphur dioxide and various sulphites including sodium sulphite, potassium sulphite, sodium bisulphate, potassium bisulphate, sodium metabisulphite and potassium metabisulphite form sulphurous acid which is the active antimicrobial compound. Reduction of disulphide linkages, formation of carbonyl compounds, reaction with ketone groups and inhibition of respiratory mechanisms are the important mechanisms of action of SO_2 and sulphites. In addition to their antimicrobial action, sulphites are also used to prevent enzymatic and non-enzymatic changes or discolouration in some foods. Various sulphites are used in the wine industry to sanitize equipment and to reduce the normal flora of the grape must. SO_2 is used in syrups and fruit juices. Fumes of burning sulphur are used to treat most light-coloured dehydrated fruits. Dehydrated vegetables are exposed to a spray of neutral bisulphites and sulphites before drying. In some countries, meats and fish are treated with sulphites. At present, sulphite is permitted as a preservative in all foods which are not important as a source of Vit B_1.

4.6.4 Nitrites & Nitrates

The antibacterial action of nitrite was first described in 1920s and employed unknowingly in the production of cured meat where it is also responsible for their characteristic colour and flavour. Nitrites can react with secondary and tertiary amines to form nitrosamines which are carcinogenic. Nitrites decompose to nitric acid, which forms nitrosomyoglobin when its reacts with the heme pigments in meats and thereby forms a stable red colour. Currently nitrites are added to foods in the form of sodium nitrite, potassium nitrite, sodium nitrate or potassium nitrate. The nitrates added act probably as a reservoirs for nitrite. Nitrites are inhibitory to a range of bacteria and inhibit *Clostridium botulinum* in meat products, particularly in bacon and canned or processed hams. The bacterial inhibitory activity of nitrites increases with decrease in pH value. At a pH of 6.0, nitrite at a concentration of 200mg /kg is sufficient to inhibit *Escherichia*, *Flavobacterium*, *Micrococcus* and *Pseudomonas*. However, *Salmonella* and *Lactobacillus* are found to be resistant to nitrites.

4.6.5 Acetic acid

The derivatives of acetic acid viz., monochloroacetic acid, peracetic acid, dehydroacetic acid and sodium diacetate are recommended as preservatives. Acetic acid in the form of vinegar used in pickles and pickled sausages. Acetic acid is more effective against yeasts and bacteria than molds. Its

effectiveness increases with decrease in pH level. Dehydroacetic acid has been used to impregnate wrappers for cheese to inhibit growth of molds and as a temporary preservative for squash. Sodium diacetate used in cheese spreads, malt syrups and also to treat the wrappers used on butter.

4.6.6 Antibiotics

Antibiotics are the secondary metabolites produced by microorganisms that inhibit or kill a wide spectrum of other microorganisms. Of a great number of antibiotics produced by molds and bacteria, only a few antibiotics are permitted for use as food preservatives. Two antibiotics (nisin and natamycin) are approved for use in food in a large number of countries and three others (tetracyclines, subtilin and tylosin) have been studied and found effective for various food applications. Three antibiotics namely subtilin, tylosin and nisin have been investigated extensively as heat adjuncts for canned foods. Chlortetracycline and oxytetracycline are widely studied for their application to fresh foods, while natamycin id employed as a food fungistat. The antibiotic substance used as a chemical preservative should

- kill, not inhibit, the flora and should ideally decompose into innocuous products or be destroyed on cooking for products that require cooking.
- not be inactivated by food components or products of microbial metabolism.
- not readily stimulate the appearance of resistant strains.
- not be used in foods if used therapeutically or as an animal feed additive.

Nisin – the most important antibiotic used as a food preservative and permitted under UK legislation is nisin, a peptide antibiotic produced by *Lactococcus lactis.* Structurally, nisin relates to subtilin but does not contain tryptophane residues like in subtilin. As a food preservative, nisin posses some desirable properties like

- non-toxic
- produced naturally
- heat stable and has excellent storage stability
- destroyed by digestive enzymes
- does not contribute to off-flavours and off-odours
- has a narrow spectrum of antimicrobial activity

Nisin is effective against gram-positive bacteria, primarily spore formers, and ineffective against gram-negative bacteria and fungi. Popularly used to suppress anaerobes like clostridia in cheese and cheese products.

Natamycin – this antibiotic is also known as pimaricin or tennecetin or myprozine. It is a polyene isolated from *Streptomyces natalensis* and is effective against yeasts and molds but not bacteria. It has limited EU approval for use in the treatment of cheese rind and the surfaces of dried sausages to prevent mould growth. Natamycin appears to act in the same manner as other polyene antibiotics i.e. inducing distortion of selective membrane permeability by binding to membrane sterols. It inhibits

the protein synthesis in fungal cells but has no activity against bacteria as the sterols are absent in bacteria.

Tetracyclines – Chlortetracycline and Oxytetracycline were approved by the Food & Drug Administration in 1955 and 1956, respectively, at a level of 7 ppm to control bacterial spoilage in uncooked refrigerated poultry. This treatment double the storage life of the poultry. Tetracyclines at 5 ppm is permissible only on fresh fish, shucked scallops and unpeeled shrimp. The combination of chlortetracycline with sorbate was found effective in delaying the spoilage of fish up to 14 days. The tetracyclines are both heat sensitive and storage labile in foods, and these factors are important in their acceptance for food use. However, the risks associated with their use as food preservatives in developed countries seem clearly to outweigh the benefits.

Subtilin – like nisin, subtilin is effective against gram-positive bacteria, is stable to acid, and possesses enough heat resistance to withstand destruction at 121°C for 30 to 60 minutes. Subtilin is effective in canned foods at levels of 5 to 20 ppm in preventing the outgrowth of germinating endospores , and its site of action is the same as for nisin.

Tylosin – this antibiotic is a non-polyene macrolide. It is more inhibitory than nisin or subtilin. Being a macrolide, tylosin is most effective against gram-positive bacteria. It inhibits protein synthesis by associating with the 50S ribosomal subunit and shows at least partial cross-resistance with erythromycin.

4.7 SUMMARY

Several microorganisms of different microbial groups are known to associate and cause spoilage of various foods that are consumed by human beings. They bring out various changes in foods and make the food stuffs unfit for consumption. In order to over come the spoilage problem, a number of methods are in practice to preserve the foods from spoilage. These preservation methods may employ physical agents or chemical agents. Irrespective of type, all the preservation methods more or less function with basic principles of inhibiting the activity of microorganisms, delaying the onset of growth of microorganisms in foods, inhibiting the activity of endogenous enzymes etc., that may alter the quality of foods in terms of colour, taste, odour etc. The most common preservation methods that are in regular practice include using - low temperature, high temperature, drying, irradiation and chemical preservatives. By their mode of actions, the preservation methods may create conditions in foods which are inhibitory to the growth of microorganisms or directly may interact with different components of the microorganisms that render them killed. Though each and individual method is having its own potentiality, for hundred percent safety, combination of two or more methods is advisable.

4.8 MODEL QUESTIONS

1. Discuss the different types of food preservation methods.

2. What are the basic principles of food preservation? Write about the use of temperatures as preservation methods.

3. Describe the drying and irradiation methods of food preservation.

4. Give an account on chemical preservatives.

5. Short answers:-

   a) Sun-drying b) Freeze-drying c) Chilling d) Radappertization
   e) Nitrites and nitrates as preservatives

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M.Sc. MICROBIOLOGY (Final)

Lesson No. 5

DAIRY MICROBIOLOGY

5.0 Objective
5.1 Introduction
5.2 Microflora of milk
5.3 Pasteurization of milk
5.4 Quality testing of milk
5.4.1 Methylene blue reduction test
5.4.2 Resazurin test
5.4.3 Phosphatase test
5.5 Microbial spoilage of milk
5.6 Summary
5.7 Model questions
5.8 References

5.0 OBJECTIVE

This lesson plan is intended to analyse the reader about the chances of microbial contamination and the detection of quality of milk, which is the most common and important food in every body's diet.

5.1 INTRODUCTION

Milk is the fluid, excluding colostrum, secreted by mammary glands of female mammals for the nourishment of their young. It contains almost all the nutrients necessary to sustain life. In the market, the term milk is now used to refer to cow's milk exclusively while those of others are specifically named such as goat milk or sheep milk. Milk is a white or yellowish white, opaque emulsion of fat globules dispersed in an aqueous solution of proteins, sugar and minerals. The specific density of cow's milk ranges from 1.029 to 1.039. The specific density of milk decreases with increasing fat content and increases with increasing amounts of proteins, sugar and salts. The pH of fresh milk is in the range of 6.5 to 6.75.

The principal constituents of milk including fat, protein (primarily casein), milk sugar or lactose and the minerals of milk are found in varying proportions in the milk of different animals. The high degree of variability of composition of milk from different species is important in processing operations. Milk obtained from cows and buffaloes are the major sources for dairy operations. The total solids in the milk minus the fat content is generally referred to as solids-not-fat or milk-solids-not-fat or serum solids and the minerals in milk are collectively referred to as ash. The general composition of milk for different species is given in table 5.1.

Constituents	Cow	Buffalo	Goat	Human
Water	87.1	82.1	83.5	87.0
Fat	3.9	7.4	6.0	3.7
Protein	3.3	4.8	4.0	2.1
Lactose	5.0	4.8	6.0	7.0
Ash	0.7	0.8	0.5	0.2

Table 5.1 – Composition of Milk from Different Species

Fresh milk is the starting point for a number of other food products and some of them are shown in figure 5.1 given below.

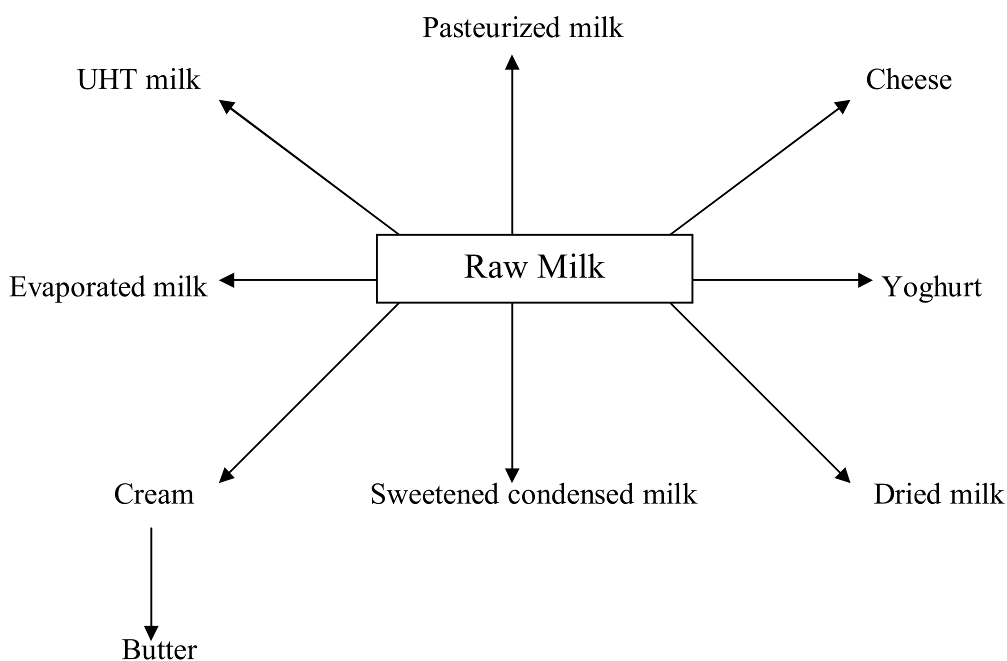

Figure-5.1 : Milk and milk products

5.2 MICROFLORA OF MILK

Milk secreted by a healthy udder is sterile but yet a large number of species of microorganisms have been identified as contaminants of milk drawn from healthy cows. The udder canal and teat surface have their own microflora so that milk drawn aseptically from the cow is not sterile but contains micrococci, streptococci and corynebacteria. During the normal milking operation, however, milk is subject to contamination from the animal, especially the exterior of the udder and adjacent areas. Bacteria found in manure soil and water may enter from this source. Such contamination is reduced by clipping the cow, especially the flanks and udder, grooming the cow, and washing the udder with water or a germicidal solution before milking. Contamination of the cow with soil, water and manure

is reduced by paving and draining barnyards, keeping cows from stagnant pools, and cleaning manure from the barns or milking parlours.

The two most probable and significant sources of contamination of milk are dairy utensils and milk-contact surfaces, including the milk pail or milking machines, as the case may be, strainers, milk cans or pipelines, and the bulk-milk cooler. If dairy utensisls or the milk-contact surfaces are inadequately cleaned, sanitized, and dried, bacterial may develop in large numbers in the dilute milk-like residue and enter the next milk to touch these surfaces. The milk contaminants from all these sources include

- Gram-negative rods – *Pseudomonas, Alcaligenes, Acinetobacter* and *Flavobacterium*;
- Enterobacteriaceae;
- Gram-positive spore forming rods – *Bacillus* and *Clostridium*;
- Lactic acid bacteria – *Streptococcus*, *Lactococcus*, *Lactobacillus* and *Leuconostoc*;
- Thermodurics that survive pasteurization – micrococci, enterococci, bacilli and brevibacteria
- Yeasts and molds.

The organisms most commonly isolated are micrococci, streptococci and the diptheroid *Corynebacterium bovis*. Counts are frequently higher though due to mastitis, an inflammatory disease of the mammary tissue, which is a major cause of economic loss in the dairy industry. Many organisms can cause mastitis, the most important being *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus agalactiae*, *Streptococcus uberis*, *Pseudomonas aeruginosa* and *Corynebacterium pyogenes*. The first three of these are all potential human pathogens and a number of other human pathogens such as Salmonella, *Listeria monocytogenes*, *Mycobacterium bovis* and *Mycobacterium tuberculosis* are also occasionally reported. Other possible sources of contamination are the hands and arms of the milker or dairy workers, the air of the barn or milking parlor, and flies. Normally these sources would contribute very few bacteria, but they might be a source of pathogens or spoilage microorganisms. The quality of farm water supply used in the milking parlor for cleaning, rinsing, etc., will have some effect on the quality of the milk.

The relative numbers of each type and levels of contamination depends on the type of animal feed used, whether animals are housed indoors, and the method and conditions of milking. In modern milking systems in which the milk is fully enclosed the main sources of contamination are:

- Udders soiled with animal faeces and soil
- Inadequately sanitized teat clusters
- Teat clusters dropped before use
- Inadequately cleaned and sanitized milking equipment.

Milk produced under conditions of good hygiene will contain numbers as low as 10^3/ml but under poor conditions, numbers may be as high as 10^5/ml or even greater. The exterior of the cow contributes comparatively few organisms if precautions are taken and a milking machine is used, but under very poor conditions thousands per milliliter could enter the milk.

The high water activity, moderate pH (6.4-6.6) and ample supply of nutrients make milk an excellent medium for microbial growth. This demands high standards of hygiene in tis production and processing. Milk possess a number of antimicrobial features present either to protect the udder from infection or to protect the newborn calf. Generally, these are present at too low a concentration in cow's milk to have a very marked effect on its keeping quality or safety. In some cases the antimicrobial activity is antagonized by other milk constituents such as the effect of citrate and bicarbonate on lactoferrin activity. There are some recommended measures to minimize the milk contamination from the udder exterior which include –

- Providing enough clean bedding and replacing it as necessary
- Removing slurry (faeces and urine) from concrete areas at least twice daily
- Preventing muddy areas wherever possible
- Shaving udders and trimming tails
- Washing teats with warm water containing disinfectant and drying individually with paper towels
- Keeping the milking parlour floor clean during milking
- Thoroughly cleaning teat cups if they fall off during milking and discarding fore milk.

Although such procedures certainly improve the microbiological quality of milk, economic constraints such as increasing size of individual dairy herds and decreased manning levels in milking parlours encouraged their neglect.

5.3 PASTEURIZATION OF MILK

Originally the main health concerns associated with milk were tuberculosis caused by *Mycobacterium bovis* and *Mycobacterium tuberculosis* and brucellosis cause by *Brucella* spp. In some parts of the world milk is still a significant source of these infections but in the UK and some other countries they have now been effectively eliminated from the national dairy herd by a programme of regular testing and culling of infected animals. Such programmes must be constantly maintained to be effective and there have been occasional problems. Enteric pathogens such as *Salmonella* and *Campylobacter* are still, however, prevalent in raw milk and pasteurization remains the most effective measure for their control.

Pasteurization is a comparatively low order of heat treatment or mild treatment done at temperatures below the boiling point of water and normally within the range between $60 - 80^{o}C$. Historically, milk first received heat treatments to increase its shelf life and since then it became a necessary safeguard against food-borne illness caused by milk. The objectives of milk pasteurization are to (1) kill all the pathogens that may enter the milk and be transmitted to people and (2) improve the keeping quality of milk. Milk pasteurization will remove *Mycobacterium tuberculosis* and other less heat resistant pathogens, including *Salmonella* and *Shigella*. The secondary effect of the process is improving storage life at chill temperatures by removing Gram negative spoilage organisms. Ideally, this treatment should be accomplished without deleteriously affecting the flavor, appearance, nutritional properties, or creaming of the milk.

When milk is pasteurized for the manufacture of cheese or cream is pasteurized for making butter, a third objective is to destroy microorganisms that would interfere with the activities of desirable organisms, such as the starter culture, or cause inferiority or spoilage of the product. The cheese maker is also concerned that the heat treatment does not harm the curdling properties of the milk. The heat treatment of cream also destroys lipases that may cause deterioration of butter during storage. Milk in bulk samples may be pasteurized in batches in stirred, jacketed stainless steel vessels using steam or hot water. Rapid cooling of pasteurized milk is necessary to limit the growth of thermophilic organisms and hence milk is passed through a cooler.

The first widely used pasteurization process for milk involved heating the milk in large tanks or vats to $60^{o}C$ for at least 20 minutes. This holding method was subsequently changed to $61.7^{o}C$ for 30 minutes and finally $62.8^{o}C$ fir 30 minutes to eliminate *Coxiella burnetii*, a rickettsia responsible for Q-fever which can be transmitted in milk. This was referred to as vat pasteurization. The use of plate heat exchangers and a continuous operation involves the high-temperature-short-time (HTST) pasteurization process at a temperature of at least $72^{o}C$ for al least 15 seconds. The HTST system is the most widely used commercial pasteurization process today.

Heat-treatment processes in excess of pasteurization for milk and milk products have been designated as very high temperature (VHT) systems and ultrahigh-temperature (UHT) systems. A precise definition for VHT system is difficult. However, according to the International Dairy Federation, UHT processes usually refer to pasteurization techniques with temperatures of at least $130^{o}C$ in a continuous flow, with holding times of approximately 1 second or more. UHT systems have been more extensively used in Europe than in the United States for fluid milk. The most popular of UHT systems are the direct-heating methods, including a steam-injection-into-milk process and a milk-injected-into-steam process, referred to as a steam-injection technique and a steam-infusion technique, respectively. In both these systems, the added steam or excess of water is removed following the heat treatment in a sterile vacuum chamber. The combination of this type of heat treatment with aseptic packaging results in a category of products usually referred to as 'sterilized milk' or 'sterilized cream'.

The efficiency of milk pasteurization or the percentage of reduction of numbers of microorganisms in milk during pasteurization depends on (1) the temperature of pasteurization, (2) the holding time, (3) the total numbers of bacteria, and (4) the proportion of the total microbial load that are spore-formers or thermoduric organisms. In general, the conventional HTST system may reduce the number of microorganisms in milk by at least 90 to 99 per cent. Following pasteurization, milk is cooled rapidly to at least $7.2^{o}C$ or less. The shelf life of pasteurized milk depends on the temperature of storage and the numbers and types of microorganisms surviving the pasteurization process.

Conventional pasteurization should kill all yeasts and molds and most vegetative cells of bacteria in the milk. The surviving bacteria, termed thermodurics, belong to a number of different groups of bacteria, of which a few more important ones include (1) the high-temperature lactis, e.g., the enterococci; *Streptococcus thermophilus*, high-temperature lactobacilli such as *Lactobacillus bulgaricus*, and *L. lactis*; and species of *Microbacterium*, and (2) certain species of *Micrococcus*. Some species of *Streptococcus* and *Lactobacillus* are thermophilic as well as thermoduric. The spore-forming thermodurics fall into two main groups : (1) species of *Bacillus* viz., *B. cereus*,

B.subtilis, B. licheniformis, , *B. coagulans*, *B. polymyxa* , and (2) species of *Clostridium* viz., *C. butyricum* and *C. sporogenes*. Other miscellaneous bacteria may survive pasteurization but do not grow well in milk.

5.4 QUALITY TESTING OF MILK

A set of tests which have been used for some time in the dairy industry depend on the response of a number of redox dyes to the presence of metabolically active microorganisms. These tests are relatively simple and rapid to carryout at low cost. The redox dyes are able to take u electrons from an active biological system and this results in a change of colour. Usually, the redox dyes in oxidized from are coloured and in reduced form are colourless. Most widely used dye reduction tests are methylene blue reduction test and resazurin test. Besides these two, phosphatase test is also used for quality testing to know whether the milk sample is completely pasteurized or not.

5.4.1 Methylene blue reduction test (MBRT) :

Large numbers of bacteria present in raw milk, especially Escherichia coli and Streptococcus lactis, are indicative of poor methods of production handling. Bacteria present in the milk utilize the oxygen, which is present in small amount, producing a reduced environment. This reductase test is the based upon the oxidation-reduction (O/R) activities of the bacteria present in the milk. Methylene blue test is a rapid and an inexpensive way of indicating quality of milk that had been unrefrigerated. In this test, methylene blue, which is color sensitive to oxygen concentration is added to the milk. The indicator is blue in the oxidized state and white in the reduced condition. The speed of color disappearance of MB (i.e. methylene-blue reduction time, MBRT) which is proportional to the number of bacteria present, is taken as an indication of the bacterial load, i.e., the more the bacteria present, the faster the reduction. An MBRT of six hours is very good. Milk with an MBRT of 30 minutes is indicative of very poor quality of the sample.

The basic method of this test involves the procedural steps viz.,

- Preparation of methylene blue solution by dissolving 1 mg MB in 250 ml of sterile distilled water
- Transfer of 10 ml milk sample to be tested into test tube followed by the addition of 1 ml of methylene blue solution
- Stoppering of tubes and inversion of tube upside down fro 2-3 times
- Incubation of tubes in a water bath at 37°C for 6hrs.
- Observation of tubes after incubation for the colour change of sample from blue to white and recording of time required for the decolorization in the milk sample
- Classification of milk according to the time decolorization relationship given in the table 5.2.

Methylene blue reduction reduction time (MBRT)	Classification of milk sample	Approximate number of bacteria/ml
0 to 30 minutes	Very poor quality	>20,000,000
31 to 120 minutes	Poor quality	>4,000,000
121 to 360 minutes	Fair quality	>500,000
361 to 480 minutes	Good quality	<500,000

Table-2: MBRT and quality relationship of milk

5.4.2 Resazurin Test :

This test is one of the rapid methods for detecting milk supplies of poor quality. Resazurin is a azo dye and hence acts as an oxidation-reduction indicator which is blue in oxidized stage and pink in reduced stage. Upon reduction due to bacterial contamination or other causes it is changed into blue to pink coloured compound and then into the colourless compound dihydroresorufin. The rate of reduction from blue to pink depends upon the extent of contamination. Hence, the bacteriological quality of milk is based on duration (minutes) i.e., how much time is taken in reduction of resazurin. This test completes within 10 minutes which shows the presence of excessive bacterial numbers indicating poor quality of milk.

The steps of the test are:

- Transfer of 10 ml of milk sample into the test tube
- Addition of 1 ml of 0.5% resazurin solution to the test sample
- Treatment of the dye added sample in water bath for 10 minutes
- Comparison of the colour of the test milk sample with natural colour i.e., control milk and with standard resazurin discs
- If the colour falls between natural and coloured one, the milk is of poor quality

5.4.3 Phosphatase test :

The phosphatase test if applied to determine whether milk has been properly pasteurized. Milk contains the enzyme alkaline phosphatase which is inactivated by the time/temperature combinations applied during pasteurization. To determine whether a milk sample has been satisfactorily pasteurized and is free from contaminating raw milk, a chromogenic substance is added. If active phosphatase is still present then it will hydrolyse the substrate producing a colour which can be compared to standards to determine whether the milk is acceptable or not.

The milk samples are diluted with sodium carbonate-bicarbonate buffer at pH 10.2 and incubated at a temperature $37^{o}C$ for 2 hours. Any alkaline phosphatase present in the milk sample will liberate p-nitrophenol from artificially added buffer substrate, disodium p-nitrophenyl phosphate which can be compared with the standard colour discs.

5.5 SPOILAGE OF MILK

Dairy products such as milk, butter, cream, and cheese are all susceptible to microbial spoilage because of their chemical composition. Milk is an excellent growth medium for all of the common spoilage organisms, including molds and yeasts. Fresh, non-pasteurized milk generally contains varying numbers of microorganisms, depending on the case employed in milking, cleaning, and handling of milk utensils. Raw milk held at refrigerator temperatures for several days invariably shows the present of several or all bacteria of the genera namely *Enterococcus, Lactococcus, Streptococcus, Leuconostoc*, *Lactobacillus, Microbacterium, Propionibacterium, Micrococcus, Proteus, Pseudomonas*, *Bacillus* and others. Psychrotrophic organisms quickly dominate the microflora of milk held at chill temperatures with Pseudomonas, particularly *Pseudomonas fluorescens*, the organism primarily responsible for spoilage. The spoilage symptoms of off-odour and off-flavour variously described as metallic, unclean, bitter, and putrid are produced by the breakdown of amino acids used as energy sources by the organism. When milk is not held at refrigeration temperatures at the farm or during transport to the dairy and ambient temperatures are high (above 25^oC), mesophilic organisms grow rapidly. Lactose fermenting mesophiles namely streptococci, lactobacilli and enterobacteriaceae produce lactic acid from the lactose present in the milk, causing the milk to sour.

The pasteurization process eliminates all but thermoduric strains, primarily streptococci and lactobacilli, and spore-formers of the genus Bacillus. The spoilage of pasteurized milk is caused by the growth of heat-resistant streptococci utilizing lactose to produce lactic acid. Psychrotrophic Gram-negative rods recontaminate the pasteurized milk during filling so that in the normal course of events symptoms associated with the spoilage of pasteurized milk held at chill temperature are similar to those of raw milk. Pasteurized milk held at high ambient temperatures is likely to sour via the activity of mesophiles that have either survived the heat process or have contaminated the milk during filling. *Bacillus circulans* and *Bacillus sphaericus* that survived the heat process and occurred in pasteurized milk produce bitter flavours in the milk and cause sweet curdling i.e., coagulation of milk protein caused by rennin-type enzymes.

The common type of milk spoilage is the ropiness and sliminess. This condition can occur in milk, cream, or whey but are important mostly in market milk and cream. The bacterial ropiness is caused by slimy capsular material from the cells, usually gums or mucins, and ordinarily develops best at low storage temperatures. The ropiness usually decreases as the acidity of the milk increases. There are two main types of bacterial ropiness, one in which the milk is most ropy at the top and the other in which the milk becomes ropy throughout. Surface ropiness is caused most often by *Alcaligenes viscolactis*, an organism chiefly from water or soil that can grow fairly well in the vicinity of 10^oC. Some of the thermoduric micrococci, e.g., *Micrococcus freudenreichii*, can cause surface ropiness. Ropiness throughout the milk may be caused by any of a number of kinds of bacteria. Besides the ropiness, the other types of spoilage may include the changes in flavour, color etc., carried out by different microorganisms.

5.6 SUMMARY

The microbial flora of raw milk consists of those organisms that may be present on the cow's udder and hide and on milking utensils or lines. Fresh, non-pasteurized milk generally contains varying numbers of microorganisms, depending on the care employed in milking, cleaning, and handling of milk utensils. Under proper handling and storage conditions, the predominant flora is gram positive. While yeasts, molds, and gram negative bacteria may be found along with lactic acid bacteria, most or all of these types are more heat sensitive than gram positives and are more likely to be destroyed during pasteurization. The presence of psychrotrophic *Bacillus* and *Clostridium* species in raw milk is not surprising since they are quite abundant in soils. Also abundant in soils are psychrotrophic pseudomonads, and they are not uncommon in raw milks. Dairy products such as milk, cream, butter and cheese are all susceptible to microbial spoilage because of their chemical composition. Milk is an excellent growth medium for all of the common spoilage organisms, including molds and yeasts. Milk may be spoiled by different microorganisms in different ways and the slime and ropiness are important. The association of different bacteria with milk may result in the change of the milk colour due the pigmentation of bacteria.

5.7 MODEL QUESTIONS

1. Give an account on microorganisms associated with milk and microbial spoilage of milk.
2. What is pasteurization? Explain the methods of pasteurization and quality testing of milk.
3. Short answers:
   i) Pasteurization
   ii) MBRT
   iii) Resazurin test
   iv) Phosphatase test

5.8 REFERENCES

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M.Sc. MICROBIOLOGY (Final)

Lesson No. 6

FERMENTED FOODS

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6.2.1 Flow chart of production
6.2.2 Manufacturing process
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6.3.1 Cheddar cheese
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6.0 OBJECTIVE

To know about the manufacturing processes of some fermented food products.

