FOOD AND INDUSTRIAL MICROBIOLOGY

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Food and Industrial Microbiology

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COURSE OUTLINE

Module 1. The Trajectory of Food Microbiology

Lesson 1

Introduction to Food Microbiology: Part-I: Bacteria

1.1 Introduction

Food science is a discipline concerned with all aspects of food - beginning after harvesting, and ending with consumption by the consumer. It is considered one of the agricultural sciences, and it is a field which is entirely distinct from the field of nutrition. The field of food microbiology is a very broad one, encompassing the study of microorganisms which have both beneficial and deleterious effects on the quality and safety of raw and processed foods. It is important to understand the relationships among the various microorganisms making up the microflora of a food. Infact food microbiologists are concerned with the practical implications of the microflora of the food and the food microorganisms that can cause spoilage of food and disease in humans. The primary tool of microbiologists is the ability to identify and quantitate food-borne microorganisms. However, the inherent inaccuracies in enumeration processes, and the natural variation found in all bacterial populations complicate the microbiologist's job. Moreover, they may be important from the aesthetic point of view. Of course, some useful bacteria may be important because they change the functional properties of food stuffs resulting in new tastes, odors or textures. Microorganisms in food include bacteria, molds, yeasts, algae, viruses, parasitic worms and protozoa. These organisms differ in size and shape and in their biochemical and cultural characteristics.

The microorganisms described below are among the most important genera and species normally found in food products. Each microorganism has its own particular nutritional and environmental requirements.

1.2 Bacteria

1.2.1 *Acinetobacter*

Acinetobacter is a genus of Gram-negative bacteria belonging to the Gammaproteobacteria . *Acinetobacter* species are non-motile and oxidase-negative, and occur in pairs as observed under magnification. Young cultures show rod shaped morphology. They are strict aerobes that do not reduce nitrates. They are important soil and water organisms and are also found on many foods especially refrigerated fresh products. *A. baumannii* is a frequent cause of nosocomial pneumonia, especially of late onset ventilator associated pneumonia. It can cause various other infections including skin and wound infections, bacteremia, and meningitis,

1.2.2 *Bacillus cereus*

B. cereus is a thick long rod shaped Gram positive, catalase positive aerobic spore former and the organism is important in food borne illness. It is a normal inhabitant of soil and is isolated from a variety of foods. It is quite often a cause of diarrheal illness due to the consumption of desserts, meat, dishes, dairy products, rice, pasta etc that are cooked and kept at room temperature as it is thermoduric. Some of the *B. cereus* strains are psychrotrophic as they grow at refrigeration temperature.

B. cereus is spread from soil and grass to cows udders and into the raw milk. It is also capable of establishing in cans. It is also capable of producing proteolytic and amyloltic enzymes and also phoslipase C (lecithinase). The production of these enzymes by these organisms can lead to the spoilage of foods. The diarrheal illness is caused by an enterotoxin produced during the vegetative growth of*B. cereus* in small intestine. The bacterium has a maximum growth temperature around 48°C to 50°C and pH range 4.9 to 9.3. Like other spores of mesophilic *Bacillus* species, spores of *B. cereus* are also resistant to heat and survive pasteurization temperature.

Fig. 1.1 ***Bacillus cereus***

1.2.3 *Bacillus subtilis*

Bacillus subtilis , known also as the hay bacillus or grass bacillus , is a Gram-positive, catalase-positive bacterium commonly found in soil. A member of the genus *Bacillus*, *B. subtilis* is thin short rod-shaped, and has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions. *B. subtilis* produces the proteolytic enzyme subtilisin. *B. subtilis*spores can survive the extreme heat during cooking. *B. subtilis* is responsible for causing ropiness a sticky, stringy consistency caused by bacterial production of long-chain polysaccharides in spoiled bread dough. A strain of *B. subtilis* formerly known as *Bacillus natto*is used in the commercial production of the Japanese food *natto*, as well as the similar Korean food *cheonggukjang*. It is used to produce amylase and also used to produce hyaluronic acid, which is useful in the joint-care sector in healthcare.