6.1 INTRODUCTION

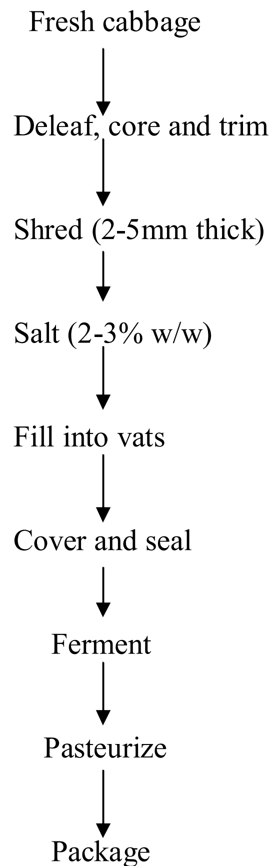
In the biochemical sense, the term fermentation refers to a metabolic process in which organic compounds are broken down to release energy without the involvement of a terminal electron acceptor such as oxygen. The term fermentation can also be applied to any industrial process that produces a material that is useful to humans and if the process depends on the activity of one or more microorganisms. These processes, known as industrial fermentations are usually carried out on a large scale and in vessels in which the organisms are normally grown in liquid media. A vast range of materials are produced by industrial fermentations.

Fermented foods are those foods produced by the modification of a raw material of either animal or vegetable origin by the activities of microorganisms. Bacteria, yeast and molds can be used to produce a diverse range of products that differ in flavour, texture and stability from the original raw material. The production of many fermented foods involves organisms that are biochemically fermentative. Lactic acid bacteria that ferment carbohydrates to produce lactic acid are particularly important, but yeasts also play a major role in some food fermentations, fermenting carbohydrate to produce ethanol and other organic chemicals. Mold fungi that do not ferment carbohydrates play an essential role in some food fermentations, for example, the production of blue cheeses and soy sauce. Many hundreds of different types of fermented foods are found worldwide, prepared from various raw materials like vegetables, fruits, milk etc.

6.2 SAUERKRAUT

The Sauerkraut is one of the fermented vegetable products. Its production is thought to have been brought to Europe from China. Like a number of other traditional fermentations, the commercial process is technologically simple. It is produced from cabbage vegetable and special cabbage cultivars are grown exclusively for preparation of sauerkraut in the areas where it is produced commercially. These cabbages are tend to have a higher solids content than normal and so minimize production of liquid waste during processing.

6.2.1 Flow chart



6.2.2 Manufacturing of Sauerkraut :

The closely filled, fully matured heads of variety of cabbage preferred for kraut making are wilted for 1-2 days to bring the cabbage to a uniform temperature and to facilitate shredding. Spoiled spots and defective outer leaves are trimmed off, the heads are washed with pure water, the core is shredded to slim sized pieces. The shredded cabbage is then salted with 2.25 –2.5% salt by weight. The level of salting is critical to obtain a satisfactory product. Too little salt (<2%) makes the product soften unacceptably and too much salt (>3%) leads to the undesirable quality of the product. The main purposes of salting are

- to extract moisture from the shredded cabbage by osmosis to form the brine in which the fermentation takes place
- it helps to inhibit some of the natural microflora of cabbage such as pseudomonads which would otherwise cause spoilage and helps to select for the lactic acid bacteria
- it helps to maintain the crisp texture of the cabbage by withdrawing water and inhibiting endogenous pectolytic enzymes which cause the product to soften
- it contributes to the flavour of the product

The salted cabbage is packed into vats, for the fermentation stage, tamped down and finally weightened down so that a layer of expressed, brined juice stands on the surface. A covering of some kind should protect the surface from contamination with dirt and insects. Traditionally, fermentation vats have been made of wood but nowadays are more often of concrete with a synthetic polymer lining to protect from attack by the acid brine. The tanks are sealed by covering the salted cabbage with plastic sheeting. The temperature, during fermentation, is maintained between 21 – 24°C. If temperature is below 15.6^{o}C, slow and incomplete fermentation occurs and if the temperature is between 26 – 29°C results in abnormal fermentation. When desired acidity is achieved, fermentation is stopped.

During fermentation, anaerobic conditions develop rapidly in the salted, shredded cabbage and the surrounding juice, chiefly as a result of the removal of O_2 by the respiration of the plant cells but with some help from bacteria. The juice contains the natural flora of the cabbage plus contaminants from soil and water. At first, different kinds of bacteria begin to grow, but the acid-forming types soon predominate. At beginning, coliform bacteria *Enterobacter cloacae* and some lactic acid bacteria develop. Soon after, *Leuconostoc mesenteroids* begin to outgrow all other microorganisms and continue acid production up to 0.7 –1.0%. This bacterium attacks sugars to form lactic acid, acetic acid, ethanol, mannitol, dextran, esters, and CO_2 which contribute to the flavour of good sauerkraut. Next to this, non-gas forming lactobacilli, *Lactobacillus plantarum*, continue the production of acid and can raise the acidity to 1.5 – 2.0%. A final acidity of 1.7% is most desirable and fermentation can be stopped at this stage. The total fermentation lasts for about 4-8 weeks. The final sauerkraut is packaged and stored. A good grade sauerkraut should possess light colour, crispy nature, 1.7% acidity, cleanness and acid flavour.

6.3 CHEESE

Cheese one of the oldest foods of mankind. Cheese is defined as “a product made from the curd obtained from milk by coagulating the casein with the help of rennet or similar enzymes in the presence of lactic acid produced by added or adventitious microorganisms, from which part of the moisture has been removed by cutting, cooking and /or pressing, which has been shaped in a mould, and then ripened by holding it for some time at suitable temperatures and humidities”. It can also be defined simply as a “consolidated curd of milk solids in which milk fat is entrapped by coagulated casein”. Cheese is a valuable means of conserving many of the nutrients in milk. Today, cheese making is a major industry worldwide, producing about 14 million tones per annum. The large scale industrialized production is dominated by one variety i.e., cheddar cheese. All cheeses, irrespective of country of origin and methods of manufacture, possess certain characteristics in common. They are –

- Made from milk of certain mammals
- First stage is souring / ripening
- Second stage is clotting/coagulation by rennet/similar enzyme preparation
- Third stage is cutting/break up of the coagulum to release the whey
- Fourth stage is consolidation/matting of the curd
- Fifth stage is maturing/curing

However, some chief factors are responsible for differences in final cheese and are –

- Type of milk
- Degree of souring and type of souring organisms added
- Temperature of rennetting and subsequent cooking of the curd in the whey
- Method and fineness of cutting or of breaking up the curd
- Treatment of the curd after separation from the whey
- Milling and salting of the curd before placing it in the mould
- Pressure applied to cheese
- Time, temperature and RH of curing
- Special treatment such as pricking/stabbing the cheese, bathing in brine, and surface treatment to produce a certain type of coat

Today, in the world, about 20 distinct classes of cheese are reported, and they are given over a thousand different names. For example, 78 different types of blue cheeses and 36 cammembert cheeses have been reported. Cheeses can be classified according to the following systems –
Geographical considerations : Country, Valley, Institution, Town/region where first produced or marketed.

- Type of milk : Cow, Sheep, Goat and Buffalo
- Method of manufacture: Temperature of cooking, degree of acidity, fineness of cutting etc., which effect moisture retention which in turn affects firmness i.e. hardness or softness and also the rate of ripening.
- General appearance: Flavour, Size, Colour and Keeping quality
- Physical properties: Very hard, hard, semi-hard and soft
- Chemical analysis: Water, calcium, sodium chloride, casein, lactose, fat, acidity contents
- Microbiological properties: Bacteria-ripened, Mould-ripened and Unripened

Cheese has high food and nutritive value. It is

- an excellent source of milk proteins
- a rich source of Calcium and Phosphorus
- an excellent source for several fat-soluble vitamins such as A,D,E,K
- a concentrated form of energy (eg. Cheddar gives 400 calories/100gr)
- palatable and digestible

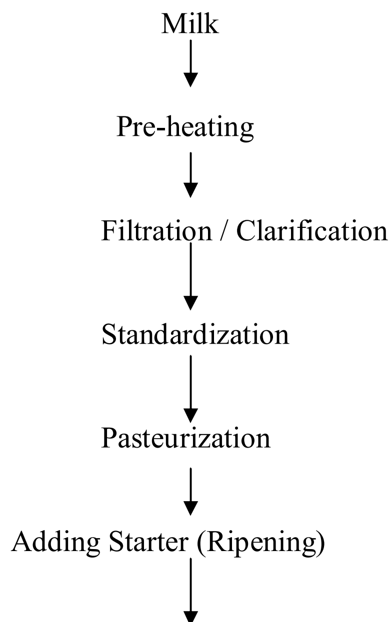
Cheese varieties: based on the moisture content, cheeses can be grouped into different varieties as given in the table below.

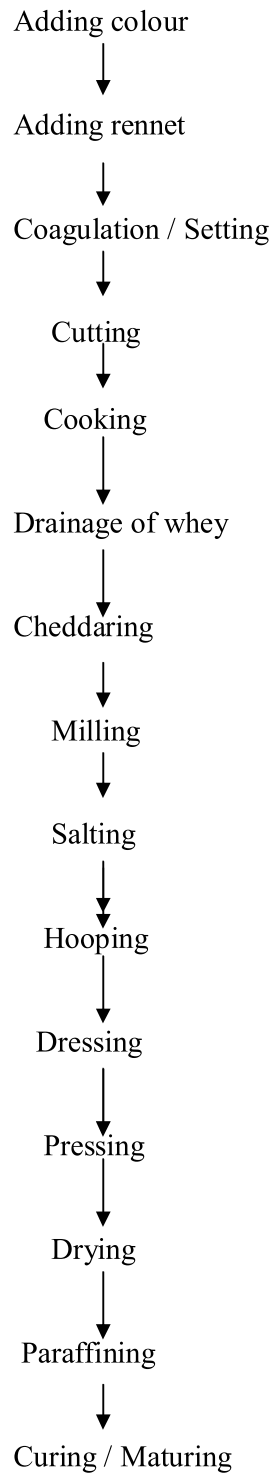
Moisture %	Cheese variety and examples
50-80%	Soft Cheeses Unripened → Cottage, Quark, Cream Mozzarella Ripened → Cammembert, Brie, Caciotta, Cooked Salt-cured or pickled → Feta, Domiati
39-50%	Semi-soft cheeses Ripened principally by internal mould growth → Roquefort, Stilton, Danish blue Ripened by bacteria and surface microorganisms → Limburger, Brick, Trappist, Port salut Ripened primarily by bacteria → Bel paesa, Brickm Pasta filata, Gouda, Edam, Provolone
< 39%	Hard Cheeses Without eyes, ripened by bacteria → Cheddar, Caciocavallo With eyes, ripened by bacteria → Emmental, Gruyere
< 34%	Very Hard Cheeses → Asiago Old, Parmesan, Romano, Grana

6.3.1 Cheddar Cheese

This cheddar cheese is originated in the town of Cheddar located in the county of Somerset in South-western England. Cheddar is probably the best-known cheese in the world and many variants are now recognized such as American, Australian, New Zealand, Canadian and Indian cheddar. The cheddar cheese is particularly valued for its smooth texture and good keeping qualities.

6.3.1.1 Flow-chart for cheddar cheese production:




Adding colour
Adding rennet
Coagulation / Setting
Cutting
Cooking
Drainage of whey
Cheddaring
Milling
Salting
Hooping
Dressing
Pressing
Drying
Paraffining
Curing / Maturing

6.3.1.2 Manufacturing of cheddar cheese

Cow's milk used for cheese production must be free from antibiotics and sanitizing agents that might interfere with the fermentation. A heat treatment equivalent to pasteurization is usually applied at the start of processing. This helps to ensure a sage product and a reliable fermentation, although cheeses made from unpasteurized milk have been claimed to possess a better flavour. The milk is then cooled to the fermentation temperature i.e. 29-31°C. The mesophilic *Streptococcus cremoris* or *Streptococcus lactis* is used as a starter culture. The role of starter organisms in cheese making is both crucial and complex. Their central function is the fermentation of the milk sugar lactose to lactic acid. This and the resulting decrease in pH contribute to the shelf-life and safety of the cheese and gives a sharp, fresh flavour to the curd. The starter culture produce acidity, aroma and special effects like eyes in cheese. A bad culture may result in the production of low quality cheese. At this stage, a colouring matter usually carotene extract may be added to give a pale orange colour.

After about 30 minutes, a mildly acidic condition of about 0.2% acidity exists and rennin, in the form of a dilute solution or a commercial form i.e. rennet is used. Rennet is a preparation of the enzyme rennin, also called as chymosin, obtained from the lining of the fourth stomach of calves. Microbial rennets from *Mucor meihei* or proteases may also be used. The amount of rennet to be added depends on (i) strength of rennet, (ii) temperature of milk, (iii) acidity of milk and (iv) composition of milk. After 30-45 min, coagulation of the milk is complete and the process of whey expulsion is started by cutting the curds into approximately 1 cm cubes. Whey expulsion is further assisted by the process known as scalding when the curds, heated to 38-42°C, shrink the become firmer. The starter organisms are not inhibited by such temperatures and continue to produce acid which aids curd shrinkage. When the acidity has reached the desired level, the whey is run off from the cheese vat. At this stage the process known as cheddaring occurs. The curd is formed into blocks which are piled up to compress and fuse the curds, expelling more whey.

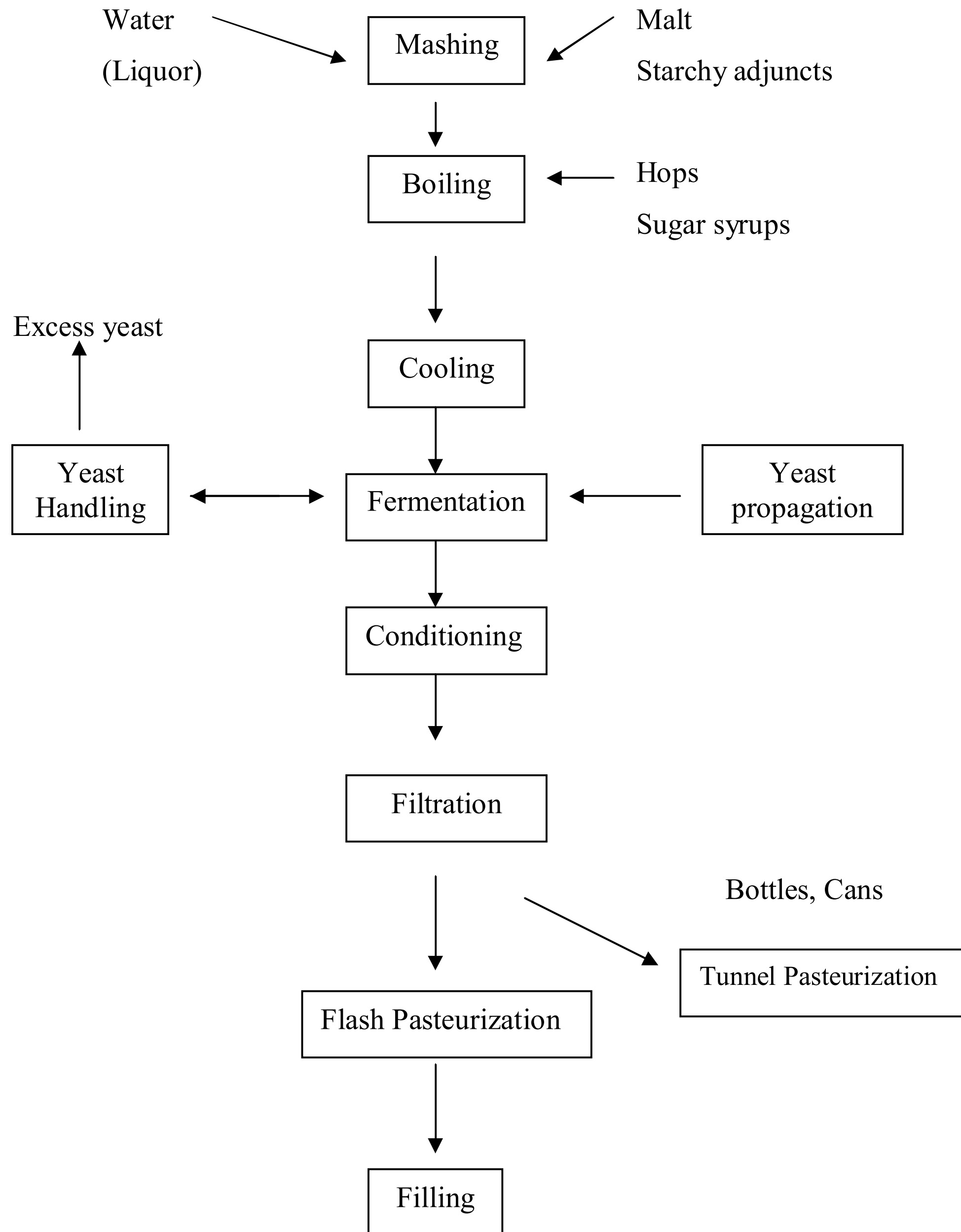
At the end of cheddaring, the curd has a characteristic fibrous appearance resembling cooked chicken breast. The blocks of curd are then milled into small chips. This facilitates the even distribution of salt which, in cheddar, is added at a level of between 1.5 and 2% w/w. The salted curd is formed into blocks which are then pressed to expel trapped air and whey. Finally the cheese is ripened or matured at 10°C to allow flavour development. During this stage, which can last up to five months to produce a mild cheddar, the microflora is dominated by non-starter lactobacilli and a complex combination of bacteria and enzymic reactions give the cheese its characteristic flavour. In particular, proteases and peptidases from the starter culture continue to act, even though the organism can no longer grow. With other proteases from the rennet, they release free amino acids, principally glutamic acid and leucine in cheddar, and peptides which contribute to the cheese flavour.

6.4 BEER

Beer is one of the principal malt beverages which belongs to one the three classes of alcoholic beverages. Beer is made from barley malt to which hops (dried flowers of the hop plant) and cereal or malt adjuncts (starch or sugars obtained from corn, rice, wheat, soybean, potato, cassava or barley) are added and fermented. Beer is manufactured by the brewing process. Brewing is thought to have originated in Mesopotamia where it is said that as much as 40% of total cereal production was used

for this purpose. Variations in beer types are manufactured by varying the concentration of malt and hops, aging time, initial concentration of fermentable solids and temperature of fermentation to yield beers differing in alcohol content, colour and residual fermentable carbohydrate content.

6.4.1 Flow Chart



6.4.2 MANUFACTURE OF BEER

The steps involved in the brewing process are

- malting
- mashing to prepare wort (unfermented beer)
- boiling the wort with hops,
- fermentation
- maturation or aging
- finishing

6.4.2.1 Malting :

Malting is controlled germination of cereal grains (barley) to activate the enzymes of the resting grain in order to convert the starch to fermentable sugars. Barley for brewing should have low nitrogen content compared to barley used in distilleries. The barley grains are kiln dried to control the moisture content to about 10-14% and stored for about three weeks to allow secondary ripening. The stored grains are then soaked or 'steeped' in water at 10-16°C, germinated at 16-21°C for about five to seven days and kiln dried to arrest the enzyme activity without destroying it. Most of the sprout or germs are removed to yield the remaining mass called the *malt*. The malt is a rich source of amylases and proteases required for the brewing process.

6.4.2.2 Mashing :

Mashing is carried out to initiate hydrolysis and solubilize the polysaccharides. The malt is crushed and mixed with water at temperatures in the range of 38-50°C. Starchy cereal or malt adjuncts are cooked at about 100°C under steam pressure and added to the malt mash. The addition raises the temperature of the mash to about 65-70° C at which saccharification (production of sugars from starch) occurs rapidly. The mashing conditions are regulated with respect to concentration of malt, malt adjuncts and temperature to vary the proportion of various carbohydrates. The temperature of the mash is then raised to 75°C to inactivate the enzymes. The mixture is then allowed to settle the insoluble solids which are filtered off to yield a clear liquid called *wort*.

6.4.2.3 Brewing :

This involves boiling the wort with hops in a brew kettle. The various objectives of this step include concentration and sterilization of the wort, inactivation of enzymes, extraction of soluble substances from hops for imparting flavour, bitterness, stability and head retention to beer, caramelization of sugars to a slight extent, coagulation and precipitation of proteins and other insolubles such as tannins to prevent haze formation of beer, and contribution of antiseptic substances called alpha resins such as humulone, cohumulone and adhumulone.

6.4.2.4 Fermentation :

A special beer yeast of bottom type, *Saccharomyces carlsbergensis* is used for inoculation or 'pitching' of the cooled wort. The wort temperature is maintained at 3-14°C and fermentation is continued for about eight to fourteen days. During fermentation, yeast converts sugars mainly into alcohol and carbon dioxide and small amounts of glycerol and acetic acid. Higher alcohols and acids are formed from proteins and fats. The acids and alcohols combine to yield esters contributing to the aroma. As the carbon dioxide evolution increases foaming increases and finally the bottom yeasts 'break' by flocculating and settling to the bottom of the tank. The mixture is filtered to yield 'green' beer.

6.4.2.5 Maturing :

The young or green beer is chilled and stored or lagered in vats at about 0°C for several weeks and even to months. During aging, proteins, yeast, resins and other undesirable substances precipitate out and the beer becomes clear and mellowed or matured. Esters and other compounds are produced to contribute to the taste and aroma. The body changes from harsh to smooth.

6.4.2.6 Finishing :

The lager beer obtained by aging is carbonated to a carbon dioxide content of about 0.5% by means of gas collected during fermentation. The beer is then cooled, clarified or filtered and packaged into barrels or smaller bottles and cans. The beer for cans and bottles may be pasteurized at 60°C or filtered through membranes to remove all the remaining yeasts. The alcohol content in beer is about 3.8%.

6.4.2.7 Types of beers :

- malt liquor ---- contains higher alcohol content
- bock beer ----- dark beer with higher alcohol content
- pilsener beer --- low calorie, light coloured beer without carbohydrate
- Sake ---- a beer of Japanese origin with 14-17% alcohol content
- Sonti ---- a rice beer of Indian origin
- Ginger beer ---- an acidic and mild beer with ginger flavour

6.4.2.8 Beer defects and spoilage :

Beer defects are certain undesirable characteristics such as turbidity due to unstable proteins, tannins and resins and development of off-flavours due to poor quality ingredients or contact with metals. Beer infections or diseases are primarily due to growth and activity of undesirable microorganisms during fermentation. Butyric acid fermentation by *Clostridium* sp. or lactic acid fermentation by lactics may result in off flavours. Yeasts grow in beer and cause turbidity or cloudiness and bitterness, e.g., *Saccharomyces pastorianus*. Bacteria of general *Pediococcus*, *Lactobacillus*, *Flavobacterium* and *Acetobacter* cause beer diseases resulting in sourness, turbidity, ropiness and

bad odour. However, most of the yeasts and bacteria are killed by boiling the wort and hops and maintaining aseptic conditions during subsequent stages of brewing.

6.5 WINE

The wine is principally a fermented grape juice. It may also be produced by fermentation of juices of fruits such as apple (cider), peaches, apricots, plums, pears, cherries and berries and honey (mead or honey wine). Grape wine is either red or white depending on whether the skins of purple or red varieties of grapes have been fermented to impart the red colour of the pigment, or white grapes or expressed juices of other grapes without the skin have been fermented.

The various steps involved in the manufacture of grape wine include (i) juice extraction, (ii) fermentation and (iii) aging

6.5.1 Juice extraction :

Grapes, specially adapted to wine making, are harvested at a stage when the sugar content is about 15-25% depending on the variety and ripeness. The composition of grapes varies due to the climatic conditions prevailing during their cultivation and hence the quality of wine varies from one year to another. The harvested grapes are stemmed and crushed to yield the expressed juice called ‘must’. The must is then treated with sulphur dioxide at 75-200 ppm concentration or equivalent amount of potassium bisulphite to inhibit the growth of undesirable microorganisms.

6.5.2 Fermentation :

The must is inoculated with a ‘natural inoculum’ of yeast present on the grapes or a special wine yeast strain of *Saccharomyces cerevisiae var. ellipsoideus*. The primary or active fermentation is initiated by stirring the mixture with aeration to facilitate the growth of yeast and extraction of the pigment from the skins. Alternatively, the red pigments may also be extracted separately and added to the must. Later mixing is stopped to facilitate anaerobic fermentation to yield alcohol. The primary fermentation occurs over a period of 3-5 days at an optimum temperature of 24-27° C for red wines while for white wines the fermentation period is about 7-14 days at a temperature of 10-21°C. Heat is liberated during fermentation and hence cooling the must is necessary.

After the primary fermentation advances sufficiently the fermented juice is drawn off to separate it from the residue and is allowed to undergo secondary fermentation in a tank under a light pressure of carbon dioxide. During the secondary fermentation which occurs over a period of 7-11 days at 21-29°C the remaining sugar is fermented to yield a dry wine. Clear wine is siphoned off from the sediment at the bottom of the tank and filled or ‘racked’ into barrels for aging.

6.5.3 Aging :

The wine is cooled for several days, filtered and transferred into wooden (white oak or redwood) or plastic lined concrete tanks for aging. The tanks are filled completely and sealed to keep out air. The

wine is periodically racked off from the bottom sediment. Final aging in bottles for long periods of months to years produces desirable changes in body and flavour of wine. The characteristic aroma and bouquet develop due to the various esters and alcohols. In addition, malic acid of grape juice is fermented by lactobacilli during aging to give lactic acid and carbon dioxide and also decrease the acidity. After the process of aging is complete, the wine is filtered or clarified, bottled and pasteurized. The final alcohol content varies between 8-13% by volume.

6.5.4 Types of wines

- Still wines --- do not retain any carbon dioxide produced during fermentation
- Sparkling wines ---- contain considerable amounts of carbon dioxide
- Dry wines ---- do not contain any fermentable sugars
- Fortified wines ---- contain added wine spirits or brandy to increase the alcohol content to about 20% by volume
- Table wines ---- contain lower level of alcohol with little sugar
- Dessert wines ---- are the fortified sweet wines

6.5.5 Wine defects and spoilage :

Wine defects include turbidity, cloudiness, precipitation and loss of colour or colouration due to metals such as iron, tin and copper and their salts. Microorganisms, both aerobic and facultative, cause wine spoilage by imparting cloudiness, bitter characteristics, ropiness, undesirable flavours, high volatile acidity and low alcohol content. Wild yeasts, molds and bacteria of the genera *Acetobacter*, *Lactobacillus*, *Leuconostoc* and *Pediococcus* cause spoilage of wine.

6.6 VINEGAR :

The term vinegar is originated from the French word “vinaigre” which means ‘sour wine’. Vinegar is defined as a condiment made from sugary or starchy materials by an alcoholic fermentation followed by an acetous one. It must contain at least 4 g of acetic acid per 100 ml. Vinegar may be classified on the basis of the materials from which it has been made. Vinegar may be made from the juices of fruits such as apples, grapes, oranges; from starch vegetables such as potatoes after hydrolyzing their starch into sugars; from malted cereals of barley, rye, wheat or corn; from molasses, honey or from waste alcoholic liquor.

Manufacture of vinegar involves two steps : (i) fermentation of sugar to ethanol followed by (ii) oxidation of alcohol to acetic acid. The first step is an anaerobic process carried out by *Saccharomyces cerevisiae var. ellipsoideus*. The second step involves aerobic oxidation by acetic acid bacteria of genera *Acetobacter* and *Gluconobacter*.

6.6.1 Alcoholic Fermentation :

During this step, sugar-containing materials such as fruit juices, honey or hydrolyzed starchy materials are fermented to ethyl alcohol. This bioconversion is brought about by the agency of

enzymes of yeast, *S.cerevisae var. ellipsoideus*. The concentration of fermentable sugar in the raw material should be in the range of 8-20% and one gram of glucose should yield 0.5114 g of ethyl alcohol. The fermentation should be carried out at 75-80°F temperature. This alcoholic fermentation step is carried out in two stages – Primary stage ---- lasts for 3-7 days and carried out in open fermentation vats Secondary stage ---- lasts over a period of several weeks and carried out in closed fermentors At the start of the fermentation and prior to the addition of the starter culture of yeast, SO_2 at a level of 50-100 mg/litre is added to production medium, to inhibit the growth and activity of unwanted yeasts and bacteria. After fermentation, a clarified wine is obtained by racking it from the sediment.

6.6.2 Acetic acid Fermentation :

There are three main processes for the microbial conversion of dilute ethanolic solution to acetic acid viz., Orleans process, Quick vinegar process and Deep fermentation process. Of these three, the quick vinegar process remains of considerable industrial importance.

Quick vinegar process – this process uses "Frings generator". The generator is an air tight tank of 14 ft diameter and 15 ft height with a wooden grating near the bottom. Near the top of the tank, a sprayer or distributing arm called as sparger is fitted. At the top, a 4- inch vent with a damper connected to a vapour-liquid separator is placed to control the air supply. Near the level of the wooden grate and around the tank, air intakes containing air filters are situated. The tank is also equipped with tubular cooling coils at the bottom of the tank in order to control the temperature. The generator is packed with beech wood shavings up to about 1.5 ft from the top of the generator. *Acetobacter* species is inoculated onto the beech wood shavings. The vinegar stock or mix is allowed to undergo circulation repeatedly through the beech wood shavings. Thus, the alcoholic solution undergoes more and more oxidation and this operation continues till vinegar of the desired strength has been obtained.

For successful acetic acid fermentation, several factors should be under control like that of nature of packing material, nature of vinegar stock used , rate of flow of ethanolic solution, rate of air flow and temperature.

6.7 SUMMARY

Fermentation involves the use of microbial metabolic process in which carbohydrates and other nutrients are oxidized partially to a variety of breakdown products such as alcohols, acids, amino acids, other metabolites and antibiotics and a small amount of energy. Fermentation is carried out for (i) the modification / production of new or desired form of foods such as cheese, bread, idli, dosa, tofu, coffee, alcoholic beverages, soya sauce, pickled vegetables such as sauerkraut and meat sausages (ii) production of flavours as in the case of cheese, butter milk and other dairy products, (iii) preservation of foods as in the case of sauerkraut, cucumber, carrot, olive, mixed vegetables, green tomatoes and hot cherry pepper pickles and (iv) production of food chemicals and additives such as proteins, enzymes, vinegar, amino acids, citric acid, etc.

Fermented foods have several advantages such as appetizing flavour and aroma as well as textural characteristics. They also have longer shelf life compared to the stability of starting materials from which they have been derived. The digestibility of such foods is also greater. Microorganisms used in food fermentation may be added as pure or mixed cultures as in the manufacture of dairy products such as fermented milk, butter and cheese, bread, malt beverages, wine, distilled liquor and vinegar. In some cases, the desired microorganism may be present in sufficient numbers in the original raw material as in the manufacture of sauerkraut, fermented pickles, green olives and processing of cocoa, coffee, etc.

6.8 MODEL QUESTIONS

1. Describe the production method of Sauerkraut.
2. Give a detailed account on the manufacturing of cheddar cheese.
3. Write an essay on the production of beer from malt.
4. Explain the process of commercial production of wine.
5. Describe the method of vinegar production.
6. Short answers:
   i) Types of cheeses on moisture content.
   ii) Brewing process
   iii) Acetic acid fermentation
   iv) Malting
   v) Spoilage of beer and wine

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M.Sc. MICROBIOLOGY (Final)

Lesson No. 7

MICROBES AS FOOD

7.0 Objective
7.1 Introduction
7.2 Single cell protein (SCP)
7.2.1 Microorganisms used in production
7.2.2 Substrates used in production
7.2.3 Production of SCP
7.2.4 Biomass recovery
7.2.5 Nutritive value
7.2.6 Advantages of SCP
7.3 Mushrooms
7.3.1 Nutritive value
7.3.2 Cultivation method
7.3.3 Recipes of edible mushroom
7.3.4 Advantages of mushroom
7.4 Summary
7.5 Model questions
7.6 References

7.0 OBJECTIVE

To understand the cultivation methods for Single-cell protein and mushrooms and their usages and advantages.

7.1 INTRODUCTION

Microorganisms may serve as food for human beings or feed for animals, as a source of enzymes to be used in the processing of foods, or as manufacturers of products to be added to foods. The dried cells of microorganisms (algae, bacteria, actinomycetes and fungi) used as food or feed are collectively known as ‘microbial protein’. Since the ancient times a number of microorganisms have been used as a part of diet. The term ‘microbial protein’ was replaced by a new term “single cell protein” (SCP) during the First International Conference on microbial protein held in 1967 at the Massachusetts Institute of Technology, Cambridge, Massachusetts, USA, to depict the idea of microorganisms as food sources. Single cell protein could be a food source that is nutritionally complete and requires minimum of land, time and cost to produce. The SCP can be produced on a variety of waste materials.

Importance of mass production of microorganisms as a direct source of microbial protein was realized during World War I in Germany and consequently, baker's yeast (*S.cerevisae*) was produced in an aerated molasses medium supplemented with ammonium salts. During World war II, the aerobic yeasts (e.g. *Candida utilis*) were produced for food and feed in Germany. Since then, considerable efforts has been made to develop technologies for mass cultivation of SCP by formulating different types of growth media and improved culture of microorganisms. In India, relatively little attention has been paid on the production of SCP, though mushroom cultivation started in the early 1950s. However, work on mushroom culture at Solan (Himachal Pradesh) form 1970 onward has brought satisfactory results.

7.2 SINGLE CELL PROTEIN (SCP)

The term 'single cell protein' refers to microbial cells grown and harvested primarily for use as human or animal food. The development of a microorganism as a direct primary food for human consumptions is based on the conversion of the abundant carbohydrates (starch and sugar) of plants into protein. SCP offers a possible solution to meet a shortage of protein under unusual conditions when agriculture and fishing may not be able to produce sufficient quantities of protein so as to meet the demand. Any new protein food must satisfy the conditions of safety and organoleptic characteristics fit for human consumption. A number of microorganisms have been identified as potential food for humans and animals. Bacteria, yeasts, molds and algae may be used for production of SCP, but till today, only yeasts have been produced commercially and used as food. Recently *Spirulina maxima*, an algae is being grown commercially as a nutrient rich food. Organisms may be grown directly for the purpose of food termed primary or they may be recovered as by-product of a fermentation termed secondary.

The more obvious advantages of the use of SCP include

- The possibility of using a non-human food for the substrate of SCP production
- The inherent high protein content of microorganisms-on a dried-weight basis, protein content might approach 60 to 70 percent of the cell
- The rapid increase in cells (protein) because of the extremely short generation times
- Lack of dependence of the SCP production process on climatic conditions.

SCP is mainly produced as an animal feed. There are problems with using SCP for direct human consumption because of high concentrations (6-11%) of nucleic acids. This may result in increased serum levels of uric acid causing kidney stone formation or gout, allergic ions and possible gastrointestinal reactions. Chicken and animals, however, can be grown on SCP helping to meet the world food needs.

7.2.1 Microorganisms used in production

Algae, Fungi (filamentous), Yeasts and Bacteria are used for SCP production. The advantages of microbial SCP over plant and animal sources of protein are:

- Microorganisms have a very short generation time and can thus provide a rapid mass increase
- Microorganisms can be easily modified genetically to produce cells that bring about desirable results
- The protein content is high
- SCP production can be based on raw materials readily available
- SCP production can be carried out in continuous culture and thus be independent of climatic changes.

The microorganisms use for SCP production must be:

- non-pathogenic to plants, animals and man
- of good nutritional value
- easily and cheaply produced on scale
- toxin-free
- fast-growing
- easy to separate from medium and to dry etc.

Algae – Members of the genera *Chlorella, Scenedesmus* and *Spirulina* are generally grown in ponds or tanks. They use CO_2 and sunlight as substrate which are without any cost. Generally, the limiting factor in their large scale production is illumination. Algal SCP has about 60% crude protein, which is generally good in amino acid composition except for some deficiency in sulphur-containing amino acids. They are suitable for animal feed as protein-rich supplement. The disadvantages of algal SCP are:

- rich chlorophyll content which is not suitable for human use
- serious problems when *Chlorella* and *Scenedesmus* are used in human diet
- low cell density, e.g., 1-2 g dry weight / liter
- serious risk of contamination
- costly recovery methods for unicellular algae

Filamentous fungi – Filamentous fungi have been used to produce SCP mainly from polysaccharide hydrolysates, e.g., starch hydrolysates, sulphite liquor from wood pulp industries etc. these are usually grown as submerged cultures in which they grow as yeast-like cells, in filamentous form or in pellets. They have crude protein content of 50-55%; the protein is low in S-containing amino acids, but otherwise is excellent in amino acid composition. The recovery of filamentous and pellet-forms is rather easy by filtration. The problems associated with fungi are:

- slower growth rates than bacteria and yeast, but some microfungi may be comparable to yeast
- contamination by yeast may be frequent if sterility is not maintained, while that by bacteria can be minimized by keeping the pH of broth below 5
- they have high nucleic acid content (up to 15% RNA) which must be reduced
- the strains have to be thoroughly evaluated for mycotoxin production.

Yeasts – Members of *Saccharomyces*, *Candida* and *Torulopsis* have been widely studied for SCP production and those of the first two genera are used for some commercial processes using various

substrates. The SCP has 55-60% crude protein which has good amino acid balance except for a deficiency in S-containing amino acids. It is usually very rich in B-group of vitamins. The SCP is used both for human food and animal feed supplementation. The risk of bacterial contamination is low and recovery by continuous centrifugation is easy. The difficulties in use of yeasts as SCP are:

- slower growth rates than fastest growing bacteria
- high nucleic acid content (up to 15%) which need to be reduced
- methionine supplementation may be done to overcome S-containing amino acid deficiency of its proteins

Bacteria – A large number of bacterial species have been evaluated for SCP production (i) using a wide variety of substrates, (ii) some of these are used for production at commercial scales, e.g., *Methylophilus methylotrophus* using methanol. SCP has very high crude protein (over 80%) of good amino acid composition, although in some cases a small deficit of sulphur-containing amino acids may be encountered. The disadvantages of using bacteria for SCP production are:

- high nucleic acid, especially, RNA content (c. 20%) which must be reduced
- sterility must be maintained during the production process since pH of cultures is kept between 5 and 7
- risk of contamination by pathogenic bacteria is considerable
- recovery of cells is problematic
- careful evaluation for endotoxin production is essential particularly when gram-negative bacteria are used

7.2.2 Substrates used in production

A variety of raw materials can be used as substrates by the organisms for SCP productions. These substrates include:

- molasses from sugar manufacture or hydrolysis of starch
- spent sulfite liquor, which is a waste product of the sulfite-pulping process in the paper industry
- the acid hydrolyzate of wood
- agricultural wastes, e.g., whey from the dairy industry, hydrolyzed starchy foods, e.g., grains and cull potatoes, and fruit wastes, e.g., fruit juice or citrus-peel hydrolyzate
- methane
- methanol and ethanol, as a carbon source for yeast
- paraffin or alkanes
- gas oil, the petroleum fraction between lubricating oil and diesel fuel
- combustion gas, a source of carbon dioxide for algal cultures

Some examples of the microorganisms that use different substrates are listed in the table7.1.

Substrates	Microorganisms
CO_2 and Sunlight	*Chlorella pyrenoidosa, Scenedesmus quadricaudata, Spirulina maxima*
N-alkanes, kerosene	*Candida intermedia, C. lipolytica, C. tropicalis, Nocardia spp.*
Methane	*Methylomonas spp., Methylococcus capsulatus, Trichoderma spp.*
H_2 and CO_2	*Alcaligenes eutrophus*
Gas oil	*Acinetobacter, Candida lipolytica*
Sulphite liquor waste	*Candida utilis*
Cellulose	*Cellulomonas spp., Trichoderma viride*
Starches	*Endomycopsis fibuligera*
Sugars	*Saccharomyces cerevisiae, Kluyveromyces fragilis*

Table – 7.1: Some substrates that support the microbial growth in SCP production

7.2.3 Production of SCP

The process of SCP production from any microorganism or substrate would have some basic steps that include –

- Provision of a carbon source; it may need physical and /or chemical pretreatments
- Addition, to the carbon source, of sources of nitrogen, phosphorus and other nutrients needed to support optimal growth of the selected microorganism
- Prevention of contamination by maintaining sterile or hygienic conditions. The medium components may be heated or sterilized by filtration and fermentation equipments may be sterilized
- The selected microorganism is inoculated in a pure state
- SCP processes are highly aerobic (except those using algae). Therefore, adequate aeration must be provided. In addition, cooling is necessary as considerable heat is generated
- The microbial biomass is recovered from the medium
- Processing of the biomass for enhancing its usefulness and / or storability

The biomass production is ordinarily carried out in the continuous mode to maximize yields and economic returns.

7.2.4 Biomass Recovery

The general approaches for the recovery of biomass are as follows :

- Bacteria – flocculation and floatation combined with centrifugation
- Yeast – centrifugation
- Filamentous organisms – filtration

It is important to reduce water as much as possible to reduce drying costs. Sun-drying is cheap but it reduces the quality of SCP. Since recovery processes are not done under sterile conditions, adequate hygiene must be maintained and care should be taken to prevent growth of undesirable microorganisms. Heat treatments are used during the final stages of harvesting to inactivate heat-sensitive organisms and reduce RNA content. The cell walls must be broken to enhance the nutritional value of SCP. The biomass may be further processed or even the protein may be isolated and purified.

7.2.5 Nutritive value of SCP

Nutritive value varies with the microoganism used. Protein digestibility values, expressed as a percentage, range from 65 to 96 for the various cultures tested. Protein efficiency ratio (PER) values range from 0.6 to 2.6. The method of harvesting, drying, and processing has an effect on the nutritive value of the finished product. Food yeast is high in protein and in most of the B complex vitamins but bay be deficient in methinone and perhaps cysteine. Also, the thiamine content may be lower than in secondary yeast, and there is a deficiency of vitamin B_{12} . Furnished by food yeast in varying amounts are thiamine, riboflavine, biotin, niacin, pantothenic acid, pyridoxine, choline, streptogenin, glutathione, and perhaps folic acid and p-aminobenzoic acid.

7.2.6 Advantages of SCP:

The SCP processes and products offer several advantages –

- The SCP is rich in high quality protein and is rater poor in fats, which is rather desirable
- They can be produced all the year round and are not dependent of the climate
- The microbes are very fast growing and produce large quantities of SCP from relatively very small area of land
- They use low cost substrates and, in some cases, such substrates which are being wasted and causing pollution to the environment
- When the substrate used for SCP process is a source of pollution, SCP production helps reduce pollution
- Strains having high biomass yields and a desirable amino acid composition can be easily selected or produced by genetic engineering
- Some SCPs are good sources of vitamins particularly B-group of vitamins, as well
- Mushrooms are considered as delicacy in the human diet
- At present, SCP appears to be the only feasible approach to bridge the gap between requirements and supply of proteins

7.3 MUSHROOMS

Mushrooms are the members of higher fungi, belonging to the class Ascomycetes (e.g., *Morchella*, *Tuber*, etc.) and Basidiomycetes (e.g. *Agaricus*, *Auricularia*, *Tremella*, etc.). They are characterized by having heterotrophic mode of nutrition. Mushrooms include both edible and poisonous mushrooms. The common edible mushrooms are: *Agaricus compestris, A. arvensis, Morchella esculanta, Volvaria terastius, Lepiota naucina, L. procera, Amanita rubescens, Armillaria mellea* etc. The poisonous mushrooms include *Amanita phalloides, A. muscaria, Clitocybe illudens, Lepiota*

morgani, Boletus satanas etc. the poisonous mushrooms differ from edible mushrooms due to the presence of the following features –

- Poisonous mushrooms have brightly-coloured fruit bodies
- They have an unpleasant taste and odour
- They generally grow on woody materials
- They produce pink coloured spores
- They do not thrive well in open places
- They grow well on leaf litter under the dense stories of plants

Mushrooms contain a large proportion of proteins, vitamins, organic acids and minerals. They contain a very low amount of fats, so that they form an important protein-rich food for diabetic patients and also for heart patients. They also contain a very low quantity of carbohydrates. Mushrooms are often named as vegetable meat. Use of mushrooms can contribute positively in facing the challenge of world-wide food shortage, originating with rapidly expanding human population at the rate of more than 2 lakh per day. Their value as food accessory is beyond the computation of the chemists and physiologists. They are among the most appetizing of the table delicacies and aid greatly to palatability of many food when cooked with them. The great value in promoting the cultivation of mushrooms lies in their ability to grow on cheap carbohydrate materials and to transform various waste materials. Different substrates used for the cultivation of variety of mushrooms are given in table 7.2.

Mushrooms	Substrate	Temp. for fruiting body formation
Agaricus bisporus (button or common edible mushroom)	Compost (horse manure, rice straw)	10—20°C
Auricularia spp.	Saw dust, rice bran	20—30°C
Coprinus spp.	Straw	20—40°C
Lentinus edodes (Shii-take)	Logs, saw dust, rice bran	12—20°C
Pleurotus ostreatus	Straw, paper, saw dust	10—20°C
Volvariella volvceae (Chinese or straw mushroom)	Straw, cotton	30—35°C

Table – 7.2 : Some of the mushrooms and their substrates

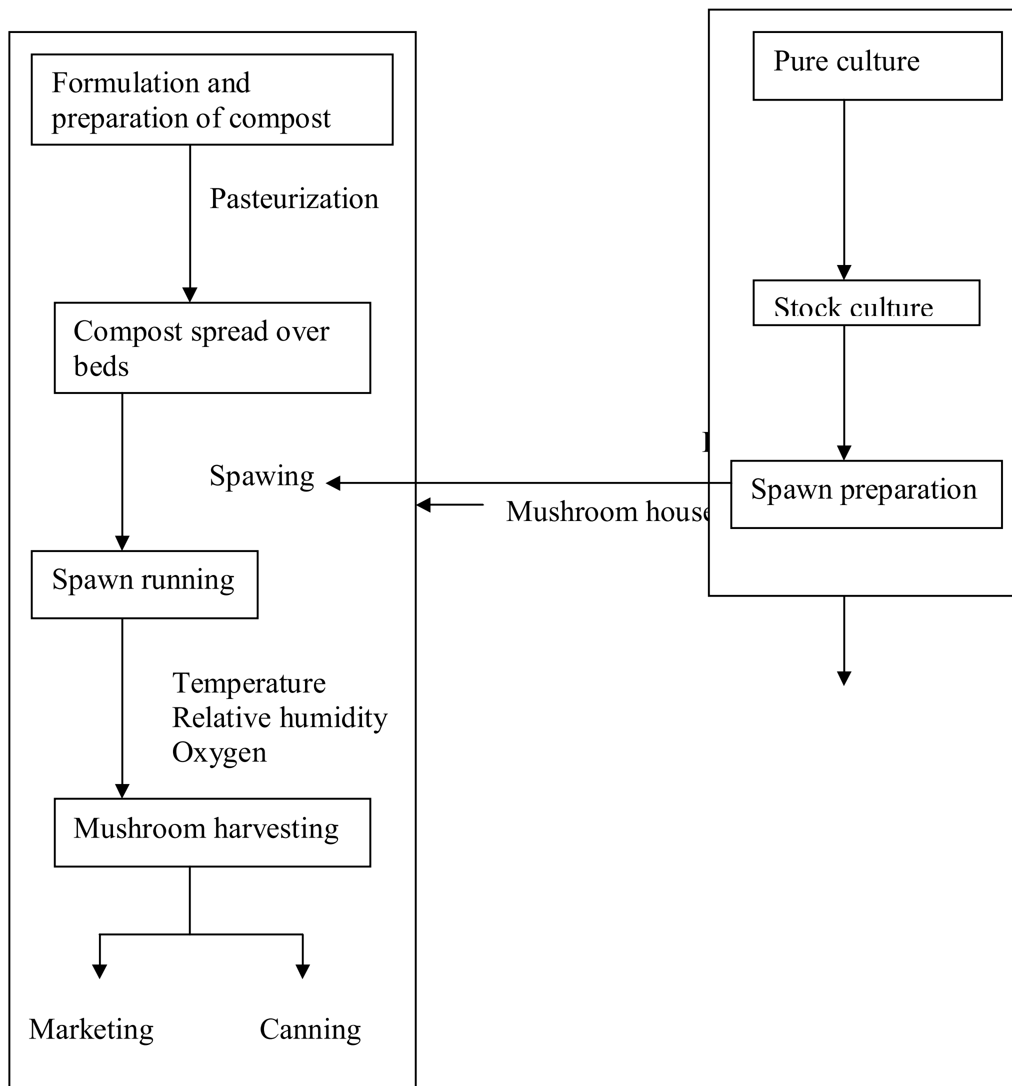
7.3.1 Nutritional value

Generally, mushroom contains 85-90% water of its dry matter. However, amount of water is greatly influenced by relative humidity and temperature during growth and storage. Protein is the most

critical component which contributes to a lot of nutritional value of food. Not all, only 34-89% mushroom proteins are digestible. Amount of protein varies from 34% to 44% of total dry weight in *Agaricus* spp. The crude fat content ranges from 1-20% of total dry weight. Besides protein, a large variety of free and combined fatty acids also occur in *A.bisporus* with high concentration of palmic acid, stearic acid and oleic acid. Fresh mushroom contains relatively large amount of carbohydrates i.e. 3-28%, particularly pentoses, hexoses, disaccharides, and trehlose. They appear as a good source of several vitamins (thiamine, riboflavin, niacin, biotin, ascorbic acid, vitamin A,B,C,D), minerals (sodium, potassium, calcium, iron, etc), essential amino acids (methionine, citralline, ornithin) and several undesirable elements (cadmium, selenium, chromium etc.).

7.3.2 Cultivation methods

The basic scheme of procedure for the cultivation of mushroom is given in the following figure 7.1.



Different countries have adopted different cultivation methods in accordance with facilities available and cost affecting gross production. In India, most of the edible mushrooms are cultured on paddy straw. The paddy straw is cut into small pieces of 3-5 cm long. The pieces of paddy straw are soaked in clean water for about 10 hours, during which the pieces of paddy straw become wetted. The wet paddy straw is hen properly sterilized by keeping it in boiling water for about 30 minutes. After proper sterilization, the sterile paddy straw is removed from the boiling water and allowed to drain off the excess water from itself. This sterile raw is used in the culture of mushrooms.

The spawn or seed is prepared from mature mushrooms and mixed with flour of sorghum to deep the spores in a viable condition. The seeds are also obtained from agricultural colleges or from agricultural universities. The sterile, sufficiently –wetted paddy straw is poured into a polythene bag, till the height of paddy straw reaches 5 cm in the bag. A bottle full of spawn is divided into 2 parts. Each half is used to culture one bag of mushrooms. It is again divided into 4 parts; of the four parts one portion is sprinkled over the layer of paddy straw in the polythene bag. Again paddy straw is filled to a height of 10 cm on the layer of spawn and again the second portion of spawn is sprinkled over the straw. This process is repeated 3 times. The mouth of the bag is then tied properly with a nylon thread. Similarly, a large number of culture bags are prepared.

After the preparation of the culture bags, they are kept in open wooden racks in a dark room. After a week, the polythene bags are cut longitudinally with a sterile knife. The culture is sufficiently wetted to favour the growth of the fungi. The temperature inside the dark room is adjusted around 25°C by timely spraying water in the room. Some workers simply make holes in the culture bags and have the bags hung in the dark room. After 18-20 days, small button-like fruit bodies will appear around the stock of paddy straw. After proper maturation, the mushrooms are harvested and readily marketed. Generally, mushrooms are harvested 3 times from each culture stack. Usually, harvesting can be carried out at a regular interval of one week. The yield is affected by compost, sawn, temperature, moisture etc.

7.3.3 Recipes of Edible mushrooms

Mushrooms are taken out from the mushroom house or procured from the market, washed with water to remove the adhering soil particles and outer portion is gently removed if stipe is hard. It is air-dried, if required dilute vinegar is mixed to check the blackening due to cutting with knife. Delicious recipes of mushrooms are prepared in different ways as required. Some of the recipes that include are mushroom puree, mushroom paneer, mushroom rice, mushroom omelette, mush room soup etc.

7.3.4 Advantages of mushrooms

Mushrooms offer some advantages like—

- They utilize substrates of poor value and are easy to produce
- They are delicious to eat and have good flavour
- They are rich in protein (about 51% on dry weight basis)

- They are rich in vitamins, particularly, nicotinic acid and riboflavin, and their vitamins are well preserved during cooking, drying etc.
- They are devoid of starch which makes them suitable for diets of diabetic individuals

7.4 SUMMARY

Microorganisms, besides their actions in fermentation as agents of physical and biological change, they themselves can be used as a food source. A variety of bacteria, yeasts, and other fungi have been used as animal and human food sources. Mushrooms are one of the most important fungi used directly as a food source. Large caves are used to maintain optimal conditions for the production of this delicacy. Microorganisms can be used directly as a food source or as a supplement to other foods and are then called single-cell protein. One of more popular microbial food supplements is the cyanobacterium, *Spirulina*. It is used as a food source in Africa and is now being sold world wide as a dried cake or powdered product. Therefore, in the light of protein shortage, microorganisms offer many possibilities for protein production. They can be used to replace totally or partially the valuable amount of conventional vegetable and animal protein feed. For this, development of technologies to utilize the waste products would play a major role for the production of SCP.

7.5 MODEL QUESTIONS

1. Write an essay on the production of SCP.
2. Describe the cultivation method of mushroom in India.
3. Explain the advantages and disadvantages of SCP in diet.

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M.Sc. MICROBIOLOGY (Final)

Lesson No. 8

FOOD POISONING AND FOOD-BORNE INFECTIONS

8.0 Objective
8.1 Introduction
8.2 Botulism
8.3 *Satphylococcus* food intoxication
8.4 Salmonellosis
8.5 *Clostridium perfringens* gastroenteritis
8.6 Vibriosis
8.7 Summary
8.8 Model questions
8.9 References

8.0 OBJECTIVE

This lesson plan is aimed to aware of how disease can be spread by foods and how such transmission can be prevented. So, highlights the contemporary agents of food-borne illness, including their habitat, source in foods, foods frequently involved, mechanism of pathogenicity and means of control of some important food borne diseases.

8.1 INTRODUCTION

The term "food poisoning" as applied to diseases caused by microorganisms, ordinarily used very loosely to include both illnesses caused by the ingestion of toxins elaborated by the organisms and those resulting from infection of the host through the intestinal tract. The disease that results from the ingestion of food is called as food-borne disease or food-borne illness. Most commonly food poisoning is defined as an acute gastroenteritis caused by the ingestion of food.

All food-borne diseases are sub-divided into poisonings and infections. Food poisonings can be the result of either chemical poisoning or the ingestion of a toxicant (intoxication). The toxicant might be found naturally in certain plants or animals or be a toxic metabolic product excreted by a microorganism. A bacterial food intoxication therefore refers to food-borne illnesses caused by the presence of a bacterial toxin formed in the food. The bacterial toxins that produce intoxications are exotoxins that may be either enterotoxins affecting the gut or neurotoxins affecting nervous system. A bacterial food infection refers to food-borne illnesses caused by the entrance of bacteria into the body through ingestion of contaminated foods and the reaction of the body to their presence or to their metabolites.

According to this classification, there are two chief kinds of food intoxications caused by bacteria – (1) botulism, caused by the presence in food of toxin produced by *Clostridium botulinum*, and (2) staphylococcal intoxication, caused by a toxin in the food from *Staphylococcus aureus.* On the other hand, food infections can be divided into two types namely – (1) those in which food does not ordinarily support growth of the pathogens but merely carries them, i.e., pathogens such as those causing tuberculosis, diphtheria, the dysenteries, typhoid fever, brucellosis, cholera, infectious hepatitis, Q-fever etc., and (2) those in which food can serve as a culture medium for growth of the pathogens to numbers that will increase the likelihood of infection of the consumer of the food. These pathogens include *Salmonella* spp., *Vibrio parahaemolyticus*, and enteropathogenic *E.coli*. Outbreaks of food infections of the second type are likely to be more explosive than outbreaks caused by other intestinal pathogens.

Botulism and staphylococcal intoxication are the best examples of intoxication category of food-borne diseases caused by bacteria. Of the bacterial food-borne infections, the best examples are Salmonellosis, *Clostridium perfringens* gastroenteritis and Vibriosis.

8.2 BOTULISM

The disease botulism is caused by the ingestion of food containing the neurotoxin produced by *Clostridium botulinum*.

Organism—C. botulinum is a gram-positive, saprophytic, spore-forming, gas forming, anaerobic, rod shaped, soil bacterium. On the basis of serological specificity of the toxins produced by the organism, seven types are distinguished. Of these, Type A, B, E and F have been identified to cause botulism in human beings. Type A is more toxic than Type B to humans. Toxin of Type C causes botulism of fowls, cattle and other animals. Type D is associated with forage poisoning of cattle. C.botulinum can be divided into proteolytic type which includes all strains of Type A and some strains of Type B and F or non-proteolytic that includes all strains of Type E and some strains of Type B and F.

Growth and toxin production—Toxin production by C. botulinum depends on the ability of the cells to grow and to autolyze in the food. For the Types A,B,E, and F toxins apparently are synthesized as large, comparatively inactive proteins which become fully toxic after some hydrolysis. Therefore, the factors that influence spore germination, growth, and hence toxin production are of special interest. These factors include the composition of the food or medium, especially its nutritive properties, moisture content, pH, O-R potential, and salt content, and the temperature and time of storage of the food. The combination of these factors determines whether growth can take place and the rate and extent of that growth.

Glucose or maltose, as one of the nutrients of medium, is known to be essential for toxin production. Meat, fish and low-or medium-acid canned foods have been shown to support toxin production and to differ in potency of the toxin formed. Media containing milk or casein, glucose or maltose and corn-steep liquor yield more potent Type A toxin than other media. A near neutral pH favours the growth of organism and at a pH of 4.5 or lower prevent the toxin production in most foods. Out breaks of botulism from inadequately heat-processed canned high-acid foods including tomatoes,

tomato juices, blackberries are due to the growth of other organisms which could increase the pH of a food so that *C. botulinum* could grow. The temperature requirements of Types A and B vary but the maximum growth temperature is 48°C. The optimum temperature for toxin production is 35°C for proteolytic strains and for non-proteolytic strains it is 26-28°C.

The toxin of *C. botulinum* is a protein of high potency and is absorbed in the small intestine and paralyzes the involuntary muscles of the body. Toxin is so powerful that only a tiny amount if sufficient to cause death. An estimated 50% lethal dose for humans is 1ng/kg body weight. In the laboratory, Type A toxin will be inactivated by heat treatment at 80°C for 5 to 6 minutes and Type B toxin will be inactivated at 90°C by 15 minute treatment. The toxin can be destroyed by gamma radiation of 7 to 8 Mrad. The toxin is also unstable at pH values above 6.8. The toxin can persist in foods for long periods especially when stored at low temperature.

Disease – People are so susceptible to botulism that if appreciable amounts of toxin are present in the ingested food. Consumption of very small pieces of food, a pod of string bean or few peas can cause illness and death. The incubation period for the appearance of typical symptoms of botulism is about 12 to 36 hours. The early symptoms include acute digestive disturbance followed by nausea and vomiting, diarrhoea, fatigue, dizziness and headache. Other symptoms include double vision and dryness of mouth and constriction of throat, swollen tongue and difficulty in swallowing and speaking. The toxin paralyses the involuntary muscles and paralysis spreads to heart and respiratory system and death results due to respiratory failure. In fatal cases, death usually occurs within 3-6 days after the ingestion of poisonous food. Administration of an antitoxin, particularly before the advent of symptoms, is the only known treatment for the disease.

Outbreaks – Mortality as high as 60% have been recorded in some outbreaks. In recent outbreaks mortality rate is lowered because of early diagnosis and administration of antitoxin. The conditions that are necessary for an outbreak of botulism include –

- Presence of spores of *C. botulinum* of Type A, B and E in the food being canned or being processed in some other way
- A food in which the spores can germinate and the clostridia can grow and produce toxin
- Survival of the spores of the organism because of inadequate heating in canning or inadequate processing otherwise
- Environmental conditions after processing that will permit germination of the spores and growth and toxin production by the organism
- Insufficient cooking of the food to inactivate the toxin
- Ingestion of toxin-bearing food.