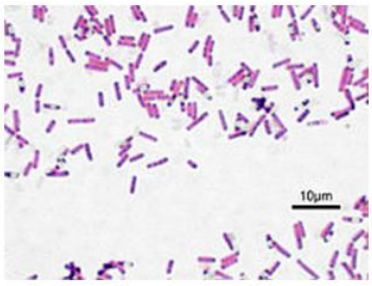

Fig-1.2: ***Bacillus subtilis***

1.2.4 *Carnobacterium*

Carnobacterium is a genus of Gram-positive bacteria within the family *Leuconostocaceae*. *C. divergens* and *C. maltaromaticum* are found in the wild and in food products and can grow anaerobically. These species are not known to be pathogenic in humans but may cause disease in fish. The genus *Carnobacterium* contains nine species, but only *C. divergens* and *C. maltaromaticum* are frequently isolated from natural environments and

foods. They are tolerant to freezing/thawing and high pressure and able to grow at low temperatures, anaerobically. They metabolize arginine and various carbohydrates, including chitin, and this may improve their survival in the environment. *Carnobacterium divergens* and *C. maltaromaticum* have been extensively studied as protective cultures in order to inhibit growth of *Listeria monocytogenes* in fish and meat products. Several carnobacterial bacteriocins have been identified and described. Carnobacteria can spoil chilled foods, but spoilage activity shows intraspecies and interspecies variation. Their production of tyramine in foods is critical for susceptible individuals, but carnobacteria are not otherwise human pathogens.

1.2.5 *Corynebacterium*

Corynebacterium is a genus of Gram-positive rod-shaped bacteria. They are widely distributed in nature and are mostly innocuous. Some are useful in industrial settings such as *C. glutamicum*. Others can cause human disease. *C. diphtheriae*, for example, is the pathogen responsible for diphtheria. Some species are known for their pathogenic effects in humans and other animals. Perhaps the most notable one is *C. diphtheriae*, which acquires the capacity to produce diphtheria toxin only after interacting with a bacteriophage. Diphtheria toxin is a single, 60,000 molecular weight protein composed of two peptide chains, fragment A and fragment B, held together by a disulfide bond.

1.2.6 *Clostridium perfringens*

C. perfringens is a Gram-positive encapsulated anaerobic non-motile bacterium commonly found on meat and meat products. It has the ability to cause food borne disease. It is a toxin producing organism-produces *C. perfringens* enterotoxin and β -toxin that are active on the human GI tract.

It multiplies very rapidly in food (doubling time < 10 min). Spores are resistant to radiation, desiccation and heat and thus survive in incompletely or inadequately cooked foods.

However, it tolerates moderate exposure to air. Vegetative cells of *C. perfringens* are also somewhat heat tolerant as they have relatively high growth temperature (43°C -45 °C) and can often grow at 50°C. They are not tolerant to refrigeration and freezing. No growth occurs at 6 °C . *C. perfringens* is present in soil and the other natural environment.

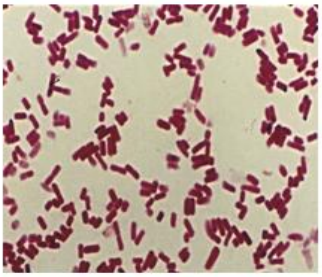

Fig. 1.3 *Clostridium* spp.

1.2.7 *Clostridium botulinum*

C. botulinum produces the most potent toxin known. It is a Gram-positive anaerobic rod shaped bacterium. Oval endospores are formed in stationary phase cultures. There are seven types of *C. botulinum* (A to G) based on the serological specificity of the neurotoxin produced. Botulism is a rare but very serious disease. The ingestion of neurotoxin produced by the organism in foods can lead to death. However, the toxin (a protein) is easily inactivated by heat. The organism can grow at temperature ranging from 10-48°C with optimum growth temperature at 37°C. Spores are highly heat resistant. The outgrowth of spores is inhibited at pH < 4.6, NaCl>

10% or water activity< 0.94. Botulinum spores are probably the most radiation resistant spores of public health concern. Contamination of foods is through soil and sediments where they are commonly present. The organism grows under obligate anaerobic conditions and produces toxin in under processed (improper canning) low acid foods at ambient temperature.