Foods associated with outbreaks – foods with the following characteristics associate usually with the outbreak of botulism –

- low acid foods with pH above 4.6
- foods inadequately heat treated or not heat treated so that spores survive and competition is low

- high water activity above 0.93
- presence of insufficient preservative in foods to prevent the growth and spore germination
- foods have been held at temperature within the growth range of the organism

Several foods like canned or bottled fruits and vegetables, fermented fish and vegetable products, home-cured hams, canned fish, vacuum packed fish, smoked fish, salad dressings, chilli sauce, tomato relish, cooked potatoes, mushrooms etc., give rise to botulism outbreaks.

Prevention of outbreaks – the methods and precautions for prevention include

- use of approved heat processes for canned foods
- rejection of all gassy or otherwise spoiled canned foods
- steps should be taken by manufacturer to prevent post process recontamination of commercially sterile low acid foods
- ensure that cured meats including those received a pasteurization heat treatment should contain levels of sodium chloride, sodium nitrite or other preservative that can prevent the growth of *C. botulinum*
- good sanitation be maintained throughout the production and handling to prevent botulism from smoked fish
- fish be frozen immediately after packaging and kept frozen
- refusal of a doubtful food even to taste

8.3 STAPHYLOCOCCUS FOOD INTOXICATION

This is most commonly occurring food poisoning caused by the ingestion of enterotoxin formed in food during the growth of certain strains of *Staphylococcus aureus*. The toxin is termed as enterotoxin because it causes gastroenteritis or inflammation of the lining of the intestinal tract.

Organism – *S. aureus* is gram-positive cocci occurring in the shape of grape clusters. Colonies are usually in yellow or golden colour but some are unpigmented. Most enterotoxin producing *S. aureus* cultures are coagulase positive and facultative in their O_2 requirement. But grow better aerobically than anaerobically. Some toxigenic cocci are very salt tolerant and also fairly well tolerant to nitrites. Therefore, if other environmental conditions are favourable, they can grow in curing solutions and on curing and cured meats. *S.aureus* is fermentative and proteolytic. Depending on the food type the range of temperature that is required for growth and toxin production is about 4-46°C. Maximum pH required for growth is about 8.0 and the minimum level of required is lower under aerobic conditions than under anaerobic conditions. Minimal a_w required is 0.86 and 0.90 in aerobic and anaerobic conditions, respectively. *S.aureus* produces six serologically distinct enterotoxins named as A, B, C, C_2, D and E that differ in their toxicity.

Enterotoxin – The staphylococcal enterotoxins are simple proteins with molecular weights between 26,000 and 30,000 daltons. The single polypeptide chains are cross-linked by a disulfide bridge to form a characteristic cystine loop, which is the toxic part of the molecule. Production of toxin occurs best at 40°C. Enterotoxins A and D more often associate with food-poisoning outbreaks.

Enterotoxins are stable toward heat and pasteurization and ultra high-temperature heating is not sufficient to inactivate the toxin. Normally cooked foods, even in the absence of like bacteria in the food, might cause poisoning as the cooking process can not destroy the toxin formed in the food. Toxin at a dose of 1ng/g is sufficient to cause disease.

Disease – Individuals differ in their susceptibility to staphylococcus poisoning. So, among the people ingested the toxin contained food, some may become ill, some may affected little and some may not at all affected. Organism causes intoxication due to the ingestion of an enterotoxin secreted into the food during its growth. The presence of the live organism in ingested food is irrelevant to disease production. The incubation period for poisoning is 2-4 hours. Time to the onset of symptoms depends on the amount of toxin consumed and individuals' susceptibility. The most common human symptoms are salivation, then nausea, vomiting, retching, abdominal cramping of varying severity, and diarrhea. In severe cases, blood and mucus may be found in stools and vomitus. Headache, muscular cramping, sweating, chills, prostration, weak pulse, shock, and shallow respiration may occur. Usually a subnormal body temperature is found rather than fever. The duration is from few hours to 3 days and later recovery occurs. Mortality is rare and extremely low.

Outbreaks – the outbreaks of food poisoning often are attributed to staphylococci on the basis of the type of food involved, the short incubation period, and perhaps the demonstration of the presence of staphylococci in the food. An actual diagnosis of the poisoning would depend, however, on isolation of staphylococci and demonstration that these produce enterotoxin or isolation and detection of the enterotoxin.

Foods involved in outbreaks –

- cooked meats and meat products – Eg: ham, liver sausage etc.
- foods containing milk or cream – Eg: sauces, cakes, custrard, cheese
- poultry meat and meat products
- pre-cooked fish and fish products
- pre-cooked crustaceans
- canned foods – Eg: peas, meat, mushrooms etc.

Conditions necessary for outbreaks

- food must contain enterotoxin producing staphylococci
- food must be a good culture medium for growth and toxin production
- temperature must be favourable to growth and enough time for toxin production
- enterotoxin containing food must be ingested

Prevention of outbreaks

- prevention of contamination of food with staphylococci
- prevention of growth of staphylococci
- killing staphylococci in foods

- avoid direct handling of foods
- ensure the high-risk foods are rapidly cooled to below 5°C after cooking
- good personal hygiene without cuts on hands or dust

8.4 SALMONELLOSIS

Salmonellosis may result following the ingestion of viable cells of Salmonella spp. It is the most frequently occurring bacterial food infection. Over 2000 serovars that are pathogenic for humans are known to be carried by a variety of animals are responsible for salmonella food poisoning.

Organism – Salmonella is gram-negative and non-spore forming rod. They can ferment glucose with gas formation but do not ferment lactose or sucrose. Salmonellae can grow over a wider range of temperature (10-45°C) and pH (4-9) in a good culture medium rather than in a poor medium. Minimum a_w required is about 0.93. Depending on the type and nature of food, organisms differ in their heat resistance. Holding the food at 66°C for about 12 minutes of time kills the cells. Primary source of organisms include human beings, animals and animal food products.

Disease – the disease caused by *Salmonella* is known as salmonellosis or salmonella enteritis or salmonella food poisoning. The organism penetrates epithelial cells of villi in the lower part of the small intestine entering the connective tissue below, where they stay and multiply. Endotoxins are released giving rise to the disease syndrome. As with the other infectious diseases, individuals differ in their susceptibility to *Salmonella* infections, but in general morbidity is high in any outbreak. Susceptibility of humans varies with the species and strain of the organism and total number of bacteria ingested. Incubation period is 12-36 hours which distinguishes from 2-4 of staphylococci poisoning. In some salmonella infections, the incubation period may be as short as 5 hours and as long as 72 hours.

The principal symptoms of a *Salmonella* gastrointestinal infection are nausea, vomiting, abdominal pain, and diarrhea that usually appear suddenly. This may be preceded by a headache and chills. Other evidences of disease are watery, greenish foul-smelling stools, prostration, muscular weakness, faintness, usually a moderate fever, restlessness and drowsiness. Intensity may vary from slight discomfort and diarrhoea to death in 2-6 days. Laboratory diagnosis of the disease is difficult unless *Salmonella* can be isolated from the suspected food and from the stools of individuals. The infective dose required is influenced by host susceptibility, virulence of *Salmonella* serovar, type of food in which organism ingested. The mortality is less, but significant in young.

Foods involved in outbreaks

- meats, poultry and their products, fish and fish products, milk and milk products
- raw and undercooked eggs and products in which egg is used, canned ham, pork, imported chocolate bars etc.

Conditions required for outbreaks

- food must contain or become contaminated with *Salmonella* bacteria
- bacteria must be there in considerable numbers due to high contamination or more growth in food
- organisms ingested should be viable

Prevention of outbreaks

- avoidance of contamination of food with salmonellae from diseased humans or animal sources and carriers and ingredients carrying the organisms
- destruction of organisms in foods by heat by pasteurization or cooking
- prevention of growth of *Salmonella* in foods by adequate refrigeration or by other means
- in prevention of contamination, care and cleanliness in food handling and preparation are important.
- food handlers should be healthy and clean and not be the carriers
- ingredients used in foods should be free of salmonellae

8.5 CLOSTRIDIUM PERFRINGENS GASTROENTERITIS

This illness is first reported in the United States in 1945. The disease occurs due to the enterotoxin released in the gut during sporulation of the cells.

Organism – *C. perfringens* is a gram-positive, non-motile, anaerobic, spore forming rod. The organism grows in the temperature range of 20 to 55°C with optimal temperature between 43 to 47°C and in a pH range of 5.0 to 9.0. Growth of the organism is inhibited by 5% NaCl. The spores of food-poisoning strains differ considerably in their heat resistance. Basing on the enterotoxin produced, five types designated as A, B, C, D, and E are recognized. *C. perfringens* type A causes the vast majority of food poisoning outbreaks. Of the other types, only type C has caused food-borne disease namely necrotic enteritis.

Disease – The disease is caused by enterotoxin that is released into the gut when mother cells release their spores. Enterotoxin is relatively heat-sensitive being inactivated at 60°C for 10 minutes. For the occurrence of disease, live cells are required. The symptoms appear usually in 8 to 24 hours following ingestion. The various symptoms of the disease include abdominal pain, diarrhea, gas. The other rare symptoms include fever, nausea and vomiting.

Outbreaks – Most outbreaks suggest that the ingestion of millions of viable cells of *Clostridium perfringens* per gram of food are required for symptoms to occur. The minimum number of cells required are 7×10^5 and as high as 10^8 or 10^9. Mortality is very low. Death is occasionally reported in the elderly and people hospitalized with other diseases. The cells will grow rapidly when other competing organisms are killed by heating.

Foods involved in outbreaks

- meats (mainly beef) or poultry that have been cooked and then held at room temperature or under warm conditions or in bulk in refrigerators in which cooling is slow
- boiled salt beef, minced beef , boiled chicken, meat and chicken curry, fish paste
- meat and poultry products account for about 3 quarters of outbreaks attributed to *Clostridium perfringens*

Conditions for outbreaks

- food contains or becomes contaminated with *Clostridium perfringens*
- usually the food is cooked and reduced conditions develop
- food is inadequately cooled and favourable temperatures and enough time are allowed for appreciable growth
- food is consumed without reheating so that large number of viable cells are ingested
- the cells sporulate in vivo and elaborate the enterotoxin

Prevention of outbreaks

- adequate and rapid cooling of cooked meats and other foods
- holding hot foods above 60°C
- reheating of leftover foods
- good personal hygiene

8.6 VIBRIOSIS

Vibriosis, a food-poisoning syndrome caused by *V. parahaemolyticus* is commonly referred as *V. parahaemolyticus* gastroenteritis and is contracted almost solely from sea food. In addition to its role in gastroenteritis, *V. parahaemolyticus* is known to cause extraintestinal infections in humans.

Organism- *V. parahaemolyticus* is a marine bacterium widely found throughout the world in warm coastal and estuarine waters, sediments and plankton. It is primarily associated with coastal inshore waters rather than the open sea. It is a Gram –ve, straight or curved rod which is motile by its polar flagella. It requires NaCl and grows optimally at 3% NaCl but will grow at levels between 0.5% and 8%. The minimum a_w for growth varies between 0.937 and 0.986. The growth temperature range from 22°C– 42°C with an optimum of 37°C. It grows best at pH values slightly above neutrality (7.5 - 8.5) but also able to grow in alkaline conditions up to a pH of 11.0 and down to a pH of 5.0. It cannot be isolated when the water temperature is below 15°C and cannot survive pressures encountered in deeper waters. The survival of the organism through winter months when water temperatures drop below 15°C has been attributed to its persistence in sediments from where it may be recovered even when water temperatures are below 10°C.

Disease – the disease is caused by *V. parahaemolyticus* by the ingestion of live organisms. The pathogenicity of *V. parahaemolyticus* strains is strongly linked to their ability to produce a 22 kDa,

thermostable, extracellular haemolysin. This haemolysin can lyse the fresh human or rabbit blood cells but not those of horse blood and this phenomenon is known as Kanagawa reaction. The haemolysin has also been shown to have enterotoxic, cytotoxic and cardiotoxic activity. Most of the strains from patients with *V. parahaemolyticus* food poisoning produce the haemolysin and are designated Kanagawa positive (Ka^+) while 99% of the environmental isolates are Kanagawa negative (Ka^-). The incubation period varies from 2 to 4 days though it is usually 9-25 h. The symptoms include diarrhoea with severe abdominal pain, nausea, vomiting, mild fever, head ache and prostration. The illness may persist up to 8 days. Normally, recovery occurs within a few days. The mortality is low and majority of deaths occur in elderly debilitated people. A dose of 10^5-10^7 viable cells may require for the infection.

Outbreaks – The outbreaks of *V. parahaemolyticus* gastroenteritis have received considerable attention in Japan, where it is one of the most commonly occurring food poisoning syndromes. About 50-70% of the outbreaks of food borne gastroenteritis that occur in Japan are found to be due to *V. parahaemolyticus*. In recent years, this organism has been responsible for several food-borne outbreaks in the United States.

Foods involved in outbreaks

- Raw fish and raw shellfish including the oysters, shrimp, crabs, clams, lobsters etc., are the main foods.
- Occasionally, cooked fish and salted vegetables have been involved when cross contamination with raw fish has taken place.

Prevention of outbreaks

- Ice or refrigerate the fish immediately after harvesting and keep at low temperature until consumed.
- Avoid eating raw fish and raw shellfish.
- Cook the fish adequately, as proper cooking readily kills the organism.
- Take care to prevent cross contamination from raw fish to cooked fish.
- Avoid the use of seawater for rinsing foods to be eaten raw or for cleaning.

8.7 SUMMARY

A Foodborne disease has been defined by the World Health Organization (WHO) as 'Any disease of an infectious or toxic nature caused by, or thought to be caused by, the consumption of food or water'. This definition includes all food and waterborne illness and is not confined to those primarily associated with the gastro-intestinal tract and exhibiting symptoms such as diarrhoea and / or vomiting. The cause of food borne diseases is mainly the ingestion of infected food. Apart from the gastrointestinal problems and related illness due to improper diet, nutritional deficiencies and overeating, the causes of food borne illness may be due to either (i) food infection or (ii) food intoxication. The entry of pathogenic organisms and parasites into the body and the reaction of the body to the presence of organisms or their metabolites cause food infection. Pathogenic organisms

make their entry through the food chain i.e. by consuming foods contaminated with such organisms. Bacterial food infection refers to food borne illness caused by the entry of bacteria into the body. Food intoxication or poisoning is caused by consuming food contaminated with toxic substances. *Staphylococcus* intoxication and Botulism of food borne intoxications and Salmonellosis, *Clostridium perfringens* illness of food borne infections are the important examples of bacterial food borne diseases.

8.8 MODEL QUESTIONS

1. Write an account on food poisoning and food borne infections with suitable examples.
2. Define food intoxication? Explain the botulism and Staphylococcus intoxications.
3. What is food borne infection? Explain it with reference to Clostridium perfringens gastroenteritis and Salmonellosis.
4. Write short notes on the following:
    1. Botulism
    2. *Staphylococcus* food intoxication
    3. Salmonellosis
    4. *Clostridium perfringens* gastroenteritis
    5. *Vibrio parahaemolyticus* gastroenteritis

8.9 REFERENCES

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M.Sc. MICROBIOLOGY (Final)

Lesson 9

FERMENTATION PROCESSES

9.0 **OBJECTIVE:**
The component parts of a fermentation process and the range of fermentation processes are described in this lesson.

9.1 INTRODUCTION

9.2 COMPONENT PARTS OF A FERMENTATION PROCESS

9.3 RANGE OF FERMENTATION PROCESSES

9.3.1 Microbial biomass

9.3.2 Microbial enzymes

9.3.3 Microbial metabolites

9.3.4 Recombinant products

9.3.5 Transformation process

9.4 SUMMARY

9.5 MODEL QUESTIONS

9.6 REFERENCE BOOKS

9.1 INTRODUCTION

Microorganisms have been used extensively to provide a wide range of products. They are proved to be particularly useful because of the ease of their mass cultivation, high growth rate, use of cheap substrates and the diversity of potential products. Their ability to readily undergo genetic manipulation has opened up many possibilities for new products and services from the fermentation industries.

The production of alcohol by the action of yeast on malt or fruit extracts has been carried out on a large scale for many years. It was the first industrial process for the production of a microbial

metabolite. In fact, the term '**fermentation'** derived from the Latin verb *fervere* (to boil) describes the appearance of the action of yeast on extracts of fruit or malted grain. The boiling appearance is due to the production of carbon dioxide bubbles caused by the anaerobic catabolism of the sugars present in the extract. Industrial microbiologists have extended the term fermentation to describe any process for the production of product by the mass culture of a microorganism. It thus includes aerobic as well as anaerobic processes.

Industrial fermentations comprise both upstream processing (USP) and downstream processing (DSP) stages. USP involves all the factors and processes leading to the fermentation. It consists of three main features:

1) Aspects related to the producer microorganism
2) Selection of suitable fermentation media
3) Fermentation process which is performed under controlled conditions.

DSP includes all the processes following the fermentation. Its main aim is to recover the target product to the required specifications (biological activity, purity etc.), while maximizing the recovery yield and minimizing the costs.

9.2 COMPONENT PARTS OF A FERMENTATION PROCESS

An established fermentation process can be divided into six basic component parts:

(1) Production of an active, pure culture of the microorganism in sufficient quantity to inoculate the production vessel.

(2) Formulation of media to be used in culturing the process microorganism.

(3) Sterilization of culture medium, fermentor and ancillary equipment.

(4) Culture the organism in the production fermentor under optimum conditions for product formation.

(5) Recovery and purification of the product produced by microorganisms.

(6) Disposal of effluents produced by the process.

A generalised schematic representation of a typical fermentation process is shown in Fig. 9.1. Traditional fermentations were performed by a mixture of wild microorganisms existing on the raw materials or in local environment. The fermentation processes which developed later mostly used monocultures. The specific microorganisms employed can be isolated from the natural environment or acquired from culture collections. Most of these microorganisms have been subsequently modified by strain improvement strategies to get high yields of the product.

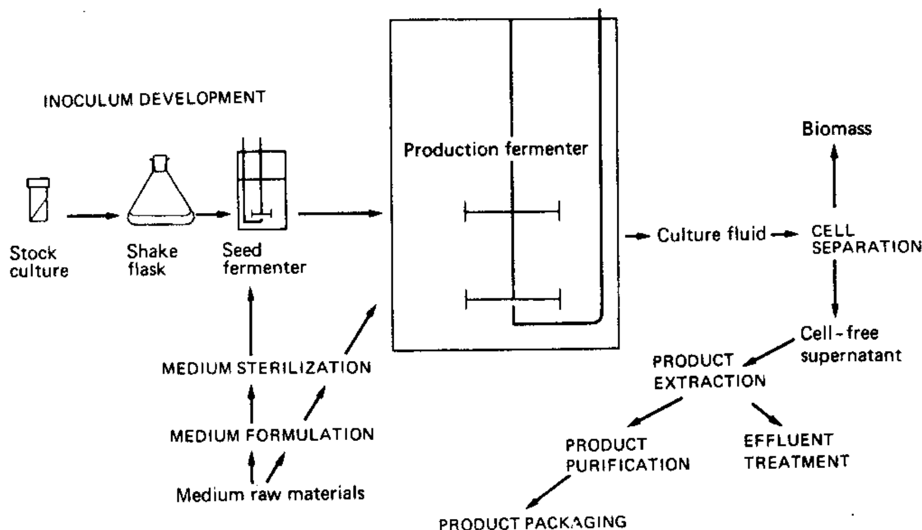

Fig. 9.1 Schematic representation of a fermentation process

The formulation of a culture medium is another essential step in the fermentation process. The constituents of a medium must satisfy the nutritional requirements of the microorganism. The nutrients should be formulated to promote the synthesis of the target product either cell biomass or a specific metabolite. In many industrial processes, the media are required in several stages. They include starter culture propagation steps, pilot-scale and the production fermentations. Most fermentations require liquid media except solid substrate fermentations in which solids are the substrates.

The vessel in which the industrial process is carried out is called a **fermentor**. Fermentors can vary in size from the small 5- to 10- liter laboratory scale to the enormous 500,000 – liter industrial scale. The size of the fermentor used depends on the process and how it is operated. Industrial fermentors can be divided into two major classes, those for aerobic processes and those for anaerobic processes. Anaerobic fermentors require little special equipment while aerobic fermentors require much more elaborate equipment to ensure adequate mixing and aeration.

A fermentation product is produced by the culture of a certain microorganism in a nutrient medium. If the fermentation is invaded by a foreign microorganism, the following consequences may occur:

a) The contaminant may outgrow the production organism and displace it from the fermentation medium.

b) It may contaminate the final product or it may produce compounds which make subsequent extraction of the final product difficult.

c) It may degrade the desired product.
d) Contamination of a bacterial fermentation with phage results in the lysis of the culture.
e) The medium would have to support the growth of production microorganism as well as contaminant resulting in a loss of productivity.

In order to avoid contamination, pure inoculum must be used to start the fermentation; sterilization of the culture medium and the fermentor vessel should be carried out and aseptic conditions should be maintained during the fermentation.

Industrial fermentations are operated as batch, fed-batch or continuous cultures. Most are batch processes (closed systems), in which there is a definite beginning and end to the process. A fermentor is loaded, sterilized and inoculated with the suitable strain. The organism is grown through a typical batch profile and the product is harvested at the end. Production of alcoholic beverages, most amino acids, enzymes, organic acids etc., can be the examples for this mode of operation. In fed-batch mode of operation, extra nutrients are added as the fermentation progresses, which increases the fermentation volume. Fed-batch operation can extend the product formation phase. Continuous culture is an open system where fresh medium is continuously added and culture is simultaneously removed at the same rate, resulting in a constant working volume. Continuous fermentations are particularly suitable for the production of biomass and growth-associated primary metabolites.

The extraction and purification of fermentation products may be difficult and costly. Recovery costs of microbial products may vary from 15 to 70% of the total manufacturing costs. The recovery and purification of many compounds may be achieved by a number of alternative routes. The decision to follow a particular approach involves comparing the factors such as capital costs, processing costs, yield potential, product quality and waste treatment needs to determine the most appropriate under a given set of circumstances.

Along with the desired product, varying amounts of waste materials are also produced during fermentations. Typical wastes include unconsumed inorganic and organic media components, microbial cells and other suspended solids, filter aids, waste wash water, water containing traces of solvents, acids, alkalis etc. Earlier, these wastes were disposed of directly to a convenient area of land or into a nearby water course. This method is not desirable as it creates pollution.

Fermentation effluents usually do not contain toxic materials which directly affect the aquatic flora or fauna. But they contain high levels of organic matter which are readily oxidized by microbial attack and drastically deplete the dissolved oxygen concentration in the receiving water. Metabolites or components of some fermentation effluents may be extremely toxic and polluting and require complete destruction before disposal. Hence, the effluent treatment is considered to be important. Fermentation wastes may be treated on site or at a sewage treatment works by any or all of the following methods: physical treatment, chemical treatment and biological treatment.

9.3 RANGE OF FERMENTATION PROCESSES

Fermentation processes have been widely used for the production of many microbial products (Table 9.1). Five major groups of commercially important fermentations are recognized. They include:

(a) Fermentations that produce biomass as the product

(b) Fermentations that produce microbial enzymes

(c) Fermentations that produce microbial metabolites

(d) Fermentations that produce recombinant products

(e) Fermentations involved in transformation process

Table 9.1. Some examples of industrial fermentation products produced by microorganisms

Products	Microorganisms
Traditional Products:	
Bread, Beer, wine and spirits	- Mostly *Saccharomyces cerevisiae*
Cheese and other dairy products	- Lactic acid bacteria
Ripening of blue and camembert-type cheese	- *Penicillium* spp.
Fermented meats and vegetables	- Mostly lactic acid bacteria
Mushrooms	- *Agaricus bisporus, Pleurotus* spp. *Volvariella volvacea*
Soy sauce	- *Aspergillus oryzae, Zygosaccharomyces rouxii*
Vinegar	- *Acetobacter* spp.
Agricultural products	
Gibberellins	- *Fusarium moniliforme*
Insecticides	- *Bacillus thuringiensis*
Silage	- Lactic acid bacteria
Amino acids:	
L-Glutamic acid	- *Corynebacterium glutamicum*
L-lysine	- *Brevibacterium lactofermentum*
L-tryptophan	- *Klebsiella aerogenes*

Enzymes:

α - amylase	- *Bacillus subtilis*
β - amylase	- *Aspergillus niger*
Amyloglucosidase	- *Aspergillus niger*
Glucose isomerase	- *Streptomyces olivaceus*
Invertase	- *Kluyveromyces* spp.
Cellulase	- *Trichoderma viride*
Alkaline proteases	- *Bacillus licheniformis*
Neutral proteases	- *Aspergillus oryzae*
Acid proteases (microbial rennet)	- *Rhizomucor miehei*

Fuels and Chemical feed stocks:

Acetic acid	- *Acetobacter* spp.
Citric acid	- *Aspergillus niger*
Fumaric acid	- *Rhizopus spp.*
Gluconic acid	- *Acetobacter suboxydans*
Itaconic acid	- *Aspergillus itaconicus*
Kojic acid	- *Aspergillus flavus*
Lactic acid	- *Lactobacillus* spp.

Pharmaceuticals and related products

Alkaloids

Ergotamine	- *Claviceps purpurea*
Ergometrine	- *Claviceps fusiformis*
D-lysergic acid	- *Claviceps paspali*

Antibiotics:

Bacitracin	- *Bacillus licheniformis*
Cephalosporins	- *Cephalosporium acremonium*
Chlortetracycline	- *Streptomyces aureofaciens*
Erythromycin	- *Streptomyces erythraeus*
Penicillins	- *Penicillium chrysogenum*
Streptomycin	- *Streptomyces griseus*

Hormones:

Human growth hormone	- Recombinant *Escherichia coli* Recombinant *Saccharomyces cerevisiae*
Insulin	- Recombinant *E. coli* Recombinant *S. cerevisiae*

Immunosuppressants

Cyclosporin	- *Trichoderma polysporum*
Interferon	- Recombinant *E. coli*
	Recombinant *S. cerevisiae*
Steroids	- *Arthrobacter* spp.
	Rhizopus spp.
Vaccines	- *Bacillus anthracis*
	Clostridium tetani
	Recombinant *E. coli*
	Salmonella typhi

Vitamins:

B12 (Cyanocobalamin)	- *Pseudomonos denitrificans*
β-carotene	- *Blakeslea trispora*
Riboflavin	- Recombinant *Bacillus subtilis*
	Ashbya gossypii

Polymers:

Alginates	- *Azotobacter vinelandii*
Dextran	- *Leuconostoc mesenteroides*
Polyhydroxybutyrate	- *Ralstonia eutropha*
Pullulan	- *Aureobasidium pullulans*
Scleroglucan	- *Sclerotium rolfsii*
Xanthan	- *Xanthomonas campestris*

Single Cell Protein	- *Candida utilis*
	Kluyveromyces marxianus
	Paecilomyces varioti
	Saccharomyces cerevisiae
	Methylococcus capsulatus
	Methylophilus methylotrophus

9.3.1 Microbial biomass

The major processes which involve the commercial production of microbial biomass include the production of yeast to be used in baking and brewing industries and the production of microbial cells to be used as single-cell protein and for several other purposes. Yeast cells can also be used as sources of food, vitamins and other growth factors.

9.3.2 Microbial enzymes

Enzymes are produced commercially from both fungi and bacteria. The microbial enzymes produced in very large amounts on an industrial basis are the bacterial proteases to be used as additives in laundry detergents. Other important enzymes manufactured commercially include amylases and glucoamylases, which are used in the production of glucose from starch. Some of the enzymes produced by microorganisms are also used in the food, dairy, pharmaceutical and textile industries.

9.3.3 Microbial metabolites

The primary metabolites such as amino acids, nucleotides, proteins, nucleic acids, lipids and carbohydrates produced by microorganisms during the log phase of the growth are commercially important and are being produced by fermentation. Amino acids have extensive uses in the food industry, in medicines, as feed additives and also as starting materials in the chemical industry. Glutamic acid is the most important commercial amino acid which is used as a flavor enhancer. Lysine, an essential amino acid for humans is commercially produced by *Brevibacterium*. Two other important amino acids, aspartic acid and phenylalanine, are the ingredients of the artificial sweetener aspartame.

A number of organic chemicals are produced commercially by use of microorganisms. For example, citric acid (used widely in foods and beverages), itaconic acid (used in the manufacture of acrylic resins) and gluconic acid (used in the form of calcium gluconate to treat calcium deficiencies in humans) are produced by fungi and bacteria. Vinegar is the product resulting from the conversion of ethyl alcohol to acetic acid by acetic acid bacteria *(Acetobacter* and *Gluconobacter*). Vinegar is used as a flavoring ingredient in salads and other foods. It is also used in pickling because of its high acidity.

Vitamins are used as supplements for human food and animal feed. Vitamin B_{12} is synthesized in nature exclusively by microorganisms. Members of the bacterial genera *Propionibacterium* and *Pseudomonas* are the main commercial producers of Vitamin B_{12}. Riboflavin is synthesized by many microorganisms including bacteria, yeasts and fungi. *Ashbya gossypii* produces huge amounts of this vitamin and is therefore used for commercial processes.

During the stationary phase, some microbial cultures synthesize compounds which are not produced during the trophophase (equivalent to the log or exponential phase). These compounds are called secondary metabolites and the phase in which they are produced is referred to as the idiophase (equivalent to the stationary phase). Many secondary metabolites have antimicrobial activity, others are specific enzyme inhibitors and some are growth promoters.

Antibiotics are chemical substances produced by microorganisms that kill or inhibit the growth of other microorganisms. Antibiotics are typical secondary metabolites. Commercially useful antibiotics are produced by Actinomycetes and filamentous fungi. The best known and probably the

most medically important antibiotics are the β-lactams (penicillins and cephalosporins) along with aminoglycosides (streptomycin and tetracyclines). Some antibiotics like actinomycin, mitomycin etc., have roles as antitumor agents. Some can be used for controlling microbial diseases of crop plants or as tools in biochemistry and molecular biology research. Several antibiotics are also added to animal feed as growth promoters.

9.3.4 Recombinant Products

The transfer of DNA between different species of bacteria has been achieved experimentally using both *in vivo* and *in vitro* techniques. Thus, genetic material derived from one species may be incorporated into another where it may be expressed. The construction of strains capable of synthesizing foreign proteins is one of the important applications of recombinant DNA technology in the context of fermentation. Genes from higher organisms may be introduced in to microbial cells such that the recipients are capable of synthesizing foreign (or heterologous) proteins.

A wide range of microbial cells have been used as hosts for these systems including *Escherichia coli, Saccharomyces cerevisiae* and filamentous fungi. Interferon, insulin, human growth hormone, erythropoietin, calf chymosin and bovine somatostatin are some of the products produced by the genetically engineered microorganisms.

9.3.5 Transformation Processes

Microorganisms have the ability to chemically modify a wide variety of organic compounds. Microorganisms can behave as chiral catalysts with high positional specificity and stereospecificity. The reactions which may be catalysed include dehydrogenation, oxidation, hydroxylation, dehydration and condensation, decarboxylation, amination, deamination and isomerization. Microbial processes have the aditional advantage over chemical reagents of operating at relatively low temperature and pressures.

The use of microorganisms in the biotransformation of compounds has been particularly successful in the manufacture of therapeutic steroids that are used for the treatment of allergies, inflammation, skin diseases and as oral contraceptives. Many steroids are now manufactured using a combination of chemical and microbial transformation steps (Table 9.2).

The microorganisms involved in these processes are mostly filamentous fungi such as *Aspergillus, Curvularia, Fusarium* and *Rhizopus* and mycobacteria, in the form of suspensions or immobilized cells, spores and cell-free extracts. Microbial processes are highly specific and enable the addition, removal or modification of functional groups at specific sites on a complex molecule. These processes have the additional advantage over chemical reagents of operating at relatively low temperatures and pressures.

Table 9.2. Some examples of microbial transformation processes

Biotransformation	**Substrate**	**Product**	**Microorganism**
I-Dehydrogenation	Hydrocortisone	Prednisolone	*Arthrobacter simplex*
II α-Hydroxylation	Progesterone	II α-Hydroxy-Progesterone	*Rhizopus nigricans*
II β-Hydroxylation	Reichstein compound S	Hydrocortisone	*Curvulava lunata*
Side-chain cleavage	β-sitosterol	Androstadienedione	*Mycobacterium* species

9.4 SUMMARY

The term fermentation has been used to describe any process in which a product can be produced by mass culturing of a microorganism. A wide range of products such as antibiotics, amino acids, enzymes, organic acids, polymers and vitamins can be obtained from microorganisms. The advent of recombinant DNA technology has extended the range of potential fermentation products. Products produced by the genetically engineered microorganisms include interferon, insulin, human growth hormone, calf chymosin and bovine somatostatin. Production of high-value compounds such as steroids, antibiotics and prostaglandins can be made possible through microbial transformations.

9.5 MODEL QUESTIONS

1. Discuss the component parts of a fermentation process.
2. Describe the range of products produced by microbial fermentations.
3. Write short notes on:
   (a) Microbial enzymes
   (b) Recombinant products
   (c) Transformation processes

9.6 REFERENCE BOOKS

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M.Sc. MICROBIOLOGY (Final)

Lesson 10

F E R M E N T O R

10.0 **OBJECTIVE:**

In this lesson, design of fermentor and different types of fermentors employed for culturing microorganisms in industrial processes are discussed.

10.1 INTRODUCTION
10.2 DESIGN OF A FERMENTOR
10.3 BODY CONSTRUCTION
10.4 TEMPERATURE CONTROL
10.5 AERATION AND AGITATION
10.6 ACHIEVEMENT AND MAINTENANCE OF ASEPTIC CONDITIONS
10.7 VALVES AND STEAM TRAPS
10.8 AIR-LIFT FERMENTORS
10.9 BUBBLE COLUMN BIOREACTORS
10.10 SUMMARY
10.11 MODEL QUESTIONS
10.12 REFERENCE BOOKS

10.1 INTRODUCTION

In industrial processes, microorganisms are cultured in specially designed vessels called Fermentors. The main function of a fermentor is to provide a controlled environment for the growth of microorganisms to obtain a desired product which may be cell biomass, a metabolite or bioconversion product. The performance of any fermentor depends on many factors, but the key physical and chemical parameters that must be controlled are agitation rate, oxygen supply, pH, temperature and foam production.

10.2 DESIGN OF A FERMENTOR

The design of the fermentor depends upon the purpose for which it is to be utilized. Many fermentors have been designed for use in submerged fermentation on a laboratory, pilot-plant and industrial scale. Fermentors are usually complicated in design, as they have to provide a suitable environment in which an organism can efficiently produce a target product. While designing and constructing a fermentor a number of points must be considered.

(a) The vessel should be capable of being operated aseptically for a number of days. It should be reliable in long-term operation.

(b) It should provide adequate aeration and agitation to meet the metabolic requirements of the microorganism.

(c) It should be provided with systems for temperature control, pH control and for sampling facility.

(d) It should be suitable for a range of fermentation processes.

(e) It should be designed to require the minimal use of labour in processes such as operation, harvesting, cleaning and maintenance.

The design of a fermentor is a multi-disciplinary approach as it involves cooperation between experts in microbiology, biochemistry, chemical engineering, mechanical engineering and costing. Industrial fermentors can be divided into two major classes, those for anaerobic processes and those for aerobic processes. Anaerobic fermentors require little special equipment whereas aerobic fermentors require much more elaborate equipment to ensure aeration and adequate mixing.

Traditional fermentors used in brewing were open vats made of wood or slate or in more recent times of stainless steel. Modern brewing vessels are closed cylindrical stainless steel vessels with a conical base and dished (domed) top (Fig. 10.1). Enclosing such vessels allows CO_2 to be collected and reused and prevents contamination of the contents from the air.

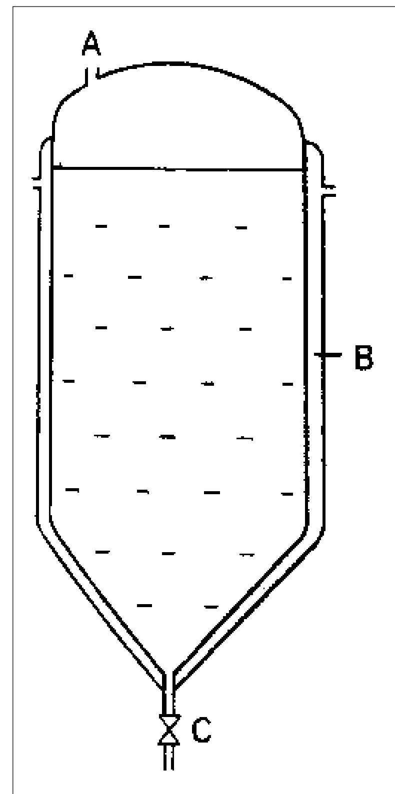

Fig. 10.1 A modern brewing vessel
A-air outlet, B-jacket, C-valve on outlet

Although many types of fermentors have been described for culturing aerobes, very few have proved to be satisfactory for industrial aerobic fermentations. The most commonly used ones are Stirred Tank Reactors (STRs). These vessels can be produced in a range of sizes from one dm^3 to thousands of dm^3. STRs have been adopted for a wide range of fermentation processes (Fig. 10.2).

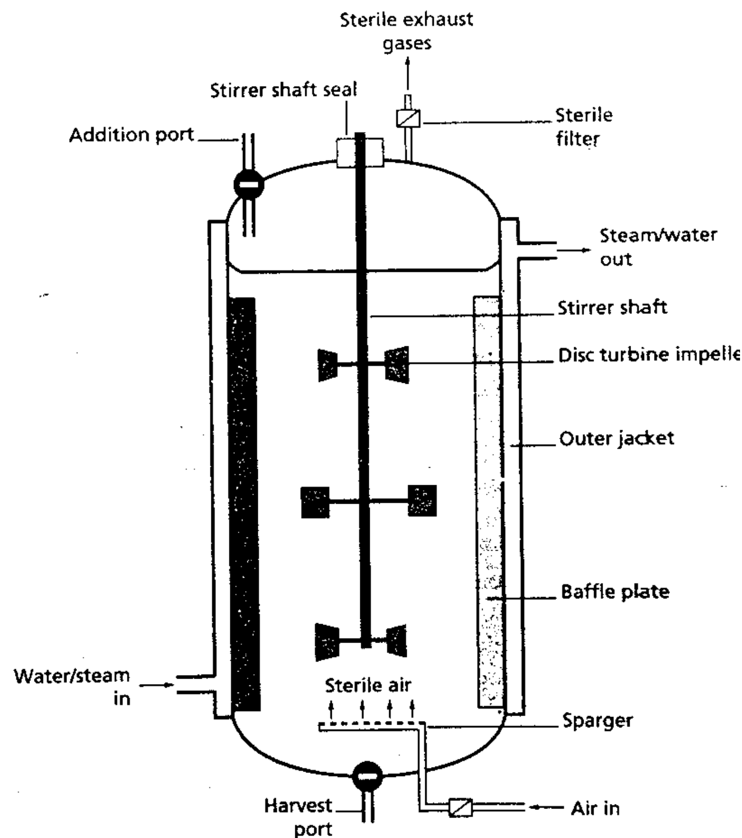

Fig. 10.2 Diagram of a stirred tank reactor

10.3 BODY CONSTRUCTION

In fermentations with aseptic requirements, it is important to select materials that can withstand repeated steam sterilization cycles. On a small scale (1 to 30 dm^3), glass and/or stainless steel may be used for constructing the body of the fermentor. Glass is useful as it provides smooth surfaces. It is also non-toxic and corrosion proof and it is easy to examine the interior of the vessel.

Pilot-scale and industrial-scale vessels are normally constructed with stainless steel or at least have a stainless steel cladding to limit corrosion. The corrosion resistance of stainless steel depends on the existence of a thin hydrous oxide film on the surface of the metal. The composition of this film varies with different steel alloys and different manufacturing processes.

10.4 TEMPERATURE CONTROL

In the design and construction of a fermentor, there must be adequate provision for temperature control. Heat is produced by microbial activity and mechanical agitation. This heat needs to be dissipated by cooling. Heat transfer is primarily achieved using an external jacket or via the internal coils. No direct contact exists between the cooling system and the fermentation medium. The heat is conducted through the vessel wall, coils and baffles. These systems are also used to sterilize the vessel and contents before inoculation, by the injection of pressurized steam.

10.5 AERATION AND AGITATION

The main purpose of aeration is to provide microorganisms in submerged culture with sufficient oxygen for metabolic requirements. Agitation of suspended cell fermentations is performed to mix the three phases in a fermentor. The liquid phase contains dissolved nutrients and metabolites, the gaseous phase is predominantly oxygen and carbon dioxide and the solid phase is made up of the cells and any solid substrates that may be present. Mixing should produce homogeneous conditions and promote nutrient, gas and heat transfer. The type of aeration - agitation system used in a particular fermentor depends on the characteristics of the fermentation process. The structural components of this system include:

a) The agitator (Impeller)
b) Stirrer glands and bearings
c) Baffles
d) Sparger

The agitator (Impeller): The agitator is required to maintain a uniform environment through out the vessel contents. Agitators may be classified as disc turbines, vaned discs, open turbines of variable pitch and propellers (Fig. 10.3).

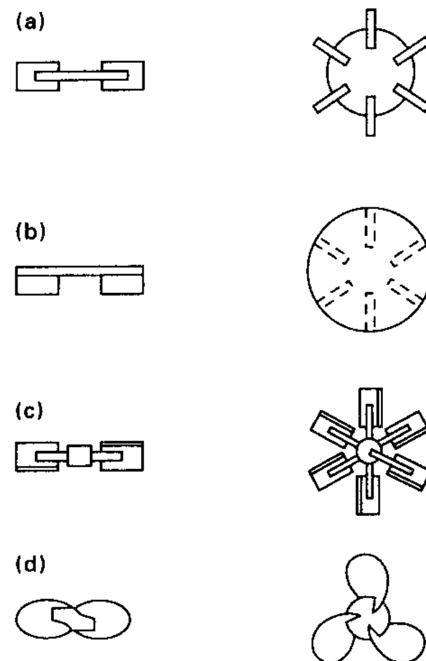

Fig. 10.3 Types of agitators
(a) disc turbine; (b) vaned disc;
(c) open turbine, variable pitch; (d) marine propeller →

The disc turbine consists of a disc with a series of rectangular vanes set in a vertical plane around the circumference. It is most suitable in a fermentor since it could break up a fast air stream without itself becoming flooded in air bubbles. Disc turbine of one-third the fermentor diameter is considered the optimum design for use in many fermentation processes.

Vaned disc has a series of rectangular vanes attached vertically to the underside. Air from the sparger hits the underside of the disc and is displaced towards the vanes. The vanes of a variable pitch open turbine and the blades of a marine propeller are attached directly to a boss on the agitator shaft.

Within each vessel the impeller is connected to an external motor which drives the stirrer system. In high viscosity broths, good mixing and aeration may be achieved by a dual impeller combination. In such cases, the lower impeller acts as the gas disperser and the upper impeller acts as a device for aiding circulation of vessel contents.

Stirrer glands and bearings: One of the difficult problems to overcome in the construction of fermentor is the satisfactory sealing of the stirrer shaft assembly. The stirrer shaft may enter the vessel from the top, side or bottom of the vessel. Top entry is most commonly used. Four basic types of seal assembly have been used:

(a) the stuffing box (packed-gland seal)

(b) the simple bush seal

(c) the mechanical seal

(d) the magnetic drive

The mechanical seal assembly is now commonly used in both small and large fermentors.

Baffles: Baffles are metal strips attached radially to the wall of fermentor. To improve aeration efficiency and to prevent vortex, four baffles are normally incorporated into agitated vessels. Six or eight baffles may be used in vessels over 3-dm^3. The width of a baffle may be about one-tenth of the vessel diameter.

Sparger: It is a device used for introducing air into the liquid in a fermentor. Three basic types of spargers viz., the porous sparger, the orifice sparger and the nozzle sparger have been used. The porous sparger of sintered glass, ceramic or metal, has been used mainly on a laboratory scale in non-agitated vessels. Orifice sparger is a perforated pipe, which is arranged below the impeller in the form of crosses or rings in small stirred fermentors. Nozzle sparger is a single open or partially closed pipe which provides the stream of air bubbles. It has been used in many modern mechanically stirred fermentors.

Sterile filtered air or oxygen normally enters the fermentor through a sparger. Air flow rates for large fermentors rarely exceed 0.5 – 1.0 volumes of air per volume of medium per minute (vvm). To promote aeration in stirred tanks, the sparger is located directly below the impeller. Sparger structure can affect the over all transfer of oxygen into the medium, as it influences the size of the gas bubbles produced. Small bubbles are desirable because the smaller the bubble, the larger the surface area to volume ratio which provides high oxygen transfer.

The bubbles of air rise to the surface of the medium and break up (disengage) releasing the gases they contain. To allow these processes, fermentors are not filled completely, but a space (the headspace) of about 20% of the capacity of the vessel is left free of medium. The actual volume of medium in the fermentor is called the working volume. Gases released into the headspace pass out of the fermentor through an air outlet at the top of the headspace.

10.6 ACHIEVEMENT AND MAINTENANCE OF ASEPTIC CONDITIONS

In order to achieve and maintain aseptic conditions during a fermentation, the following operations may have to be performed according to certain specifications.

(a) Sterilization of the fermentor

(b) Sterilization of the air supply and the exhaust gas

(c) Aeration and agitation

(d) Addition of inoculum, nutrients and other supplements

(e) Sampling

(f) Foam control

(g) Monitoring and control of various parameters

Most industrial fermentation processes operate using pure cultures. So the fermentor and its contents must be sterilized prior to inoculation with a pure culture of the organism and asepsis maintained thereafter. Hence, the fermentor should be designed in such a way that it may be steam sterilized under pressure. The medium may be sterilized in the vessel or separately and subsequently added aseptically. Steam is introduced into the fermentor coils or jacket to achieve sterilization (Fig. 10.4). Steam should be introduced through all the entry end exit points as every point of entry to and exit from the fermentor is a potential source of contamination.

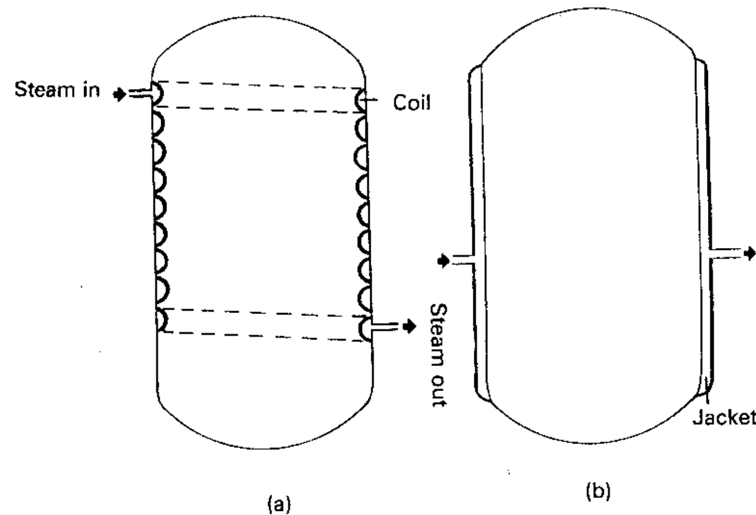

Fig. 10.4 Methods for introducing steam into the fermentor
a) Fermentor with coils
b) Fermentor with a jacket

Aerobic fermentations require the continuous addition of considerable quantities of sterile air. There are a number of ways for sterilizing air, but only two (heat and filtration) have found permanent application. Heat is generally costly for full-scale operations. Glass wool, glass fibre or mineral slag wool have been used filter material, but modern fermentors are fitted with cartridge type filters. The filter must also be sterilized in association with the fermentor before it is used. Sterilization of the exhaust gas can be achieved by 0.2 μm filters on the outlet pipe.

The addition of inoculum, nutrients and other supplements must be carried out under aseptic conditions. To prevent contamination, it is essential that both the addition vessel and the fermentor should be maintained at a positive pressure. After inoculation, the contents of the fermentor are sampled regularly, so that the culture can be examined for signs of contamination and the concentration of the product.

Aseptic sampling is performed using a steam-locked port which is a T-shaped arrangement of piping with a valve in each arm (Fig. 10.5). When not in use, the port is maintained in a sterile condition by passage of a gentle stream of pressurized steam along the outer arm of the T (with the valve next to the fermentor closed). For sampling, the valve to the steam supply is shut off and other valves opened, allowing liquid to pass out. After sampling the valves are reset to their original condition and thus the system is resterilized.

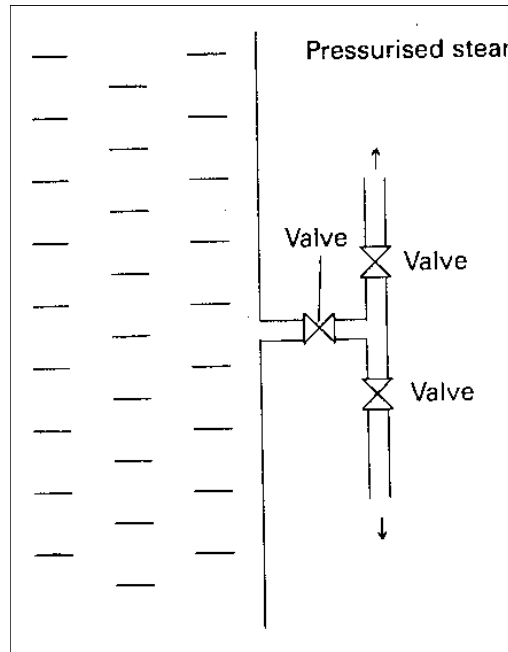

Fig. 10.5 Simple design for a sampling port

In any fermentation, it is important to minimize foaming. When foaming becomes excessive, the filters become wet resulting in contamination. The foam can be controlled by employing antifoam agents or mechanical foam breakers. It is a common practice to add an antifoam to a fermentor when the culture starts foaming above a certain predetermined level. The methods used for foam sensing and antifoam additions mainly depend on process and economic considerations.

Foam breakers are used exclusively if the presence of antifoam in the product is not acceptable. A number of mechanical antifoam devices have been described including discs, propellers, brushes or hollow cones attached to the agitator shaft above the surface of the broth. The foam is broken down when it is thrown against the walls of the fermentor.

Fermentation systems must be efficiently controlled in order to optimize productivity and product yield. The key physical and chemical parameters involved mostly depend on the bioreactor, its mode of operation and the microorganism being used. They are primarily aeration, mixing, temperature, pH and foam control. Control and maintenance at optimum levels inside the reactor is mediated by sensors (electrodes) along with compatible control systems and data logging (Table 10.1).

Internal sensors that are in or above the fermentation medium (pH, oxygen, foam, redox, medium analysis and pressure probes) should be steam sterilizable and robust. Some sensors do not

come into direct contact with any internal component of the bioreactor and do not need sterilization; for example agitator shaft power and speed meters and external sensors used to analyze samples withdrawn from the fermentor.

Table 10.1 The sensors used in fermentor monitoring and control

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

Control of pH is usually a major factor as many fermentations yield products that can alter the pH of the growth media. Fermentation media contain buffering salts usually phosphates. Addition of acid or alkali may be required if the capacity of buffering salts to control pH is not sufficient. The pH can be maintained at the desired value by the automatic addition of acid or alkali in response to changes recorded by the pH electrode.

Monitoring of temperature is by resistance thermometers and thermistors linked to automatic heating or cooling systems. Levels of dissolved O_2 and CO_2 are determined using O_2 and CO_2 electrodes. The basic principle of control involves a sensory system linked to a control system and feedback loop (Fig. 10.6). Sensors are used to measure and record the events within the bioreactor. Overall control can be manual or automated.

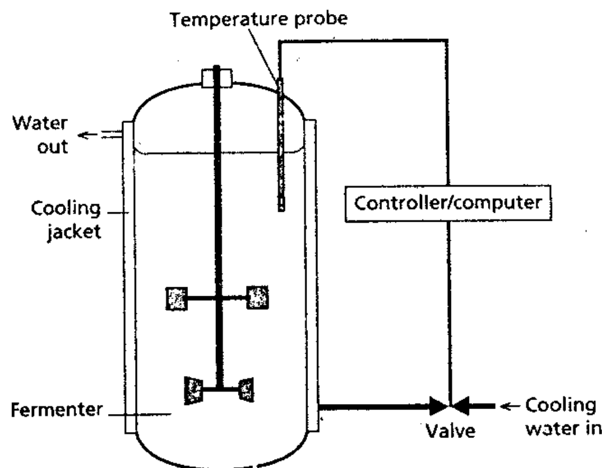

Fig. 10.6 A scheme for controlling the temperature of a fermentor

10.7 VALVES AND STEAM TRAPS

Valves attached to fermentors are used to control the flow of liquids and gases in several ways. The valves are of different types:

(a) Simple ON/OFF valves which are either fully open or fully closed.

(b) Valves that provide coarse control of flow rates.

(c) Valves that provide accurate control of flow rates.

(d) Safety valves which allow the flow of liquids or gases in only one direction.

Valves such as gate valves, globe valves, piston valves, needle valves, plug valves, ball valves, butterfly valves, pinch valves and diaphragm valves may be attached to fermentors that carry out several types of fermentation processes.

In all steam lines, it is essential to remove any steam condensate which accumulates in the piping. This can be achieved by incorporating steam traps which will collect and remove automatically any condensate at appropriate points in steam lines. A steam trap has two elements. One is a valve and seat assembly which provides an opening to ensure effective removal of any condensate. The second element is a device which will open or close the valve by measuring some parameter of the condensate reaching it to determine whether it should be discharged.

10.8 AIR-LIFT FERMENTOR

Sometimes it would be uneconomical to use a mechanically stirred fermentor. In such cases, air-lift fermentors can be used. An air-lift fermentor contains a gas tight baffled riser tube (liquid ascending) connected to a down comer tube (liquid descending). Air-lift fermentors with an external riser and internal riser are shown in figures 10.7 and 10.8 respectively. The advantage of the air-lift system is that it has no moving parts making operation and maintenance easier.

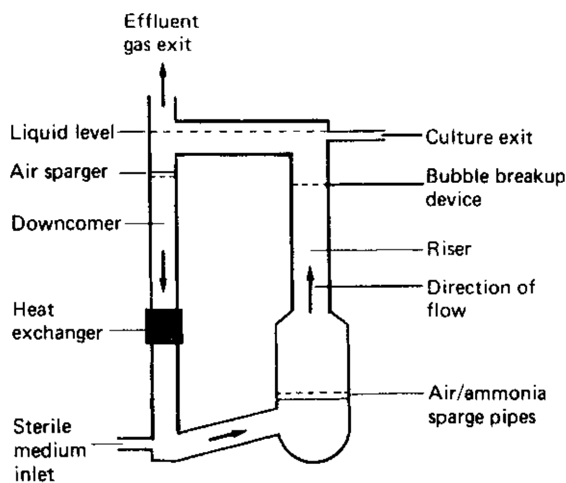

Fig. 10.7 Air-lift fermentor with outer loop

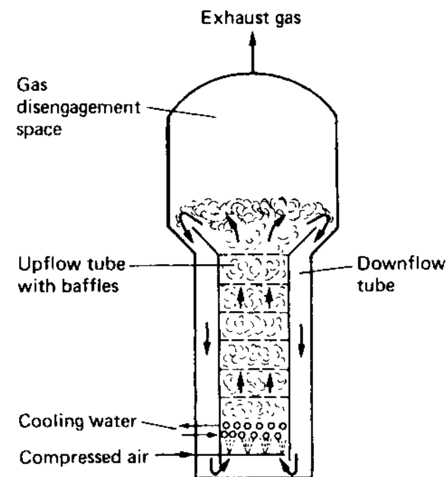

Fig. 10.8 Air-lift fermentor with inner loop

In this fermentor air is introduced into the base of the riser by a sparger. The driving force for circulation of medium in the vessel is produced by the difference in density between the liquid column in the riser (excess air bubbles in the medium) and the liquid column in the downcomer (depleted in air bubbles after release at the top of the loop). All performance characteristics of air-lift fermentors are linked to the gas injection rate and the resulting rate of liquid circulation. Air-lift bioreactors are highly energy-efficient relative to stirred fermentors, yet the productivites of both types are comparable.

10.9 BUBBLE COLUMN BIOREACTOR

A bubble column bioreactor is shown in Fig. 10.9. Gas is sparged at the base of the column through perforated pipes, perforated plates or sintered glass or metal micro-porous spargers.

Oxygen transfer, mixing and other performance factors are influenced mainly by the gas flow rate and the rheological properties of the fluid.

Other fermentation vessels such as Waldhof-type fermentor, tower fermentor, the deep-jet fermentor and the cyclone column have been developed for specific purposes and have limited applications.

10.10 SUMMARY

Microorganisms are cultured in specially designed vessels called fermentors. The main function of a fermentor is to provide a controlled environment for the growth of microorganisms to obtain a desired product. Anaerobic fermentors require little special equipment as compared to aerobic ones which require much more elaborative equipment to ensure adequate aeration and mixing. The performance of any fermentor depends on many factors, but the key physical and chemical parameters that must be controlled are agitation rate, oxygen transfer, pH, temperature and foam production. A number of aspects such as operation of the fermentor under aseptic conditions, its reliability in long-term operation, aeration and agitation systems in the fermentor, providing systems for controlling temperature and pH and sampling facility must be considered while designing and constructing a fermentor. Stirred tank reactors are the most commonly used vessels and have been adopted for a wide range of fermentation processes.

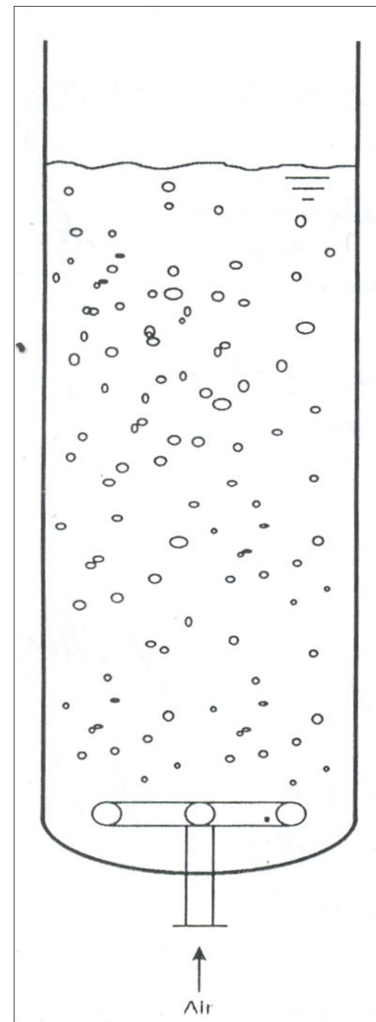

Fig. 10.9 A bubble column bioreactor

10.11 MODEL QUESTIONS

1. Give a detailed account on the design and construction of fermentor.
2. What are the operations required to achieve and maintain aseptic conditions during fermentation.
3. Write short notes on:

a) Aeration-agitation system
b) Stirred tank reactor
c) Air-lift fermentor
d) Sampling port

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FERMENTOR

M.Sc. MICROBIOLOGY (Final)

LESSON 11

FERMENTATION MEDIA

11.0 Objective: The various components of fermentation media employed for culturing microorganisms are discussed.

11.1 INTRODUCTION

11.2 CARBON SOURCES
11.2.1 Molasses
11.2.2 Malt
11.2.3 Starch and dextrins
11.2.4 Sulphite waste liquor
11.2.5 Cellulose
11.2.6 Whey
11.2.7 Oils and fats
11.2.8 Hydrocarbons and their derivatives

11.3 NITROGEN SOURCES
11.3.1 Corn-steep liquor
11.3.2 Yeast extracts
11.3.3 Peptones
11.3.4 Soyabean meal

11.4 MINERALS
11.5 GROWTH FACTORS
11.6 CHELATORS
11.7 BUFFERS
11.8 PRECURSORS
11.9 INDUCERS
11.10 INHIBITORS
11.11 CELL PERMEABILITY MODIFIERS
11.12 OXYGEN
11.13 ANTIFOAMS
11.14 SUMMARY
11.15 MODEL QUESTIONS
11.16 REFERENCE BOOKS

11.1 INTRODUCTION

Fermentation media must satisfy the nutritional requirements of the microorganisms. The nutrients should be formulated to promote the synthesis of the target product, either biomass or a specific metabolite. Over the years, substantial progress has been made in developing fermentation media.