Fig-1.4: *Clostridium botulinum*

1.2.8 *Campylobacter*

Campylobacter are Gram negative nonspore forming rods. *Campyloleacter jejuni* is an important food borne pathogen. It is one of the many species within the genus *Campylobacter*. *Campylobacter* species *C. jejuni* and *C. coli* cause diarrhea in humans. The organism is heat sensitive (destroyed by milk pasteurization temperature). It is also sensitive to freezing. The organism belongs to the family *Campylobactereaceae*. The organisms are curved, S-shaped, or spiral rods that may form spherical or coccoids forms in old cultures or cultures exposed to air for prolonged periods. Most of the species are microaerophilic. It is oxidase and catalase positive and does not grow in the presence of 3.5% NaCl or at 25 °C or below. The incidences reported for gastro enteritis by this organism are as high as in case of *Salmonella*.

The organism is commonly present in raw milk, poultry products, fresh meats, pork sausages and ground beef. The infective dose of *C.jejuni* may be <1,000 organisms.

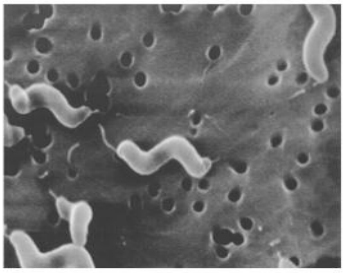

Fig. 1.5 Campylobacter

1.2.9 *Erwinia*

Erwinia is a genus of the family *Enterobacteriaceae* bacteria containing mostly plant pathogenic species. The organisms was named after the first phytobacteriologist, Erwin Smith. It is a Gram negative bacterium related to *E. coli*, *Shigella*, *Salmonella* and *Yersinia*. It is primarily a rod-shaped bacterium. A well-known member of this genus is the species *E. amylovora*, which causes fire blight on apple, pear and other Rosaceous crops. *Erwinia carotovora* (also known as *Pectobacterium carotovorum*) is another species, which causes diseases in many plants. These species produce pectolytic enzymes that hydrolyze pectin between individual plant cells. . Decay caused by *E. carotovora* is often referred to as bacterial soft rot (BSR). Most plants or plant parts can resist invasion by the bacteria, unless some type of wound is present. High humidity and temperatures around 30°C favor development of decay.

1.2.10 *Enterococcus* (*E. faecium*, *E. faecalis*)

Enterococcus is a genus of lactic acid bacteria. Enterococci are Gram positive cocci that often occur in pairs (diplococci) or short chains and are difficult to distinguish from streptococci on physical characters mentioned above. The two species are commensal organisms in the intestine of humans.

The Enterococci are facultative anaerobic organisms non spore forming that grows optimally at 35°C . However, they tolerate wide range of environmental conditions (10-45°C) pH (4.5 to 10.5) quite high NaCl concentration (.6.5%) and can survive heating at 60°C for 30 min.

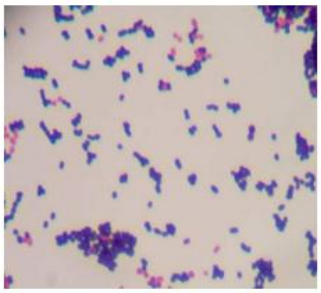

Fig. 1.6 *Enterococcus* spp.

Catalase-negative, oxidase negative-bacteria of the genes *Enterococcus* are ubiquitous organisms that often occur in large numbers on vegetables, plant materials and foods especially those of animal origin such as meat and dairy products. Enterococci also constitute a large preparation of autochthonous bacteria associated with the mammalian gastro-intestinal tract.

The resistance of enterococci to pasteurization temperatures and their adaptability to different substrates and growth conditions in food products manufactured from raw materials and in heat treated food products is of great significance.

Enterococci may constitute an important part of the microflora of fermented cheese and meats.

1.2.11 *Escherichia coli*

E. coli strains are associated with food borne gastroenteritis. These are Gram-negative asprogeneous rods that ferment lactose and produce dark colonies with a metallic sheen on Endo agar. The organism grows well on a large number of media and in many foods. They grow over a wide range of temperature (4 to 46 °C) and pH (4.4 to 9.0).

However, they grow very slowly in foods held at refrigerator temp. (5 °C). They belong to the family *Enterobacteriaceae*. The organism is also an indicator of fecal pollution. The organism is also capable of producing acid and gas and off-flavours in foods. *E. coli* strains involved in foodborne-illness can be placed into five groups: enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enterohemorrhagic (EHEC) and facultatively enteropathogenic (FEEC).

The organism also grows in the presence of bile salts. The primary habitat of *E.coli* is the intestinal tract of most warm blooded animals. *E.coli* 0157: H7 strains are unusually tolerant of acidic environments.