In most industrial fermentations where the product is something other than the cell mass itself, there are two distinct biological requirements for medium design. First, nutrients have to be supplied to establish the growth of the organism. Second, when growth is established, proper nutritional conditions have to be provided to maximize product formation.

In addition to the biological requirements, one needs to think about selection of nutrients that are cost-effective, readily available and consistent from lot to lot. As integrated approaches to fermentation and downstream processing have been developed in recent years, it has also been recognized that the fermentation medium should not unduly hinder the downstream processing and, if possible, should even facilitate downstream processing.

On a small scale, it is relatively simple to devise a medium containing pure compounds, but the resulting medium, although supporting satisfactory growth may not be suitable for use in a large scale process. On a large scale, the medium formulated should meet as many as possible of the following criteria:

(a) The yield of product or biomass per gram of substrate utilized by microorganism should be high.

(b) It will produce maximum concentration of product or biomass.

(c) It will permit the maximum rate of product formation.

(d) The yield of undesired products should be low.

(e) It will be of consistent quality and be readily available.

(f) It should not cause problems during media making, sterilization, aeration and agitation, extraction, purification and waste treatment.

The media adopted depend on the scale of fermentation. For small-scale laboratory fermentations pure chemicals are often used in well-defined media. However, this is not possible for most industrial fermentation processes, simply due to cost, as media components may account for up to 60-80% of process expenditure.

In industrial fermentations, undefined complex natural materials have been used. Most are derived from natural plant and animal materials, often byproducts of other industries, with varied and variable composition. Crude substrates provide initial cost savings, but their high levels of impurities

may necessitate costly and complex recovery and purification steps. The components of defined media are expensive, but they give predictable yields from batch to batch and steps in recovery and purification are also simple.

All microorganisms require water, sources of energy, carbon, nitrogen, mineral elements and possibly vitamins. If the organism is aerobic, it also requires oxygen. Most fermentations, except those involving solid substrates, require large quantities of water in which the medium is prepared. It is also needed in many of the ancillary services such as heating, cooling, cleaning and rinsing. When assessing the suitability of water supply, it is important to consider pH, dissolved salts and effluent contamination. As water is becoming increasingly expensive, its recycle/reuse is essential wherever possible.

11.2 CARBON SOURCES

A carbon source is required for all biosynthetic processes. In most fermentations it also serves as the energy source. Carbon requirements may be estimated from the cellular yield coefficient (Y), an index of the efficiency of conversion of a substrate into a cellular material.

$$\text{Cellular yield coefficient (Y)} = \frac{\text{Quantity of cell dry matter produced}}{\text{Quantity of carbon substrate utilized}}$$

Carbohydrates are traditional carbon and energy sources for microbial fermentations, although other sources such as alcohols, alkanes and organic acids may be used.

11.2.1 Molasses

Molasses, a byproduct of cane and beet sugar production, is a common source of sucrose. It is a residue let after crystallization of sugar solutions in sugar refining. It is a dark coloured viscous syrup containing 50-60% (w/v) carbohydrates, primarily sucrose with 2% (w/v) nitrogenous substances, along with some vitamins and minerals.

Molasses is used in the production of high volume/low value products such as ethanol, single cell protein, organic acids and amino acids. It may also be used for the production of high value/low bulk products such as antibiotics, vaccines and fine chemicals. Hydrol molasses, a byproduct of maize starch processing can also be used. It primarily contains glucose.

11.2.2 Malt

Barley grains may be partially germinated and heat treated to prepare malt, which contains a variety of sugars besides starch. Malt extract may also be prepared from malted grain. The

composition of malt extracts varies to some extent, but they contain approximately 90% carbohydrates on a dry weight basis.

11.2.3 Starch and dextrins

These polysaccharides can be directly metabolized by amylase producing microorganisms. Starch obtained from maize grains is most widely used in fermentation media. Maize and other cereals may also be used directly in a partially ground state (maize chips). Starch may also be obtained from other cereals, potatoes and cassava. It can also be hydrolysed by dilute acids and enzymes to give a variety of glucose preparations.

11.2.4 Sulphite waste liquor

Sugar containing wastes derived from the paper pulping industry are primarily used for the culturing of yeasts. Waste liquors from coniferous trees contain 2-3% (w/v) sugar which is a mixture of hexoses (80%) and pentoses (20%). The liquors derived from deciduous trees contain mostly pentoses. Usually the liquor requires processing before use as it contains sulphur dioxide.

11.2.5 Cellulose

It 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 microoraganisms can utilize it directly. It is now mainly used in solid-substrate fermentations to produce mushrooms.

11.2.6 Whey

It is an aqueous byproduct of the dairy industry which mainly contains lactose. The use of lactose and crude lactose (whey) in media formulations is now extremely limited as relatively few microorganisms can metabolize them.

11.2.7 Oils and fats

Vegetable oils (cotton seed, linseed, maize, olive, soyabean etc.) and occasionally fish oil may be used as the primary or supplementary carbon source. Vegetable oils mostly contain oleic and linoleic acids, but linseed and soyabean oil also have a substantial amount of linolenic acid. The oils contain more energy per unit weight than carbohydrates.

11.2.8 Hydrocarbons and their derivatives

Methane, methanol and n-alkanes have been used as substrates for biomass production. n-alkanes of chain length $C_{10} - C_{20}$ are readily metabolized by some microorganisms. Mixtures rather than a single compound are usually suitable for microbial fermentations. Methane is used as a

carbon source by a few microorganisms, but its conversion product methanol is often preferred for industrial fermentations.

11.3 NITROGEN SOURCES

Many industrially used microorganisms can utilize inorganic or organic sources of nitrogen. Inorganic nitrogen may be supplied as ammonium salts, ammonia or nitrates. Ammonia can also be used for regulating pH of the culture media. Organic sources of nitrogen include amino acids, protein and urea. Nitrogen is supplied in crude forms that are essentially byproducts of other industries such as corn-steep liquor, yeast extracts, peptones, soya meal, peanut meal and cotton meal.

11.3.1 Corn-steep liquor

It is a byproduct obtained during the extraction of starch from maize. Concentrated extracts generally contain about 4% (w/v) nitrogen, including a wide range of amino acids along with vitamins and minerals.

11.3.2 Yeast extracts

Yeast extracts may be produced from baker's yeast, brewer's yeast or other strains of *Saccharomyces cerevisiae*. Alternate sources are *Kluyveromyces marxianus* and *Condida utilis*. Extracts are available as liquids containing 50-65% solids, viscous pastes or dry powders. They contain amino acids, peptides and water soluble vitamins (Table 11.1).

Table 11.1 Composition of Yeast extract

Component	Value
Total Proteins, peptides and amino acids (%)	73-75
Free amino acids	35-40
Peptides less than 600 Da	10-15
Material above 600 Da	20-30
Vitamins (μg/g)	
Thiamin	30
Riboflavin	120
Niacin	700
Pyridoxine	20
Folic acid	30
Calcium Pantothenate	300
Biotin	2.5

Note: Mineral content varies with the processing steps

11.3.3 Peptones

Peptones are prepared by acid or enzyme hydrolysis of high protein materials such as meat, casein, gelatin, keratin, peanuts, soya meal and cotton seeds. Their amino acid composition varies with the original protein source.

11.3.4 Soya bean meal

Residues remaining after extracting the oil from soya beans contain 50% protein, 8% non-protein nitrogenous compounds, 30% carbohydrates and 1% oil. The soya meal is often used as nitrogen source in antibiotic fermentations.

11.4 MINERALS

Microorganisms require certain mineral elements for their growth and metabolism. Magnesium, phosphorus, potassium, sulphur, calcium and chlorine are essential components in many media and must be added as distinct components. Others such as cobalt, copper, iron, manganese, molybdenum and zinc are also essential. They are present in the water supplies and also as impurities in other media ingredients.

11.5 GROWTH FACTORS

The growth factors most commonly required by microorganisms include vitamins, but there may also be a need for specific amino acids, fatty acids or sterols. Many bacteria can synthesize the necessary growth factors from basic elements. To culture the microorganisms which cannot synthesize the growth factors, the fermentation medium must be supplemented with them. Most natural carbon and nitrogen sources used in media formulations contain some of the required growth factors.

11.6 CHELATORS

While preparing or autoclaving many culture media, formation of precipitate of insoluble metal phosphates is a common feature. This problem can be eliminated by adding low concentrations of chelating agents such as ethylene diamine tetra acetic acid (EDTA), citric acid and poly phosphates. These chelating agents form complexes with the metal ions in a medium. The metal ions then may be gradually utilized by the microorganism.

11.7 BUFFERS

To achieve optimal productivity, regulating the pH of the fermentation medium is extremely important. A compound may be added to the medium to serve specifically as a buffer, or may also be used as a nutrient source. Many culture media are buffered at pH 7.0 by incorporating calcium carbonate. Phosphates which are part of many media also play an important role in buffering.

Some components added to the fermentation media such as precursors, inhibitors and inducers help to regulate the product formation rather than supporting the growth of the microorganism.

11.8 PRECURSORS

Specific precursors may be added to some fermentations involved in secondary metabolite production (Table 11.2). These chemicals, when added to certain fermentations are directly incorporated into the desired product. Phenylacetic acid is the most widely used precursor in penicillin production by *Penicillium chrysogenum*.

Table 11.2 Precursors used in fermentations

Precursor	**Product**	**Microorganism**
Phenylacetic acid	Penicillin G	*Penicillium chrysogenum*
Phenoxy acetic acid	Penicillin V	*P. chrysogenum*
Chloride	Chlortetracycline	*Streptomyces aureofaciens*
Chloride	Griseofulvin	*Penicillium griseofulvum*
D-Threonine	L-Isoleucine	*Serratia marcescens*

11.9 INDUCERS

When product formation is dependent upon the presence of a specific inducer, it must be incorporated into the culture medium or added at a specific point during the fermentation. The majority of enzymes which are of industrial interest are inducible. Inducible enzymes are synthesized only in response to the presence of an inducer. Inducers are often substrates such as starch or dextrins for amylases and pectin for pectinases (Table 11.3).

Table 11.3 Some examples of enzyme inducers

Enzyme	**Inducer**	**Microorganism**
α-Amylase	Starch	*Aspergillus* spp.

Pullulanase	Maltose	*Aerobacter aerogenes*
Pectinases	Pectin	*Aspergillus* spp.
Proteases	Proteins	*Bacillus* spp. *Aspergillus* spp. *Mucor* spp.
α-Mannosidase	Yeast mannans	*Streptomyces griseus*

11.10 INHIBITORS

A specific product may be produced or a metabolic intermediate may be accumulated by incorporating certain inhibitors into the medium (Table 11.4). Mostly inhibitors are effective in increasing the yield of the desired product and reducing the yield of undesirable related products.

An example of an inhibitor specifically employed to redirect metabolism is sodium bisulphite, which is used in the production of glycerol by *Saccharomyces cerevisiae*. Glycerol production depends on modifying the ethanol fermentation by removing acetaldehyde. Addition of sodium bisulphite to the medium leads to the formation of acetaldehyde bisulphite addition compound (sodiumhydroxy ethyl sulphite). As acetaldehyde is not available for reoxidation of NADH, dihydroxy acetone phosphate produced during glycolysis acts as hydrogen acceptor thereby forming glycerol-3-phosphate.

Table 11.4 Inhibitors used in fermentation processes

Product	**Inhibitor**	**Main effect**	**Microorganism**
Glycerol	Sodium bisulphite	Acetaldehyde production repressed	*Saccharomyces cerevisiae*
Tetracycline	Bromide	Chlortetracycline formation repressed	*Streptomyces aureofaciens*
Citric acid	Alkali Metal/Phosphate, pH below 2.0	Oxalic acid repressed	*Aspergillus niger*
Rifamycin B	Diethyl barbiturate	Other rifamycins inhibited	*Nocardia mediterranei*

11.11 CELL PERMEABILITY MODIFIERS

These compounds increase cell permeability thereby promoting the release of intracellular products into the fermentation medium. The best example is the use of penicillins in glutamic acid production by *Corynebacterium*.

11.12 OXYGEN

Availability of oxygen is extremely important in controlling growth rate and metabolite production in aerobic microorganisms. Depending on the amount of oxygen required by the organism, it may be supplied as air containing about 21% oxygen or occasionally as pure oxygen when requirements are high.

11.13 ANTIFOAMS

Antifoams are surface active agents which can reduce the surface tension in the foams during fermentation. Protein components of the culture medium such as corn-steep liquor, peanut meal, soyabean meal and yeast extract are the common cause for foaming. These proteins may denature at the air-broth interface and form a skin which does not rupture readily. Antifoams destabilize protein films by (a) hydrophobic bridges between two surfaces, (b) displacement of the absorbed protein, and (c) rapid spreading on the surface of the film.

An ideal antifoam should have the following properties:

(1) It should disperse readily with rapid action.

(2) It should be active at low concentrations.

(3) It should not be toxic to microorganisms, humans and animals.

(4) It should not cause handling hazards.

(5) It should be cheap.

(6) It should be compatible with other media components and the process.

Antifoams often used in different fermentation processes include alcohols, esters, fatty acids and derivatives particularly glycerides which include cotton seed oil, soya bean oil, sunflower oil, cod liver oil etc., silicones and sulphonates. Some examples of fermentation media are given in Tables 11.5 and 11.6.

Table 11.5 Fermentation medium for Penicillin

Glucose or molasses (by continuous feed)	10% of total
Corn-steep liquor	4 - 5% of total
Phenylacetic acid (by continuous feed)	0.5 – 0.8% of total
Lard oil (or vegetable oil) antifoam by continuous addition	0.5% of total

pH - 6.5 – 7.5 by acid or alkali addition	

Table 11.6 Fermentation medium for glutamic acid

Dextrose	270 g dm^{-3}
$NH_4H_2PO_4$	2 g dm^{-3}
$(NH_4)_2$ HPO_4	2 g dm^{-3}
K_2SO_4	2 g dm^{-3}
$MgSO_4$. $7H_2O$	0.5 g dm^{-3}
Mn SO_4 . H_2O	0.04 g dm^{-3}
Fe SO_4 . $7H_2O$	0.02 g dm^{-3}
Polyglycol 2000	0.3 g dm^{-3}
Biotin	12 μg dm^{-3}
Penicillin	11 μg dm^{-3}

11.14 SUMMARY

Medium formulation is an essential stage in the design of successful laboratory experiments and manufacturing processes. The constituents of a medium must satisfy the elemental requirements for cell biomass and metabolite production. The microorganisms require water, sources of energy, carbon, nitrogen, mineral elements and vitamins plus oxygen (if aerobic). Attention should be paid to all the components while formulating the culture media. Some components of a fermentation medium such as precursors, inducers and inhibitors help to regulate the product formation rather than support the growth of the microorganism.

11.15 MODEL QUESTIONS

1. Describe the components of culture media used for Industrial Fermentations.

2. Write short notes on:
   a) Carbon sources of fermentation medium.
   b) Antifoams.
   c) Precursors and Inducers used in fermentation media.

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M.Sc. MICROBIOLOGY (Final)

Lesson 13

STRAIN IMPROVEMENT OF INDUSTRIAL MICROORGANISMS

13.0 **OBJECTIVE:**
The basic principles involved in the strain improvement of industrially important microorganisms are discussed.

13.1 INTRODUCTION

13.2 MUTATIONS

13.2.1 Mutagenesis through radiation

13.2.2 Mutagenesis with chemical agents

13.3 RECOMBINATION

13.3.1 Recombination in procaryotes

13.3.2 Genetic recombination in fungi

13.3 Protoplast fusion

13.4 RECOMBINANT **DNA** TECHNOLOGY

13.5 SUMMARY

13.6 MODEL QUESTIONS

13.7 REFERENCE BOOKS

13.1 INTRODUCTION

Natural isolates usually produce commercially important products in low concentrations. Hence, attempts are made to increase the productivity of the selected organisms. Increased yields may be achieved by optimizing the culture medium and growth conditions. But this approach would be limited by the organism’s maximum ability to synthesize the product. The potential productivity of the organism is controlled by its genome. Therefore, the genome of the organism must be modified to increase the potential yield.

Genetic modification may be achieved by selecting natural variants, by selecting induced mutants and by selecting recombinants. Among the natural variants, superior producers have been selected particularly in the early stages in the development of a natural product from a newly isolated organism. The heterogeneity of some cultures can present serious problems of yield degeneration hence, it is not possible to rely on these strains. The fundamental genetic approaches for strain development are discussed below:

13.2 MUTATIONS

Changes in the genotype are caused by mutation and genetic recombination. Mutations occur *in vivo* spontaneously or after induction with mutagenic agents. The rate of spontaneous mutation is between 10^{-10} and 10^{-5} per generation and per gene. Because of the low frequency of spontaneous mutations, it is not cost-effective to isolate such mutants for industrial strain development. The mutation frequency (proportion of mutants in the population) can be significantly increased by using mutagenic agents.

Spontaneous and induced mutations arise as a result of structural changes in the genome. **Genome mutations** may cause changes in the number of chromosomes. **Chromosome mutations** may change the order of the genes within the chromosome. **Gene** or **point mutations** may change the base sequence in a gene.

Mutations used in microbial strain improvement usually are point mutations. Much information is available about point mutations. A **transition** is an exchange of a purine with another purine or a pyrimidine with another pyrimidine. A **transversion** refers to the substitution of a pyrimidine with a purine or vice-versa. **Frameshift mutations** result when one nucleotide or more is inserted or deleted, thus altering the reading frame in the following transcription and translation processes. It leads to a changed amino acid sequence in the resulting protein. The most commonly used mutagens are listed below:

13.2.1 Mutagenesis through radiation

Ultraviolet radiation (UV) and ionizing radiation are used in mutagenesis. Short-wave UV is one of the most effective mutagenic agents. The wavelengths effective for mutagenesis are between 200 and 300 nm with an optimum at 254 nm, which is the absorption maximum of DNA. The most important products of UV action are dimers formed between adjacent pyrimidines or between pyrimidines of complementary strands. Long wavelength UV radiation (300-400 nm) has less lethal and mutagenic effects than short-wavelength UV.

Ionizing radiation includes X-rays, γ-rays and β-rays which act by causing ionization of the medium through which they pass. These rays are used for mutagenesis only if other rays cannot be used. UV radiation or chemical agents are normally preferable for mutagenesis in industrial strain development.

13.2 Mutagenesis with chemical agents

The chemical mutagens can be classified into three groups according to their modes of action.

a) Mutagens which affect non-replicating DNA including nitrous acid, hydroxyl amine and alkylating agents such as ethyl methane sulfonate (EMS), Methyl methane sulfonate (MMS), N-methyl- N'-nitro-N-nitrosoguanidine (NTG) and mustard gas (Fig. 13.1).

b) Base analogs which are incorporated into replicating DNA due to their structural similarity with one of the naturally occurring bases. For example, base analogs such as 5-bromouracil (BU) or 2-amino-purine (AP) are incorporated into replicating DNA instead of the corresponding bases thymine and adenine.

c) Frameshift mutagens such as acridine dyes (acridine orange, proflavine and acriflavine) cause insertion or deletion of one or a few nucleotide pairs.

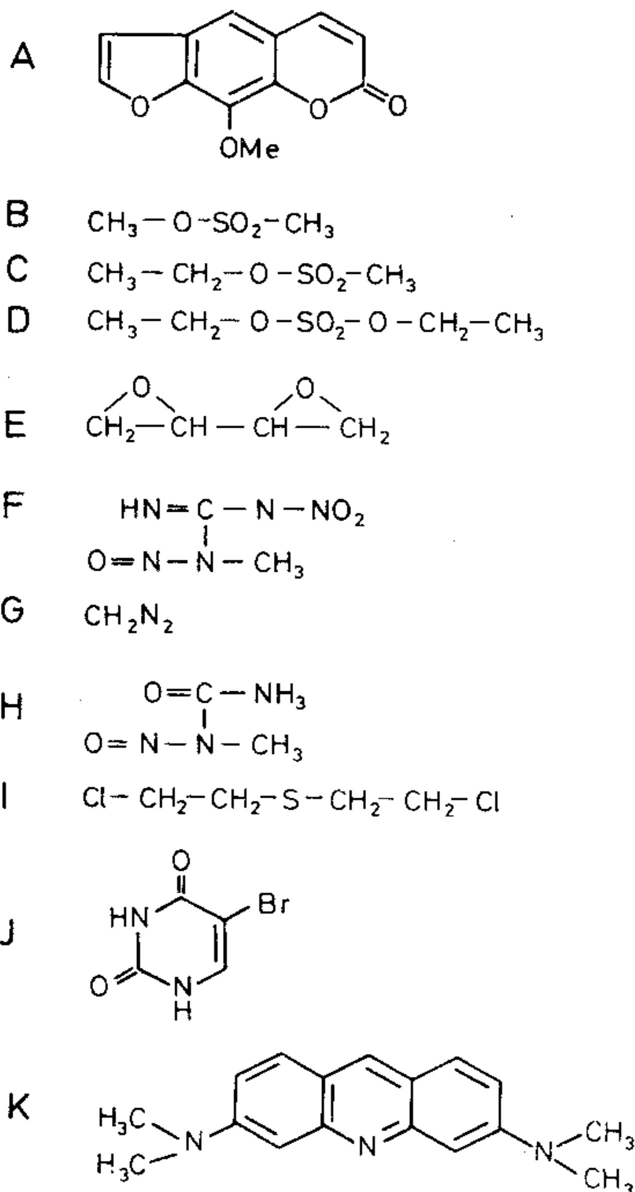

Fig. 13.1 Structures of different mutagens

A. 8-Methoxypsoralen; B. Methylmethanesulfonate (MMS); C. Ethylmethanesulfonate (EMS); D. Diethylsulfate (DES); E. Diepoxybutane (DEB); F. N-Methyl-N'-nitro-N-nitrosoguanidine (NTG);

G. Diazomethane; H. N-Methyl-N-nitrosourea (NMU); I. Di-(2-chlorethyl)-sulfide (mustard gas); J. 5-Bromouracel (BU); K. Acridine orange (AO).

The method of selection of mutants is crucial for the effective screening of mutants. There are basically two ways of screening: a random selection of survivors from a mutagenized population and selective isolation of mutants. Now-a-days, mutant strains of *Penicillium chrysogenum* (penicillin), *Streptomyces griseus* (streptomycin), *Aspergillus niger* (citric acid), *Corynebacterium glutamicum* (glutamic acid) etc. have been extensively used for the production of commercially valuable microbial metabolites.

13.3 RECOMBINATION

The genetic information from two genotypes can be brought together into a new genotype through genetic recombination. Thus recombination is an effective means of increasing the genetic variability of a population. Microorganisms carry out several types of recombination: general recombination, site-specific recombination and replicative recombination.

General recombination, the most common form, usually involves a reciprocal exchange between a pair of homologous DNA sequences. It can occur any place on the chromosome. It results from DNA strand breakage and reunion leading to cross over. Site - specific recombination is important in the integration of virus genomes into bacterial chromosome. The genetic material is not homologous with the chromosome it joins. The enzymes responsible for this event are specific for the particular virus and its host. Replicative recombination accompanies the replication of genetic material and does not depend upon the sequence homology. It is used by genetic elements that move about the chromosome.

13.3.1 Recombination in procaryotes

Recombination is a one way process in procaryotes, a piece of genetic material (the exogenote) is donated to the chromosome of a recipient cell (the endogenote) and integrated into it. The actual transfer of genetic material between bacteria usually takes place in one of three ways: Transformation, Transduction or Conjugation. *Escherichia coli* and *Pseudomonas* are gram-negative bacteria of industrial interest in which conjugation is well developed. Pseudomonads have six plasmids similar to the F factor of *E. coli*. Transduction systems are also present in these bacteria. Transformation systems exist for *Bacillus* species such as *B. licheniformis, B. pumilus* and *B. subtilis*. In addition, transduction systems have been established for *B. subtilis* with phages PBS1 and SP10.

The most common form of genetic exchange in actinomycetes is conjugation. Some examples of recombinant strains of actinomycetes are given below:

Streptomyces aureofaciens, producer of chlortetracycline,
S. griseus, producer of streptomycin,
S. rimosus, producer of oxytetracycline,

Nocardia mediterranei, producer of rifamycin,
Micromonospora purpurea, producer of gentamicin.

13.3.2 Genetic recombination in Fungi

Fungi have two distinct types of genetic recombination processes (sexual and parasexual cycles) that can be sued in a strain improvement program. Some fungi used industrially (some strains of *Aspergillus, Claviceps* and *Saccharomyces*) have a complete sexual cycle. Some of the most economically useful fungi such as *Penicillium chrysogenum* (Producer of Penicillin) and *Cephalosporium acremonium* (Producer of Cephalosporin) do not have a sexual cycle. The discovery of parasexual processes in imperfect fungi has led to the development of suitable breeding techniques. The major components of the parasexual cycle are the establishment of a heterokaryon, vegetative nuclear fusion and mitotic crossing over or haploidization resulting in the formation of a recombinant. Some examples of industrial fungi for which genetic recombination has been established are presented in Table 13.1.

Table 13.1 Antibiotic producing fungi in which recombination has been discovered

Species	**Antibiotic**	**Recombination type**
Aspergillus nidulans	Penicillin G	Sexual, parasexual
Cephalosporium acremonium	Cephalosporin C	Parasexual
Emericellopsis terricola	Cephalosporin C	Sexual
Penicillium chrysogenum	Penicillin G	Parasexual
P. patulum	Griseofulvin, Patulin	Parasexual

13.3.3 Protoplast fusion

Recombination by protoplast fusion is one of the most important developments in applied genetics in recent years. Protoplasts are cells from which the cell wall has been removed by enzyme treatment. The enzyme lysozyme is used for bacteria whereas chitinase or cellulase is used for fungi to dissolve the cell walls. The protoplasts must be stabilized against lysis by suspending them in a medium containing an osmotic stabilizing agent, such as sucrose.

Protoplast fusion does not occur normally due to the strong negative charge of the protoplast surface. In the presence of polyethylene glycol (PEG), the protoplasts aggregate and fusion occurs accompanied by DNA exchange (Fig. 13.2). After fusion, the cell wall is allowed to regenerate. In the regenerated progeny, there is a significant number of recombinants.

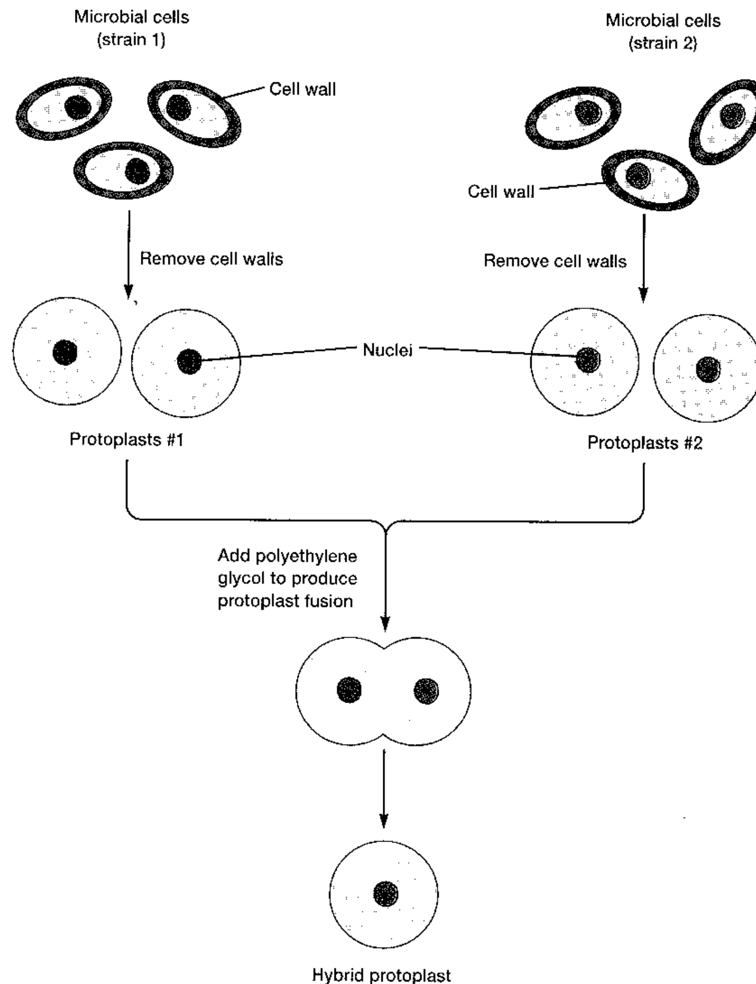

Fig. 13.2 Protoplast fusion

Besides the use of PEG to bring about fusion, the method of electric-field induced fusion of protoplasts has been developed. When cells are placed in an alternating current electrical field, transient holes develop in the plasma membrane, promoting the process of membrane merging and cell fusion. This method results in a considerably higher frequency of protoplast fusion. Protoplast fusion can be used for the following:

a) **Intraspecific recombination of strains:** This is often used for strains which lack sexual or parasexual systems or whose frequency of recombination is very low. Extensive efforts are being made to establish protoplast fusion in a wide range of microorganisms to overcome the difficulties of conventional recombination methods. Good protoplast fusion systems exist for many industrial microorganisms: for various strains of *Bacillus, Lactobacillus, Corynebacterium* or *Brevibacterium*, in fungi such as *Aspergillus, Penicillium* and *Cephalosporium*, in yeasts such as *Candida, Saccharomyces* and *Kluyveromyces* and in actinomycetes such as *Streptomyces, Micromonospora* and *Nocardia*.

b) **Interspecific hybridization:** This approach allows genetic information from different species to be combined *in vitro* in order to create new or modified products. Among the fungi, interspecific crosses have been attempted between species of Aspergillus (*A. nidulans* x *A. fumigatus*), species of *Penicillium* (*P. citrinum* x *P. cyaneofulvum*) and species of *Saccharomyces* (*S. cerevisiae* x *S. diastaticus*). Recombinants have only been obtained in the cross between *P. cyaneofulvum* and *P. citrinum*. The fusion frequency of intergeneric crosses between *Candida tropicalis* x *Saccharomyces fibuligera* is quite low.