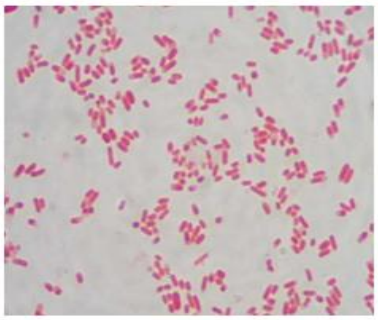

Fig. 1.7 *E. coli*

1.2.12 *Lactococcus*

L.lactis subsp. *lactis*

L.lactis subsp. *cremoris*

L.lactis subsp.*lactis biovar diaectylactis*

Lactococcus is a genus of lactic acid bacteria that were formerly included in the genus Streptococcus Group N (Group N Streptococci). They are known as homofermentors meaning that they produce a single product of glucose fermentation. They are Gram-positive, catalase negative, non-motile coccus that are found singly, in pairs or in chains. Some of the strains of lactococci are known to grow at or below 7 °C.

Lactococci are intimately associated with dairy products. These organisms are commonly used in the dairy industry in the manufacture of fermented dairy products like cheeses. They can be used in single strain starter cultures or in mixed strain cultures with other lactic acid bacteria such as *Lactobacillus* and *Streptococcus*. Their main purpose in dairy production is the rapid acidification of milk. This causes drop in the pH of fermented product which prevents the growth of spoilage and pathogenic bacteria. These bacteria also play a role in the flavor of the final product. Dairy lactococci have also been exploited for several industrial fermentations in the biotechnology industry. They are easily grown at industrial scale up on cheap whey based media.

Lactococcus lactis subsp. *lactis* includes species formerly designated as *S. lactis* subsp. *lactis*. *L. lactis* subsp. *cremoris* is distinguished from *L. Lactis* subsp. *lactis* by the inability to (i) grow at 40 °C (ii) grow in 4% NaCl (iii) hydrolyse arginine and (iv) ferment ribose.

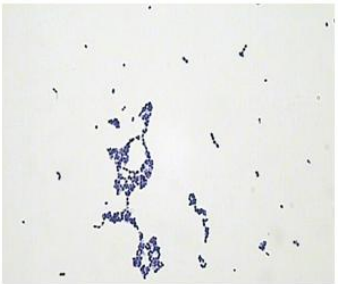

Fig-1.8: *Lactococcus* spp.

1.2.13 *Lactobacillus* (*L. bulgaricus, L. helveticus. L. plantarum,*

L. acidophilus, L. casei, L. lactis, L. fermentum)

The organisms belonging to this important genus are rods usually long and slender and in some of the species form chains. They are aerotolerant/microaerophilic but some ferment sugars chiefly to lactic acids if they are homofermentative. The hetero fermentative species, besides lactic acid, also produce small amount of acetic acid, carbon dioxide and trace amounts of volatile compounds such acetaldehyde and alcohol. The homofermentative species of *Lactobacillus* include *L. bulgaricus, L. casei, L. helveticus, L. lactis, L. acidophilus* and grow optimally at 37 °C. *L. fermentum, L. brevis* are the typical example of hetero fermentative *Lactobacillus* and grow well at higher temperatures.

Lactobacilli are of considerable importance in foods as they ferment sugar to lactic acid and other desirable flavouring compounds and are thus used in the production of fermented plant dairy and meat products. However, they are also implicated in the spoilage of wine and beer.

The organism normally occurs on plant surfaces silage, manure and dairy products. They are quite fastidious in their nutritional requirements as they are unable to synthesize certain vitamins they require and, therefore, media need to be supplemented with these vitamins for their growth.

Some of the strains are psychotrophic in nature and are thus involved in the spoilage of refrigerated meats. On the other hand thermoduric properties (resistance to pasteurization temperature) of some of the thermophilic strains of lactobacilli are quite useful in the manufacture of certain varieties of cheeses e.g. Swiss cheese. Some strains of lactobacilli also show probiotic attributes and are finding application in functional probiotic foods and in pharmaceutical preparations.

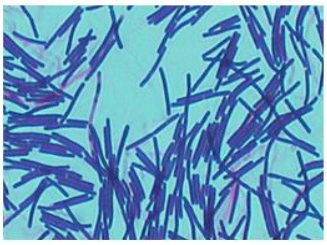

Fig-1.9: *Lactobacillus* spp.

1.2.14 *Leuconostoc*

Leuconostoc is a genus of Gram-positive bacteria, placed within the family of *Leuconostocaceae*. They are generally ovoid cocci often forming chains. *Leuconostoc* spp. are intrinsically resistant to vancomycin and are catalase-negative (which distinguishes them from staphylococci). All species within this genus are heterofermentative and are able to produce dextran from sucrose. They are generally slime-forming. Blamed for causing the 'stink' when creating a sourdough starter, some species are also capable of causing human infection.

Leuconostoc spp. along with other lactic acid bacteria such as *Pediococcus* and *Lactobacillus* spp , is responsible for the fermentation of cabbage, to sauerkraut. In this process the sugars in fresh cabbage are transformed to lactic acid which give it a sour flavour and good keeping qualities.

1.2.15 *Listeria monocytogenes*

Listeria monocytogenes in foods has attracted worldwide attention due to the serious illness it causes in human beings. The Listeria are Gram positive non spore forming, nonacid-fast rods. The organism is catalase positive and produces lactic acid from glucose and other fermentable sugars. The organism grows well in brain heart infusion (BHI), trypticase soy, and tryptose broths. However, the medium should be fortified with B. vitamins and the amino acids. It is a mesophilic organism with optimal growth temperature 37°C but it can grow at refrigerator temperature also. Strains grows over the temperature range of 1°C to 45°C and pH range 4.1 to 9.6.

Listeria monocytogenes is widely distributed in nature and can be isolated from decaying vegetation, soil, animal feces, sewage, silage and water. The organism has been found in raw milk, pork, raw poultry, ground beef and vegetables. The HTST treatment of pasteurization is good enough to destroy the organism in milk.

The most significant virulence factor associated with *L. monocytogenes* is listeriolysin O. The virulent strains produce β-hemolysis on blood agar and acid from rhamnose.

L. monocytogenes grows well in moderate salt concentrations (6.5%).

L. monocytogenes is unique among foodborne pathogens while other pathogens excrete toxins or multiply in the blood stream, *L. monocytogenes* enters the host's cells and grows inside the cell. In humans it crosses the intestinal barrier after entering by the oral route.

Ready to Eat (RTE) foods that are preserved by refrigeration pose a special challenge with regard to *L. monocytogenes* infection.

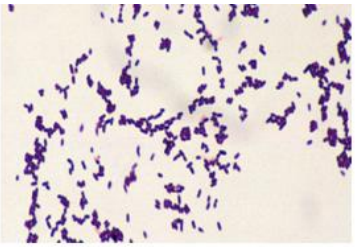

Fig-1.10: *Listeria* spp.

1.2.16 *Micrococcus*

Micrococcus occurs in a wide range of environments, including water, dust, and soil. Micrococci are Gram-positive spherical cells ranging from about 0.5 to 3 micrometers in diameter and typically appear in tetrads. *Micrococcus* has a substantial cell wall, which may comprise as much as 50% of the cell mass. Some species of *Micrococcus*, such as *M. luteusM. roseus* (red) produce yellow or pink colonies when grown on mannitol salt agar. *Micrococcus* is generally thought to be a saprophytic or commensal organism, though it can be an opportunistic pathogen, particularly in hosts with compromised immune systems, such as HIV patients.

1.2.17 *Proteus*

Since it belongs to the family of *Enterobacteriaceae*, general characters are applied on this genus: It is oxidase-negative, but catalase and nitrate reductase positive. Three species *P. vulgaris*, *P. mirabilis*, and *P. penneri* are opportunistic human pathogens. *Proteus*includes pathogens responsible for many human urinary tract infections. *P. mirabilis* causes wound and urinary tract infections. Most strains of *P. mirabilis* are sensitive to ampicillin and cephalosporins. *P. vulgaris* is not sensitive to these antibiotics. However, this organism is isolated less often in the laboratory and usually only targets immune suppressed individuals. *P. vulgaris* occurs naturally in the intestines of humans and a wide variety of animals; also manure, soil and polluted waters. *P. mirabilis*, once attached to urinary tract, infects the kidney more commonly than *E. coli*. *P. mirabili*s are often found as free-living organisms in soil and water.