13.4 RECOMBINANT DNA TECHNOLOGY

Recombinant DNA technology, which is also called gene cloning or molecular cloning includes a number of experimental protocols leading to the transfer of genetic information (DNA) from one organism to another. There is no single set of methods that can be used to meet this objective; however, a recombinant DNA experiment often follows the following steps (Fig. 13.3).

- The DNA (cloned DNA, insert DNA, target DNA, foreign DNA) from a donor organism is extracted, enzymatically cleaved (cut, digested) and joined (ligated) to another DNA entity (a cloning vector) to form a new, recombined DNA molecule (cloning vector – insert DNA construct, DNA construct).

- This cloning vector – insert DNA construct is transferred into and maintained within a host cell. The introduction of DNA into a bacterial host cell is called transformation.

- Those host cells that take up the DNA construct (transformed cells) are identified and selected from those that do not.

- If required, a DNA construct can be prepared to ensure that the protein product that is encoded by the cloned DNA sequence is produced by the host cell.

Recombinant DNA technology was developed from discoveries in molecular biology, nucleic acid enzymology and molecular genetics of both bacterial viruses and bacterial extra chromosomal DNA elements (plasmids).

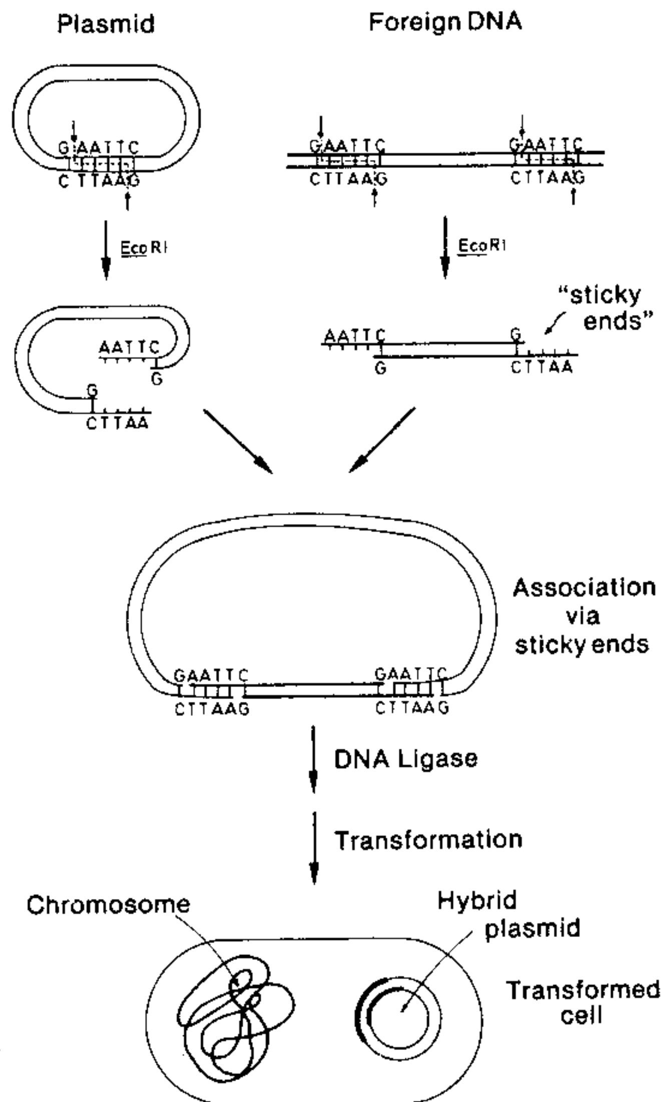

Fig. 13.3 Method for the production of recombinant DNA

One of the most important applications of recombinant DNA technology in the context of fermentation is the construction of strains capable of synthesizing foreign proteins. The first commercial heterologous (foreign) protein produced was human growth hormone (hGH) which is used to treat hypopituitary dwarfism. Prior to its manufacture by fermentation, it was extracted

from the brains of human cadevers. Naturally this source was not readily available and carried the disadvantage of the risk of contamination with human pathogens.

The successful production of recombinant hGH from *E. coli* satisfied the demand for the compound and eliminated the risks associated with the human source. The recombinant human insulin used to treat diabetes increased life expectancy of diabetics. Many other human proteins are synthesized at very low levels. The only practical way to produce them in sufficient quantities for use as therapeutic agents is as recombinant proteins. The recombinant proteins which have been licensed for therapeutic purpose are listed in Table 13.2.

Table 13.2 Recombinant proteins licensed for therapeutic use

Protein	**Clinical use**
Growth hormone	Hypopituitary dwarfism
Insulin	Diabetes
Tissue plasminogen activator	Clot lysis
Erythropoietin	Anaemia
Factor VIII	Haemophilia
Interferon	Cancers
Hepatitis B surface antigen	Hepatitis vaccine

13.5 SUMMARY

Wild strains produce commercially important metabolites in low concentrations. High yields can be achieved by optimizing the culture media and growth conditions. However, the potential productivity of the organism is controlled by its genome. Hence, the genome of the organism must be modified to enhance the potential yield. The fundamental genetic approaches for strain development include mutation and recombination.

Mutations occur spontaneously or after induction with mutagenic agents. The productivity of several strains has been improved by using mutagens. Another effective means of increasing the genetic variability of a population is Recombination in which the genetic information from two genotypes can be brought together into a new genotype.

Recombinant DNA technology permits the transfer of specific DNA sequences into procaryotic or eucaryotic organisms and the replication of these sequences. Several recombinant

proteins such as insulin, growth harmone, interferon and tissue plasminogen activator have been produced through this technology.

13.6 MODEL QUESTIONS

1. Discuss in detail about the genetic approaches for strain development.
2. Write short notes on:
   a) Strain improvement through mutations.
   b) Recombination as a means of strain improvement.
   c) Protoplast fusion
   d) Recombinant proteins.

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M.Sc. MICROBIOLOGY (Final)

Lesson 14

CULTURE SYSTEMS

14.0 **OBJECTIVE:**
In this section, the fundamental modes of culturing micro-organisms in industrial fermentation processes are discussed.

14.1 INTRODUCTION

14.2 BATCH CULTURE

14.3 FED-BATCH CULTURE

14.4 SEMI-CONTINUOUS CULTURE

14.5 CONTINUOUS CULTURE

14.5.1 Chemostat

14.5.2 Turbidostat

14.5.3 Continuous culture with cell recycle (Perfusion)

14.5.4 Multistage continuous culture

14.6 DUAL OR MULTIPLE FERMENTATIONS

14.7 SUMMARY

14.8 MODEL QUESTIONS

14.9 REFERENCE BOOKS

14.1 INTRODUCTION

In fermentation processes, the microorganisms have been carefully selected to maximise yield of product. The fermentors have been designed and engineered to provide optimal conditions for growth and product formation. In addition, the mode of operation of the fermentor (culture system used) is also extremely important in industrial fermentation processes. The four modes of culture applied in industrial fermentations include: batch culture, fed-batch culture, semi-continuous culture and continuous culture.

14.2 BATCH CULTURE

A batch fermentation is considered to be a 'closed system' in terms of nutrition. In this system, sterilized nutrient solution in the fermentor is inoculated with microorganisms and incubation is allowed to proceed under optimal physiological conditions. In the course of the entire fermentation, nothing is added except oxygen, an antifoam agent and acid or base to control the pH. The composition of the culture medium, the biomass concentration and the metabolite concentration generally change as a result of the metabolism of the cells. The four typical phases of growth observed in batch culture include – lag phase, log phase, stationary phase and death phase (Fig. 14.1).

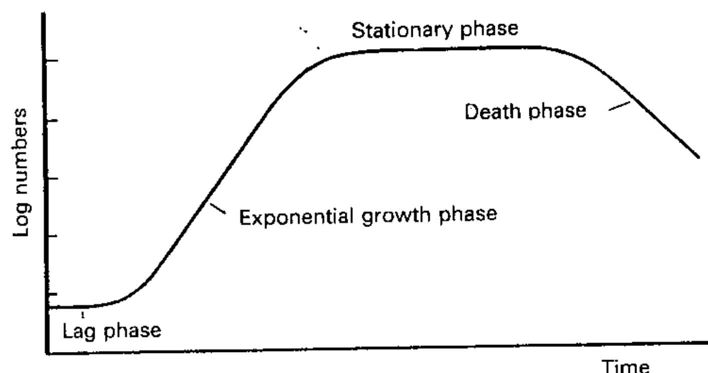

Fig. 14.1 Growth curve of a bacterial culture

Lag Phase: When cells are transferred from one medium to another, there is initially no increase in the number of cells. During this phase, the microorganisms adopt to their new environment. In a commercial process, the length of lag phase should be reduced as much as possible and this may be achieved by using a suitable inoculum.

Log Phase: The cells adapted to the new conditions of growth by the end of the log phase. Growth of cell mass can be described quantitatively as a doubling of cell number per unit time (yeasts and bacteria) or a doubling of biomass per unit time (filamentous organisms such as streptomycetes and fungi). Although the cells alter the medium through uptake of substrates and excretion of metabolic products, the growth rate remains constant during the log phase. Growth rate is independent of substrate concentration as long as excess substrate is present.

Stationary Phase: As soon as the substrate is metabolised or toxic substances have been formed, growth slows down or may be completely stopped. The biomass increases only gradually remains constant during this phase. The various metabolites formed in the stationary phase are of great biotechnological interest.

Death Phase: In this phase, the energy reserves of the cells are exhausted, resulting in cell death at an exponential rate. In commercial processes, the fermentation is usually interrupted at the end of

the log phase or before the death phase begins. For growth-linked products (including biomass) the stationary phase is non-productive. Hence, the product is recovered by the end of the log phase. For secondary metabolites, product formation occurs in the stationary phase, hence the process would be continued till the end of this phase.

In batch culture, the productive periods of a fermentor used batchwise may be relatively short compared to non-productive periods. As a result, the over all output of product is low. The advantages of batch culture on the other hand are: simplicity of operation; opportunity of using down times for maintenance; short fermentation period and reducing probability of mutation of the producing organism. An important advantage of batch culture is that it fits in well with batch processing, which is the conventional way in which industries operate.

14.3 FED-BATCH CULTURE

A fed-batch culture is a batch culture in which the nutrients are fed into the fermentor during the fermentation period without the removal of culture fluid. A fed-batch culture is established initially in batch mode and is then fed according to one of the following feed strategies:

a) The same medium used to establish the batch culture is added, resulting in an increase in volume.

b) A solution of the limiting substrate at the same concentration as that in the initial medium is added, resulting in an increase in volume.

c) A concentrated solution of the limiting substrate is added at a rate less than in (a) and (b), resulting in an increase in volume.

d) A very concentrated solution of the limiting substrate is added at a rate less than in (a), (b) and (c), resulting in an insignificant increase in volume.

Fed batch systems employing strategies (a) and (b) are described as variable volume, whereas a system employing strategy (d) is described as fixed volume. The use of strategy (c) gives a culture intermediate between the two extremes of variable and fixed volume.

Fed batch culture was used in the production of baker's yeast as early as 1915. Production of recombinant proteins from yeast may be achieved using this system. The formation of many secondary metabolites is subject to catabolite repression by high concentrations of glucose, other carbohydrates or nitrogen compounds. For this reason, in the fed-batch method, the critical elements of the nutrient solution are added in small concentrations at the beginning of the fermentation and these substrates continue to be added in small doses during the production phase. Penicillin fermentation provides an excellent example of the use of feed systems in the production of a secondary metabolite.

14.4 SEMI-CONTINUOUS CULTURE

For products which are growth linked (ethanol or biomass), most of the product is formed towards the end of the growth phase, when most growth occurs. In this system, a portion of the culture medium is removed from the fermentor at the end of growth phase and replaced by fresh medium of identical composition. Then growth of the organism will continue without non-productive log phases. This process can be repeated at appropriate intervals.

By using this system, the culture can be maintained in a condition of high biomass concentration and high rate of product formation. It results in higher over all outputs from this system compared to batch system. But this system when compared to batch system is technically more complex to operate. Long culture periods mean that contamination, mutation, wall growth and mechanical break down are more likely to occur.

14.5 CONTINUOUS CULTURE

In continuous fermentation, an open system is set up. Sterile nutrient solution is added to the fermentor continuously and an equivalent amount of converted nutrient solution with microorganisms is simultaneously taken out of the system. In a continuous process, under steady-state conditions, cell loss as a result of outflow must be balanced by growth of the organism. This system can be classified into four general types depending on the control parameter and operation mode: (a) chemostat, (b) turbidostat, (c) continuous culture with cell recycle and (d) multistage continuous culture (Fig. 14.2).

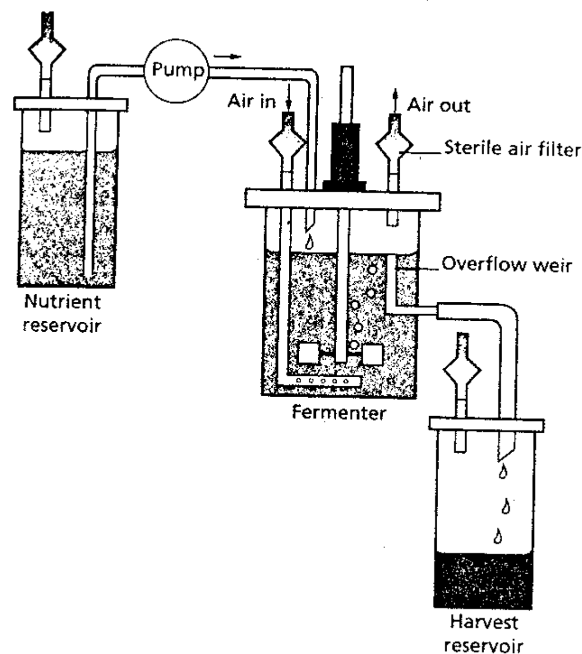

Fig. 14.1 Continuous culture apparatus

14.5.1 Chemostat

The chemostat is defined as a continuous culture system in which the feed rate is set externally and cell growth is limited by a selected nutrient. A chemostat is usually started as a batch culture. Before a nutrient becomes limiting, the nutrient feed is started. Cells grow until the chosen nutrient becomes limiting. After this, cell growth is limited by the rate of addition of medium.

The flow of medium into the vessel is related to the volume of the vessel by the term dilution rate D, defined as

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

Where

F = Flow rate ($dm^3\ h^{-1}$)

V = Volume (dm^3)

Thus, D is expressed in the units h^{-1}

The net change in cell concentration over a time period may be expressed as

dx / dt = growth - output

or $dx / dt = \mu x - Dx$

Under steady-state conditions, the cell concentration remains constant, thus $dx/dt = 0$ and

$\mu x = Dx$

and $\mu = D$

Thus, under steady-state conditions, the specific growth rate is controlled by the dilution rate.

14.5.2 Turbidostat

In the turbidostat, cell growth is kept constant by using turbidity to monitor the biomass concentration and the rate of feed of nutrient solution is approximately adjusted. This may be achieved by monitoring the biomass with a photoelectric cell and feeding the signal to a pump supplying medium to the culture such that the pump is switched on, if the biomass exceeds the set point and is switched off if the biomass falls below the set point.

Systems other than turbidity may be used to monitor the biomass concentration, such as CO_2 concentration or pH in which case it would be more correct to term the culture a biostat. The chemostat is the commonly used system because it has the advantage over the biostat of not requiring complex control systems to maintain a steady state. However, the biostat may be advantageous in continuous enrichment culture in avoiding the total wash out of the culture in its early stages.

14.5.3 Continuous culture with cell recycle (Perfusion)

Cell recycle is a useful means for increasing the concentrations of biomass and production a continuous culture. This system can be operated at a dilution rate higher than the maximum specific growth rate, leading to a much higher output of the reactor. Another property is that the dilution rate is almost independent of the growth rate. It is particularly advantageous in the following cases:

(a) the growth-limiting substrate is unavoidably dilute, for example, in the treatment of effluents;
(b) the substrate has a low solubility, such as when a gaseous substrate is used;
(c) the concentration of the growth-limiting substrate has to be limited because of the formation of inhibitory product(s); and
(d) product formation is not associated with growth.

Several methods can be used for the retention of biomass, such as filtration, sedimentation, centrifugation and immobilization. Depending on the position of the separation device inside or outside of the reactor, the methods can be further divided into internal and external systems. The concentration of culture effluent outside of the reactor by means of membrane filtration has so far found the most frequent application (Fig. 14.3). But in some cases like biological treatment of wastewater, the recycling of sludge after sedimentation has been used for a long time.

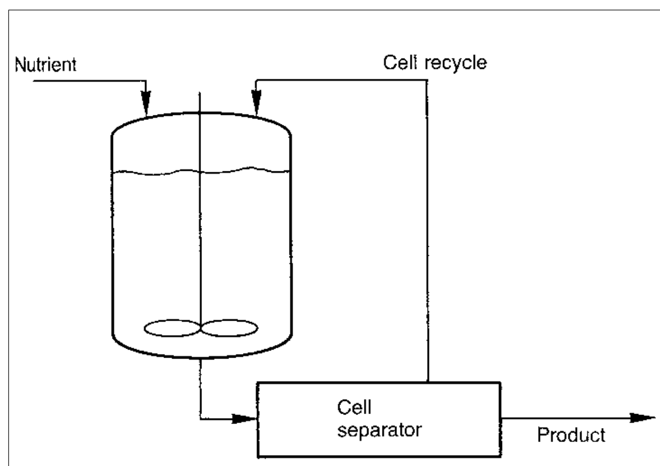

Fig. 14.3 Continuous culture with cell recycling

14.5.4 Multistage continuous culture

The two-stage continuous culture system shown in Fig. 14.4 can extend the range of application of continuous culture. For example, the second stage may be used to extend the growth rate downward to zero, and the first stage may be used to achieve stable conditions with maximum growth rate, both of which conditions may be desired in certain cases. The latter property is particularly useful when the substrate is also a growth inhibitor.

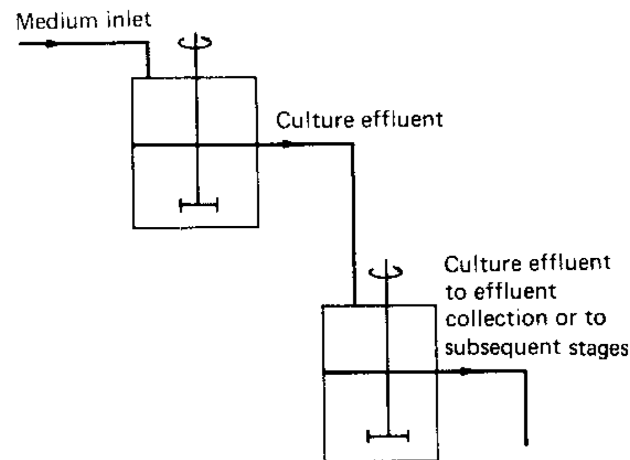

Fig. 14.4 A multistage chemostat

In the production of secondary metabolites and enzymes by continuous culture, the second stage may be used to provide a non-growing situation in which product formation occurs. For products of foreign gene expression, the second stage can be used for induction of expression. The two-steps culture system can be extended to include more stages and more feeding streams with or without biomass recycle.

The advantages of continuous culture systems are that the non-productive periods (lag phase, growth phase and down time) are avoided when the systems are up and running, giving high outputs. The disadvantages of continuous culture are: the long culture period and associated problems; the complexity of operation; the failure of the system if the dilution rate is faster than the maximum growth rate of the organism; and the fact that at high dilution rates a significant proportion of the substrate remains unused (wasted).

14.6 DUAL OR MULTIPLE FERMENTATIONS

In dual or multiple fermentations, more than one microorganism is employed. The organisms may be cultured in the following ways:

a) The organisms may be inoculated simultaneously into the growth medium.

b) One organism may be grown first in the medium, followed by the inoculation and growth of a second microorganism.

c) The organisms may be cultured separately. After growth has occurred in the original media, two separate fermentations may be combined for further fermentation activity.

The basic concept is that two or more microorganisms accomplish something that neither organism can do alone. In the state of present-day fermentation technology, this concept is more of a dream than a reality.

An example of dual simultaneous fermentation which has industrial potential is that for producing β-carotene. Two phycomycetes, *Choenephora cucurbitarum* and *Blakeslea trispora*, individually have the ability to produce this compound in aerated, submerged fermentation. However, the dual fermentation employs one or the other of these organisms, but not both. The fermentation is dual in that two different mating types, plus and minus, of the organism are grown simultaneously. The two mating types are inoculated into the medium at the same time. The resulting yields of β-carotene are found to be 15-20 times greater than the yields obtained when either mating type is grown by itself. But simultaneous growth of two microorganisms in a fermentation medium presents a problem in microbial ecology.

Dual or multiple fermentations in which one organism is grown in a medium followed by the inoculation and growth of a second organism and growth of a second organism are easier to control from a fermentation standpoint. An example for this is the production f ethanol by a yeast followed by oxidation of the ethanol to acetic acid by an *Acetobacter* species.

The combining of separate fermentations to yield further fermentation activity has already found some industrial application. This type has been used in production of L-lysine. An *Escherichia coli* auxotroph in one fermentation produces δ,ε-diaminopimelic acid; in a second fermentation, *Aerobacter aerogenes* cells are grown which contain diaminopimelic acid decarboxylase. These two fermentations are then combined so that the diaminopinelic acid decarboxylase produced by *A. aerogenes* can decarboxylate the α, ε-diaminopimelic acid from the *E. coli* auxotroph fermentation to provide L-lysine.

Toluene is added at the time of combining the fermentations so that the diaminopimelic acid decarboxylase becomes liberated from *A. aerogenes* cells, and so that the cells of both species are killed and not carry out further metabolic utilization of medium components or fermentation products.

14.7 SUMMARY

The four fundamental modes of culture that are applied in industrial fermentation processes are: a) batch culture, b) fed-batch culture, c) semi-continuous culture, and d) continuous culture. Batch culture involves adding all nutrients to the fermentor prior to inoculation; nutrients are added to, or medium removed from the fermentor during the culture phase. This system is thus a closed system in terms of nutrition.

A fed-batch culture is a batch culture in which one or more nutrient is fed into the fermentor during the fermentation period. In this way, nutrition can be added at the same rate as they are used up, so excess of nutrient can be avoided. In semi-continuous culture, a portion of the culture medium is removed from the fermentor at the end of the growth phase and replaced by fresh medium having identical composition.

Continuous culture includes removing medium (culture) from the fermentor continuously and replacing this with fresh medium, usually at the same rate. This is an open system which can be classified into four general types depending on the control parameter and the operation mode: a) Chemostat, b) Turbidostat, c) Continuous culture with cell recycle, and d) Multistage continuous culture.

14.8 MODEL QUESTIONS

1. Discuss different kinds of culture systems employed in fermentation processes.
2. Write in detail about the continuous culture system. Add a note on its advantages and disadvantages.
3. Explain the general features of batch and fed-batch culture systems.
4. Write short notes on:
   a) Semi-continuous culture
   b) Chemostat
   c) Continuous culture with cell recycle
   d) Turbidostat
   e) Multistage continuous culture
   f) Dual or multiple fermentations

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M.Sc. MICROBIOLOGY (Final)

Lesson 17

SOLID STATE FERMENTATIONS

17.0 **OBJECTIVE:**

The characteristics of solid state fermentations, process control, fermentors employed and advantages and disadvantages are discussed.

17.1 INTRODUCTION

17.2 CHARACTERISTICS OF **SSF**

17.3 PROCESS CONTROL

17.3.1 Preparation of materials and inoculation

17.3.2 Temperature and moisture control

17.3.3 Monitoring of microbial growth

17.4 FERMENTOR DESIGN FOR **SSF**

17.4.1 Tray Fermentor

17.4.2 Packed-Bed Fermentor

17.4.3 Rotary Drum Fermentor

17.4.4 Fluidized-Bed Fermentor

17.5 ADVANTAGES AND DISADVANTAGES OF **SSF**

17.6 SUMMARY

17.7 MODEL QUESTIONS

17.8 REFERENCE BOOKS

17.1 INTRODUCTION

Mostly industrial fermentations are carried out in liquid medium using bioreactor systems which range from simple stirred tank or non-stirred containers to complex aseptic, integrated systems. Besides these systems, there are some biotechnological processes that involve the growth

of microorganisms on solid and insoluble substrates in the absence or near absence of free water. These are called solid state/substrate fermentations (SSF).

In SSF, microorganisms can sometimes grow well and produce larger amounts of extracellular enzymes and other metabolites than they do in submerged (liquid) fermentation. Although SSF was developed for the manufacturing of traditional foods and alcoholic beverages, its application has been extended to the pharmaceutical and biochemical industries. The origin of SSF can be traced back to bread making in ancient Egypt. Over the years, some major advances have been established in the process engineering of SSF.

SSF played an important role in the traditional food-making processes of Oriental countries. It has been widely used in the production of traditional oriental foods and alcoholic beverages such as tempeh and ontjom in Indonesia, shaohsing wine and kaoliang (sorghum) liquor in China and miso, soy sauce and sake in Japan.

A digestive enzyme called Takadiastase was extracted from *Aspergillus oryzae* growing on wheat bran employing SSF by Takamine in 1896. This led to the application of SSF to other food and beverage industries. Now-a-days, the utilization of SSF has expanded to include composting, mushroom cultivation and the production of other foods like mold-ripened cheese. Some solid-state formulations on natural substrates are listed in Table 17.1.

Table 17.1 Examples of solid-state fermentations on natural substrates

Product	Microorganisms	Materials
Enzymes		
α-Amylase	*Aspergillus oryzae, Rhizopus* sp., *Bacillus licheniformis, Bacillus* sp.	Wheat bran, cassava
Glucoamylase	*Aspergillus niger, Aspergillus* sp., *Rizopus* sp.	Cassava, wheat bran, corn
Cellulase	*Trichoderma reesei, A. niger Penicillium* sp., *Thermoascus aurantiacus*	Wheat bran, wheat straw, Beet pulp, cellulosic biomass
Xylanase	*Aspergillus fumigatus, Thermoascus lanuginos*	Wheat bran, jute fiber + wheat germ
Pectinase	*Talaromyces flavus, A. niger, A. carbanerius*	Fruit pomace, wheat bran, coffee pulp
Glucose oxidase	*Penicillium notatum, Penicillium* sp.	
β-Galactosidase	*Kluyveromyces lactis*	Whey + corn or wheat bran
Protease	*Penicillium caseicolum, Mortierella renispora, A. orzae, A. niger*	Wheat bran, dried skim milk
Rennin	*Mucor pusillus, Mucor miehei*	Wheat bran

Product	Microorganisms	Materials
Metabolites		
Ethanol	*Saccharomyces cerevisiae*	Fruit pomace, sweet sorghum, beet, corn, carob pods
Citric acid	*A. niger*	Sugarcane bagasse, fruit pomace, wheat bran
Lactic acid	*Lactobacillus* sp., *Rhizopus oryae*	Sweet sorghum, sugarcane bagasse + glucose
Gibberellic acid	*Gibberella fujikuroi*	Wheat bran
Red pigment	*Monascus anka*	Rice, bread flake
Antibiotics		
Penicillin	*Penicillium chrysogenum*	Sugarcane bagase
Tetracycline	*Streptomyces viridifaciens*	Sweet potato residue
Cephalosporins	*Cephalosporium acremonium*	Barley
Iturin, surfactin	*Bacillus subtilis*	Soybean curd residue
Foods		
Natto	*Bacillus natto*	Soybean
Tempeh	*Rhizopus oligosporus*	Soybean
Tape	*Amylomyces rouxii, Rhizobium chinensis*	Rice, cassava, maize
Ontjom	*Neurospora sitophila*	Peanut meal
Cheese	*Penicillium roqueforti*	Milk curd
Bread dough Koji	*Saccharomyces cerevisiae, Lactobacillus sanfrancisco*	Wheat powder
Sake, shochu	*A. orzae, A. kawachii*	Rice, barley
Soy sauce	*Aspergillus sojae*	Soybean, wheat
Miso	*A. oryzae*	Soybean, rice
Shao-hsing wine	*Rhizopus* sp., *Mucor* sp. *(A. oryzae)*	Wheat (rice)
Kao-liang liquor	*Rhizopus* sp., *Mucor* sp.	Sorghum
Ragi	*Rhizopus* sp., *Saccharomycopsis* sp.	Rice
Single-cell proteins	Many yeasts and molds	Starchy or cellulosic biomass
Compost	White-rot fungi (mixed culture)	Cellulosic biomass

17.2 CHARACTERISTICS OF SSF

In **SSF**, the microbial distribution occurs on solid surface and product formation also occurs mainly on the surface. The culture environment is heterogeneous as the susbtrate is not uniform and not easily agitated.