1.2.18 *Propionibacterium* spp. (*P. freudenreichii*)

Historically, *Propionibacterium* spp. are of interest because of their use as dairy starters (especially in the production of Swiss-type cheese) and their ability to produce propionic acid during growth. The genus *Propionibacterium* is generally split into "cutaneous" and "dairy" groups. The

dairy *Propionibacterium* spp. can also be isolated primarily from dairy foods and silage. The species in dairy products include *P. jensenii, P. acidipropionici, P. theoniiP. freudenreichii*. Propionibacteria have a role in the production of flavour compounds in cheese by proteolysis and propionic acid production. Dairy strains of propionibacteria are autolytic under environmental conditions found in cheese and degrade peptides and amino acids that are present in the cheese. And The dairy species offer an interesting opportunity as novel probiotic organisms with the most obvious advantage being that they are considered safe for ingestion.

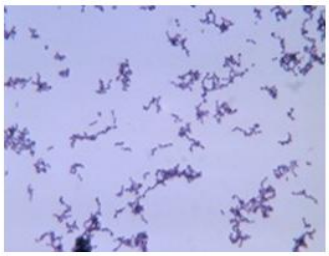

Fig-1.11: ***Propionibacterium spp***

1.2.19 *Pediococcus* spp. (*Pediococcus pentosaceus, P. acidilactici*)

Pediococci compromise a group of bacteria that are of economic importance in the brewing and food industries. Several species and strains of pediococci have been used as starter cultures in the fermentation of vegetables, meats, sausage products, fermented milks and associated with the development of flavor in Cheddar and other related cheese varieties. Some strains form capsular material that causes beer to become ropy and viscous.

They are catalase negative and exhibit a homolactic type of fermentation and produce optically inactive lactic acid i.e. a mixture of the L(+) and D(-) type. They generally appear in tetrads.

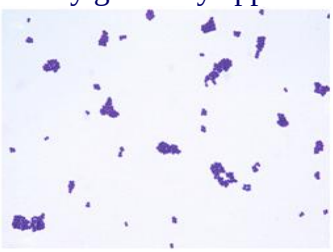

Fig-1.12: ***Pediococcus* spp.**

1.2.20 *Pseudomonas fluorescens*

Pseudomonas fluorescens is a common Gram-negative, rod-shaped, motile bacterium. The organism is psychrotrophic in nature and grows at refrigeration temperature (7°C). It has an extremely versatile metabolism, and can be found in the soil and in water. It is an obligate aerobe, but certain strains are capable of using nitrate instead of oxygen as a final electron acceptor during cellular respiration. Optimal temperature for growth of *Pseudomonas fluorescens* is 25-30 °C. It tests positive for the oxidase. *Pseudomonas fluorescens*is also a nonsaccharolytic organism. Heat-stable lipases and proteases are produced by *Pseudomonas fluorescens* and other similar pseudomonads. These enzymes cause milk to spoil, by causing bitterness, casein breakdown, and ropiness due to the production of slime and coagulation of proteins.

1.2.21 *Pseudomans aeruginosa*

It is a Gram-negative, aerobic, rod-shaped bacterium with unipolar motility. An opportunistic human pathogen, *P. aeruginosa* is also an opportunistic pathogen of plants. *P. aeruginosa* is the type species of the genus *Pseudomonas* (Migula). Gram-stained*Pseudomonas aeruginosa* bacteria (pink-red rods) secretes a variety of pigments, including pyocyanin (blue-green), pyoverdine (yellow-green and fluorescent), and

pyorubin (red-brown). *P. aeruginosa* is often preliminarily identified by its fluroscence and grape-like or tortilla-like odor *in vitro*. Definitive clinical identification of *P. aeruginosa* often includes identifying the production of pyocyanin and fluorescein, as well as its ability to grow at 42°C. *P. aeruginosa* is capable of growth in diesel and jet fuel, where it is known as a hydrocarbon-using microorganism (or "HUM bug"), causing microbial corrosion. *P. aeruginosa* is considered by many as a facultative anaerobe