The substrates of SSF are often natural materials such as cereals, soy beans, agricultural biomass and solid waste. Sometimes, the product is the entire fermented substrate as in the case of traditional foods, e.g., miso, natto and tempeh.

The moisture content of a solid substrate is normally low depending on the physical or chemical characteristics of the substrate. Further, heat derived from the metabolism and growth of the microorganism raises the temperature of the solid substrate bed and causes the loss of moisture.

The microorganisms generally used in SSF are molds. The aerial and submerged mycelia produced by these molds have different physiological activities, complicating process control. Cultivation of molds is normally stationary (except for rotating drum and fluidized-bed fermentors) since agitation of the substrate bed is very difficult and some activities are sensitive to shear stress.

17.3 PROCESS CONTROL

Preparation of the solid substrate and subsequent inoculation are extremely important as the initial condition strongly affects the entire SSF process. Among the process variables, the temperature and moisture control of the substrates are the most important for the control of SSF.

17.3.1 Preparation of Materials and Inoculation

In SSF, many types of natural solid materials are used as the substrates. These materials are usually pretreated before inoculation to facilitate mycelial penetration or to provide some chemical constituents for growth and product formation. In the case of rice koji, dehulled rice is polished to 40 to 70% of its original weight, washed and soaked for a few hours and drained overnight. Then, the material is steamed for about one hour at normal atmospheric pressure.

For inoculation, spores of *Aspergillus* species are typically used in the industrial koji process. Inoculation is done by scattering spores uniformly on the solid substrate. The solid substrate should continue to be mixed at certain intervals to obtain uniform mold growth.

17.3.2 Temperature and Moisture Control

The metabolic heat evolved in SSF raises the temperature of the solid substrate. It creates temperature gradients across the substrate bed vertically and horizontally, especially with forced aeration in which the circulated air usually flows upward from the bottom of the packed bed. The temperature gradients depend mainly on the thickness of the bed and the airflow rate.

The evolved heat reduces the moisture content of the solid substrate by evaporation. In the case of aerobic microorganisms, the catabolic heat evolution rate is regarded to be proportional to the consumption rate of oxygen. Hence, the temperature and moisture content in the solid substrate bed should be controlled.

17.3.3 Monitoring of Microbial Growth

The cell mass is a very important variable in the control of any fermentation process. However, in the case of mold, the mycelium penetrates the solid substrate and is difficult to separate from it. Methods that assay cellular components such as DNA, protein, chitin and N-acetyl glucosamine are used for monitoring microbial growth.

17.4 FERMENTOR DESIGN FOR SSF

In any fermentation process, the fermentor (bioreactor) provides the environment for the growth and activity of microbial culture being cultivated. In SSF for laboratory studies, conical flasks, petriplates, beakers, roax bottles or jars are commonly used. Fermentors used in industrial SSF are classified into several groups.

17.4.1 Tray Fermentor

Tray fermentors using wooden, plastic or metal trays are simple and widely used in traditional SSF. Cultivation is done in stationary trays with no mechanical agitation. Much labor is required for handling the trays. Since the efficiency of the heat exchange between substrate and air is not so high in this fermentor, the solid substrate cannot be highly heaped.

In the case of the rice-koji process for Japanese sake or soysauce, the maximum thickness of the solid substrate bed is about 15 cm. Usually these trays are placed in a room where the temperature and the humidity are controlled for optimal mold growth. The steamed rice inoculated with the conidia of *Aspergillus oryzae* is placed in a lump and covered with pieces of cloth overnight. After about 20 h of inoculation, the rice is spread out widely on the tray. The thickness of the substrate can be varied to facilitate temperature control.

A multiple-tray fermentor used for the production of rice koji starter is shown in Fig. 17.1. In this fermentor, all the operations are done in the cultivation vessel including steam sterilization of the substrate, inoculation, moisture supply to the substrate by a double-flow nozzle with water and air and forced aeration by a blower.

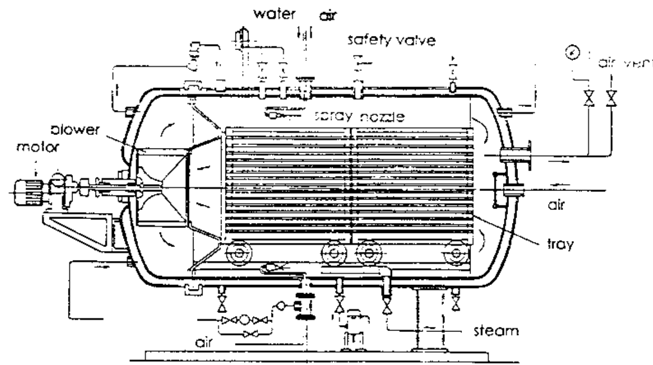

Fig. 17.1. A Tray Fermentor for the production of rice koji starter. About 3 kg of rice is loaded on a 1-m square tray. The maximum scale of cultivation is 200 kg using 63 trays.

17.4.2 Packed-Bed Fermentor

Packed-bed fermentors are usually installed with a forced aeration device. Figure 17.2 shows a simple design of packed-bed fermentor with forced aeration for rice koji making. This fermentor is placed in a cultivation room, since it is not equipped with a heater. The forced air flows upward through the substrate bed from the bottom. The time course of the cultivation temperature is programmed and aeration is done intermittently with an on-off regulation based on the temperature in the substrate bed.

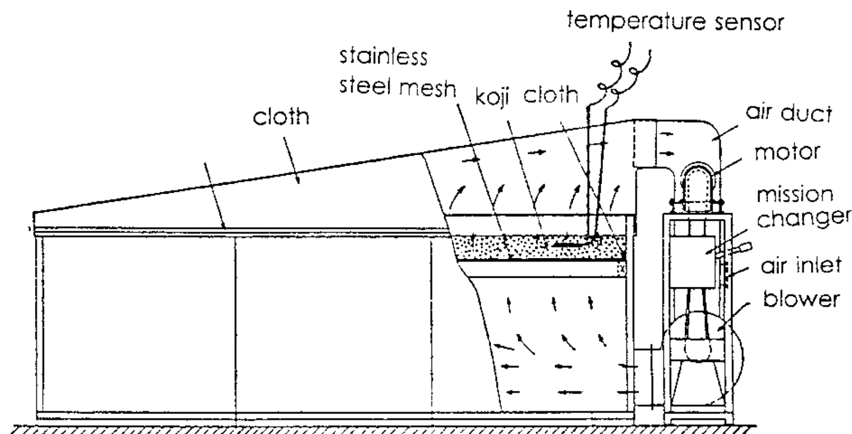

Fig. 17.2 A packed bed Fermentor with forced aeration

17.4.3 Rotary Drum Fermentor

A rotary drum fermentor is shown in Fig. 17.3 in which the drum rotates intermittently during cultivation to agitate and mix the substrate. Since this rotation is designed to work

simultaneously with aeration, contact between the substrate and the fresh air supply is accelerated, facilitating rapid heat removal and oxygen supply. Air is circulated, with the airflow varied by a damper in the air duct.

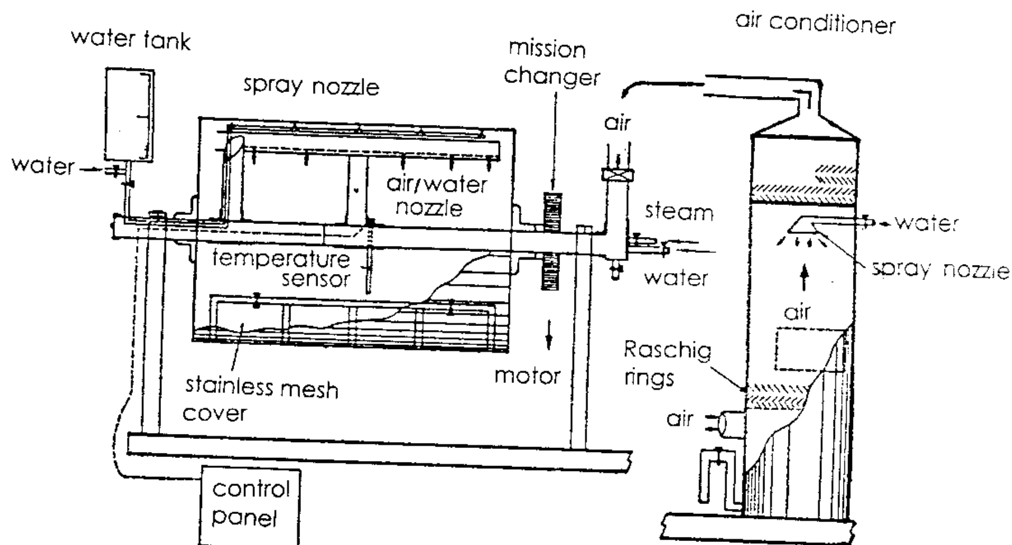

Fig. 17.3 A rotary drum fermentor

17.4.4 Fluidized-Bed Fermentor

In the fluidized-bed fermentor, the solid substrate is fluidized by upward airflow. A fluidized-bed fermentor shown in Fig. 17.4 can be used for the production of soy sauce koji using *Aspergillus sojae* or *A. oryzae* cultivated on wheat bran. It had a cylindrical shape and was installed with a wire net or a porous plate in the bottom, where the agitator rotated slowly to crush the lump of sedimented substrate.

Fluidization of the substrate offers some advantages for SSF:

(a) The effective surface area of the substrate powder is large for the microbial growth.
(b) The entire bed of solid substrate is kept under uniform conditions.
(c) Supply of water, nutrients and acid or alkali for pH control are simple.
(d) The removal of evolved heat and gas exchange of oxygen and carbon dioxide are easy.

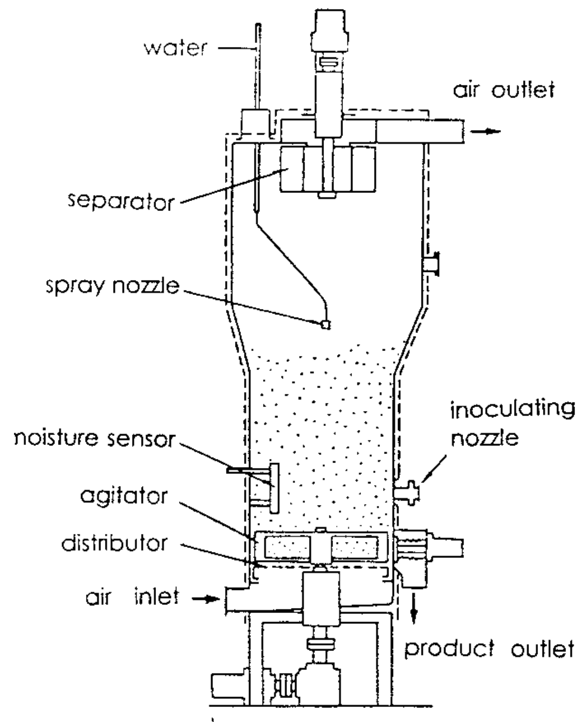

Fig. 17.4 A fluidized-bed fermentor

17.5 Advantages and disadvantages of SSF

In the industrial production of some extracellular enzymes such as amylase, pectinase and cellulase, both solid-state fermentation and submerged fermentation are used. The decision is likely based on the cost and efficiency of the process. Hence, it is important to know the advantages and disadvantages of SSF as compared with submerged fermentation.

Advantages:

- SSF is relatively resistant to bacterial contamination.
- Fermentors and the fermentative facilities are compact. The volumetric loading of the substrate is much higher in SSF than in submerged fermentation because the moisture content of the solid substrate is low.
- The extraction of the product from SSF, if necessary, requires much less solvent and lower recovery cost than from submerged fermentation.

- Treatment of the fermented residue is very simple. The fermented residue can be dried and used as animal feed or fertilizer, since its moisture content is very low.

- Microbial utilization of gaseous oxygen reduces the cost of aeration.

Disadvantages:

- Agitation of the substrate bed is difficult which leads to its heterogeneity with respect to the distribution of cell mass, nutrients, temperature, moisture content etc.

- Control of the heat evolved by microbial metabolism is very difficult.

- Rapid determination of microbial growth and other fermentative parameters is very difficult.

- Molds or other filamentous fungi can only be employed in SSF.

- Continuous operation and automation are difficult in SSF.

- Since the factors contributing to high production in SSF are not fully understood, cultivation strategy is based more or less on empirical results and the experience of the operator.

17.6 SUMMARY

Solid-state fermentation (SSF) is a microbial process in which a solid material is used as the substrate. In SSF, microbial growth and product formation mainly occur on the surface. The culture environment is heterogeneous in this type. The substrates of SSF are natural materials such as cereals, soybeans, agricultural biomass and solid waste. Molds are often employed in SSF for the production of several types of fermented foods and other products. Fermentors used in industrial SSF include tray fermentors, packed-bed fermentors, rotary drum fermentors and fluidized-bed fermentors.

17.7 MODEL QUESTIONS

1. Write in detail about solid-state fermentations.
2. Give an account of the products obtained through solid-state fermentations.
3. Write short notes on:
   a) Fermentors used in SSF.
   b) Process control in SSF
   c) Advantages and disadvantages of SSF.

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M.Sc. MICROBIOLOGY (Final)

Lesson 18

FERMENTATION ECONOMICS

18.0 **OBJECTIVE:**

Economic aspects of fermentation are discussed in this lesson.

18.1 INTRODUCTION
18.2 FERMENTOR AND ANCILLARY EQUIPMENT
18.3 RAW MATERIALS
18.4 ISOLATION OF MICROORGANISMS OF POTENTIAL INDUSTRIAL INTEREST
18.5 STRAIN IMPROVEMENT
18.6 FERMENTATION INCUBATION PERIOD
18.7 CONTAMINATION AND STERILIZATION
18.8 PRODUCT RECOVERY
18.9 MARKET POTENTIAL
18.10 OVERHEAD
18.11 EFFLUENT TREATMENT
18.12 PROCESS APPRAISAL
18.13 MODEL QUESTIONS
18.15 REFERENCE BOOKS

18.1 INTRODUCTION

Microorganisms can be considered as microscopic chemical plants that assimilate raw materials and synthesize chemical products. Over the years, the fermentation technology that uses these microbial chemical plants has changed a great deal. If a fermentation process is to yield a product at a competitive price, the chosen microorganism should give the desired end product in predictable, and economically adequate quantities. Several criteria are to be considered to develop a successful process which is economically viable.

1. The capital investment in the fermentor and ancillary equipment should be confined to a minimum, provided that the equipment is reliable and may be used in a range of fermentation processes.
2. Raw materials should be as cheap as possible and utilized efficiently.
3. High-yielding strain of microorganism should be used.
4. When a batch process is operated, the growth cycle should be as short as possible.
5. Recovery and purification steps should be as simple and rapid as possible.
6. Effluent discharge should be kept to a minimum.
7. Heat and power should be used efficiently.
8. Automation should be used where it is feasible.
9. Space requirements should be kept to a minimum.
10. All the above must comply with safety guidelines and regulations.

In any process, it is important to know the cost break down, so that it may be seen where the biggest potential savings may be achieved (Table 18.1). The four basic components that contribute to the process cost are mentioned in the decreasing order: raw materials, fixed costs, utilities, labour.

Table 18.1. Production costs breakdown expressed as percentages of total cost

Item	Product							
	Beer (Pratten. 1971)	Alcohol (batch) (Maiotella , 1984)	Acetic acid (Pape, 1977)	Citric acid (Schierhol t 1977)	Norprote in (Mogren, 1979)	SCP (Anon, 1974)	Penicilli n (Swartz 1979)	IPA (Datar *et al.* 1993)
Raw materials	38.4	76.7	42.2	39.7	70.0	62	58.0	39.8
Utilities	*	11.7	23.1	35.3	16.0	10	20.3	20.5
Labour and supervision	24.5	2.9	19.5	25.0	9.0	9	5.4	10.9
Fixed charges	7.2	4.8	10.5	--	--	19	--	--
Maintenance	29.9	--	--	--	5.0	**	14.9	--
Operating supplies	--	--	--	--	--	--	1.4@	--
Waste	--	--	--	--	--	--	--	12.0
Materials recovery	--	--	--	--	--	--	--	13.3
Other	--	--	--	--	--	--	--	3.6

Note:- *Included in 29.9% for maintenance and operating supplies.
**Included in 9% for labour and supervision.
@0.2% for laboratory costs included.

18.2 FERMENTOR AND ANCILLARY EQUIPMENT

It is most logical to build equipment as large as possible because of the economy of scale. There is an empirical relationship between the cost and size of an item of equipment. If a vessel is to be used to produce genetically engineered products, the costs will increase significantly because of the extra containment provisions which must be incorporated during construction. The designing of vessels which can be converted for multi-use may have economic advantages.

The greatest potential requirement for capital expenditure is associated with fermentation equipment and facilities. A newly developed fermentation requires the installation of newly designed equipment. Obviously, installation of this equipment adds greatly to the fermentation costs.

18.3 RAW MATERIALS

The cost of various components of a production medium can have a profound effect on the over all cost of a fermentation process. The raw materials used to formulate medium may account for 38 to 73% of the total production cost. The organic carbon source in microbial processes is usually the most expensive component contributing to the cost of the process. Attempts should be made to find an alternate or a substitute low-cost replacement for such a medium component.

The medium components do not constitute the only source of cost for a fermentation medium. Some media require pretreatment to make them acceptable for microbial growth or fermentation product accumulation. A particular medium component may also require pretreatment so that it is in a form available to the microorganism. Thus, starch must be pretreated with amylase to release fermentable sugars for yeast growth.

A medium that makes fermentation product recovery difficult also adds measurably to the overall fermentation costs, since a high fermentation yield may be meaningless if fermentation product recovery is poor or the expense of product recovery in good yield is great. Thus, medium components should be chosen to obtain the highest possible final recovery of fermentation product and not only just to obtain a high yield of product in the fermentor.

18.4 ISOLATION OF MICROORGANISMS OF POTENTIAL INDUSTRIAL INTEREST

The most appropriate microorganism for a potential process is usually found by isolation from a variety of sources, most commonly soil. The classical method of screening to obtain a suitable organism tended to be very time consuming and expensive. Eli lilly and Company Ltd. discovered three new antibiotics in 10 years while screening 4,00,000 microorganisms.

If a desired characteristic, which gives the organism a selective advantage, has already been recognized, a screen may be designed incorporating this characteristic as a selective factor. The isolation may begin with pretreatment of samples which favour the survival of the preferred

organism. This is followed by growth on selective or non-selective media and often associated with enrichment. A number of approaches are currently being used to improve isolation procedures.

It has become a common practice to obtain isolates from unusual habitats, which may include extreme environments, to ensure that the greatest microbial diversity is being examined. The screening tests which have been developed to detect new useful compounds of potential industrial interest have become much more selective and sensitive. Better knowledge of cell biochemistry has enabled the design of screens which are much more precisely targeted to detect the desired activity using specific detector strains.

18.5 STRAIN IMPROVEMENT

Strain improvement using a mutation/selection programme for improving an organism being used in a potential process can be very cost effective. Historically, mutation/selection programmes to improve strains of *Penicillium chrysogenum* were time consuming and very random because of the lack of knowledge about penicillin biosynthesis. These mutation programmes did,however, contribute significantly to increase in penicillin yields from less than 100 units cm^{-3} to over 51,000 units cm^{-3}.

A better understanding of cell metabolism and its regulation has enabled the development of more logical targeted methods to be introduced to select for mutants. Although, the main targets in strain improvement are normally to increase the product yield or specific production rates, it is also important to consider strain stability, resistance to phage infection, response to dissolved oxygen, tolerance to medium components and production of foam. A study of the range of targets mentioned in Table 18.2 has economic effects on all aspects of a fermentation process.

Table 12.2 Criteria for strain improvement (Schwab, 1988)

Target	**Impact on process or product**
Improvement of titre and/or specific production rate	General decrease of production costs, improved exploitation of reactor capacity, lower investment costs, increased efficiency in downstream processing steps.
Improvement of yield	Lower costs for substrates, decreased production of heat and CO_2, lower cooling costs, less waste and pollution.
Change in catabolic capabilities	Use of more favourable substrates (less expensive, better availability etc.), omission of pretreatment steps (e.g. enzymatic hydrolysis of polysaccharides).
Improvement of technological features of micro-organisms (e.g. flocculation behaviour, structure of mycelium, sporulation, foaming, strain stability,	Less energy costs for mixing and oxygen transfer, improved separation characteristics, fewer problems in inoculum preparation or scale-up of the process.

etc.	
Target	**Impact on process or product**
Improvement of product quality	Decreased production of specific by-products (fewer impurities), prevention of product degradation (e.g. pectinases).
Modification of products	Improvement of solubility in extraction solvents (e.g. addition of specific side chains), increased thermic stability of altered enzymatic properties of proteins.
Changing of the locus of product accumulation (e.g. intracellular to extra-cellular)	Improved product recovery (e.g. omission of cell disruption), correct products (e.g. fully processed proteins).

18.6 FERMENTATION INCUBATION PERIOD

Short incubation period fermentations are usually less costly and this applies both to inoculum build up and to production. Thus, short incubation period fermentations allow a greater turn over of fermentation equipment, that is, more fermentations can be run in the same equipment during the same period of time. Fermentations which involve long incubation periods provide additional requirements and costs for labor and also greater potential for contamination.

18.7 CONTAMINATION AND STERILIZATION

One of the most pervasive and troublesome problems that plagued the fermentation industry is microbial contamination. Once a competing microorganism infects the fermentation batch, the product yield drops and the product quality may decline to unacceptable levels. The result may be a complete loss of the contaminated fermentation batch. Although many fermentations become contaminated, few results in a total loss.

The bacterial viruses called phages can produce a second type of contamination. Phage infection usually results in a rapid lysis of the entire population of production cells and a complete loss of the fermentation. The bacteria used to produce organic and amino acids are particularly susceptible to this type of contamination and must be protected by operating the fermentation under the strict aseptic conditions. Once a contamination occurs, the facility is at increased risk of further problems. The source of contaminant must be quickly determined and all infected areas sterilized to protect against repeat contaminations.

The mutants that may spontaneously appear in microbial populations constitute a third type of contaminant. The larger the number of generations during the fermentation cycle, the more likely will be the appearance of a less productive mutant subpopulation. This is one of the principal reasons why continuous fermentation processes have not been adopted for many products.

Fermentations whose economics do not allow the cost of media sterilization usually provide some type of alternate method to check the growth of contaminants. Such methods include low pH of the medium, a substrate poorly amenable to attack by contaminants, partial heat treatment of the medium and inclusion of specific chemicals to retard contaminant growth. These provisions are not foolproof, and thus contamination costs must also be considered for these fermentations.

18.8 PRODUCT RECOVERY

The ability of a fermentation to produce high yields and to allow good product recovery is a prime consideration in fermentation economics. Yield and product recovery must be considered together, since a high yield is of little value if the product cannot be properly recovered for sale. The fermentation products are marketed at various levels of purity. The purity level required for the marketing of a fermentation product has a profound effect on the costs associated with that product.

Specific fermentation products may also be marketed at more than one concentration as well as level of purity. For example, lactic acid produced by fermentation is sold at strengths ranging from 20 to 85 per cent and at purity levels ranging from the crude technical grade to high purity edible grades. Complicated solvent extraction and fractionation procedures, successive recrystallization and other requirements for the recovery and purification of fermentation product contribute greatly to over all costs.

18.9 MARKET POTENTIAL

The ability to produce a fermentation product in high yield is only a part of the requirement for a commercially successful fermentation process. The product must be sold so as to recover costs along with an acceptable profit. However, in order to sell a fermentation product there must be a demand, or market, for the product.

Four categories of microbial products can be recognized economically and it is important to consider to which category a compound belongs.

1. Low price bulk chemicals, e.g. solvents, biomass, high fructose syrups (US \$ $10^2 – 10^3$ tonne^{-1}).

2. Mid price chemicals, e.g. organic acids, amino-acids, biopolymers (\$ $10^3 – 10^5$ tonne^{-1}).

3. High price microbial and animal -- cell products, e.g. enzymes, vitamins, antibiotics, corticosteroids, vaccines etc. (\$ $10^5 – 10^7$ tonne^{-1}).

4. Very high-price animal-cell products, e.g. monoclonals, tissue plasminogen activator etc. (\$ $10^7 – 10^9$ tonne^{-1}).

The prices of a wide range of fermentation products can be directly related to the product concentration in the fermentation broth (Fig. 18.1). The effect is particularly strong for

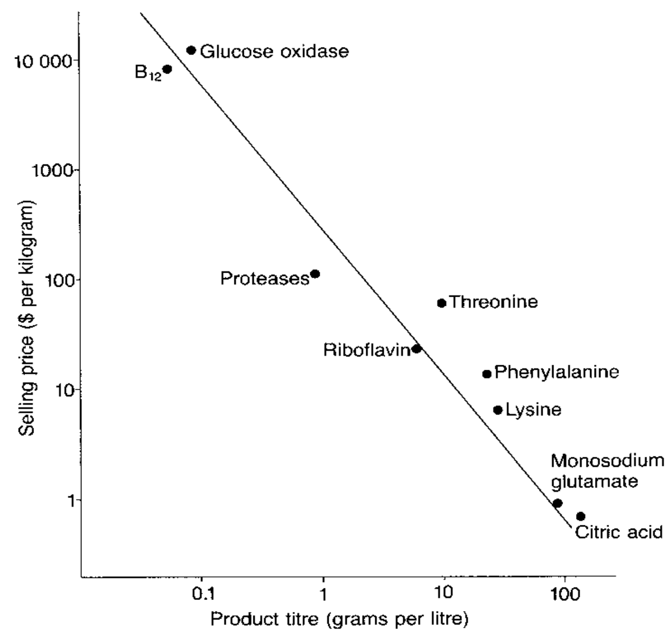

Fig. 18.1 Effect of product titre on the selling price. The higher the concentration of the product in the fermentation broth, the lower the selling price will be.

concentrations below 0.1 per cent. Above one per cent concentration, the production costs are more dependent on the special requirements of the individual product. An example is the fermentation cost for the production of an amino acid. As the titre increases above 10 grams per litre it has a diminishing effect on the production cost (Fig. 18.2).

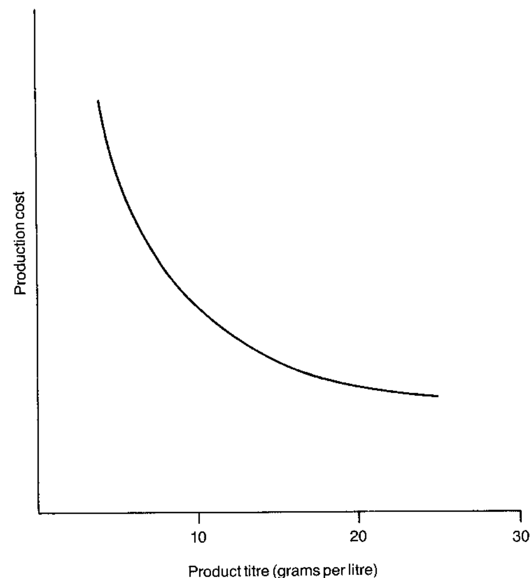

Fig. 18.2. Effect of the product titre on the production costs of an amino acid. The price comes down rapidly until the titre reaches 10 grams per litre, after which further increases in the concentration have a diminishing effect on price.

18.10 OVERHEAD

Overhead expenses refer to general expenses incurred in the running of a business. These expenses include rent, taxes, insurance, light, heat, accounting and other office expenses, depreciation etc. These expenses must be considered in the total costs of a fermentation product, but they are not tied to or do not fluctuate to any extent with a particular fermentation.

18.11 EFFLUENT TREATMENT

Fermentation wastes include wastes from the actual fermentation, wastes from recovery processes, as well as cleaning and cooling waters. Various of these wastes, particularly cooling waters, can at times be utilized in media make up for similar or other fermentations. Sometimes, it is also feasible to recover by-products from the fermentation wastes.

Disposal of effluents into streams, rivers or other bodies of water is not permissible because of state and federal regulations. In the majority of fermentation processes, it is impossible to dispose of effluents at zero cost. Whether the waste is incinerated, dumped on waste land, or discharged to sewers, rivers or tidal waters, some expenditure will be necessary for treatment that ensures that minimal harm is done to the environment.

The various alternative disposal procedures may be compared using economic considerations. The cheapest treatment method is considered to be controlled dumping, followed by waste incineration. The most expensive method is biological degradation in a waste-water-treatment plant.

18.12 PROCESS APPRAISAL

An appraisal of the economic potential for a fermentation process requires that all of the preceding considerations be evaluated both under present and future market conditions. This evaluation should be made as early as possible during process development as well as at the time of

deciding whether to enter the market with the fermentation product. The process should also be reevaluated at later dates during commercial production. For these evaluations, it is necessary to estimate present and future availability and price of fermentation substrates, costs of labor and overhead, public demand for the product, competition, potential for improving yields and product recoveries and ability and facilities to meet market demands for the product. All these points are utilized to decide whether the fermentation product can be produced and sold at an acceptable level of profit.

18.13 SUMMARY

For a fermentation process to yield a product at a competitive price, the chosen microorganism should give the desired end product in predictable and economically adequate quantities. Several criteria such as capital investment in the fermentor, raw materials used to formulate culture medium, isolation of microorganisms and strain improvement, product recovery, market potential and disposal of affluents have to be considered to develop a successful process which is economically viable.

18.14 MODEL QUESTIONS

1. Discuss the general features considered to develop a successful fermentation process.
2. Write short notes on:
   a) Market potential
   b) Sources of contamination in fermentation process.

18.15 REFERENCE BOOKS

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