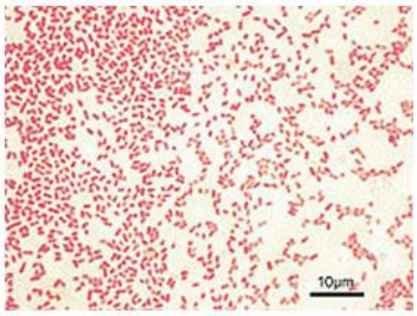

Fig-1.13: *Pseudomonas aeruginosa*

1.2.22 *Salmonella* (*S. typhimurium*, *S. typhi*, *S.enteritidis*)

Salmonella spp. have been reported to be a leading cause of foodborne illnesses in humans. Foodborne salmonellosis scores over all other foodborne bacterial illnesses in humans. Enteric fever is a serious human disease associated with typhoid and paratyphoid strains. *SalmonellaEnterobacteriaceae*. The optimum growth temperature is 37-45 °C. The organism can also grow at about 7°C in foods. I t ferments carbohydrates with its production of acid and gas. Salmonella are oxidase negative, catalase positive and grow on citrate as a sole carbon source and produce H_2S. Some *Salmonella* strains can grow at higher temperatures (54 °C) while others exhibit psychrotrophic properties. The organism has the ability to grow at pH values ranging from 4.5 to 9.5, with an optimum pH growth at 6.5 to 7.5. spp. are facultatively anaerobic, small Gram-negative, non spore forming, rod-shaped (2-4 m m) bacteria belonging to the family

Milk, meat and poultry are principle vehicles of human foodborne salmonellosis. Ingestion of only a few salmonella cells can be infectious. Low levels of salmonellae in a finished food products may, therefore, be of serious public health consequence.

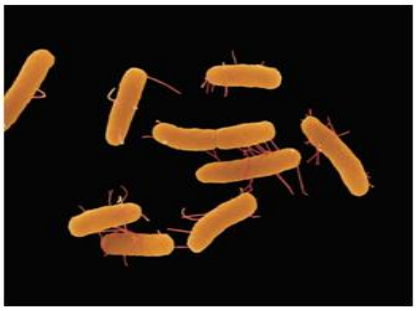

Fig-1.14: *Salmonella* spp.

1.2.23 *Serratia*

Serratia is a genus of Gram-negative, facultatively anaerobic, rod-shaped bacteria of the *Enterobacteriaceae* family. The most common species in the genus, *S. marcescens*, is normally the only pathogen and usually causes nosocomial infections. However, rare strains of *S. plymuthica*, *S. liquefaciens*, *S. rubidaea*, and *S. odoriferae* have caused diseases through infection. Members of this genus produce characteristic red pigment, prodigiosin.

1.2.24 *Streptococcus thermophilus*

The only *streptococcus* species that is associated with food technology is *S. thermophilus* which is used in the manufacture of yoghurt (in co culture with *L. bulgari*cus and Dahi).

S. thermophilus is a Gram positive facultative anaerobe and belongs to the family *Streptococcaceae*. It is catalase negative organism that is non-motile, non-spore forming and homofermentative and occurs in pairs to long chains. The spherical to avoid cells are with a diameter in the range of 0.7 to 0.9 µm. The optimum temperature for the growth of this organism is between 39°C to 45°C, although most species in the genus are able to grow at temperature ranging from 45-60°C. They do not grow at temperature below 20°C, but they can survive at 65 for 30 min. They ferment sugars with L (+) lactic acid as the major end product and produce around 0.6 to 0.8% lactic acid. They are able to grow in broth with 2.5% NaCl but fail to grow in 6.5% NaCl at pH 9.6 or in milk with 0.1% methylene blue (Bergey's Manual 1994). It is also classified as lactic acid bacteria (LAB). It is a very versatile organism. *S. thermophilus* has properties that make it one of the commercially most important lactic acid organism. *S. thermophilus* is used along with *Lactobacillus* spp., as a starter culture to manufacture several important fermented dairy foods including yoghurt and mozzarella cheese.

Though the natural habitat of *S. thermophilus* is yet to be established, most strains have been isolated from milk environments.

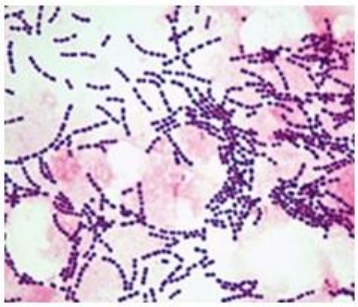

Fig-1.15: *Streptococcus thermophilus*

1.2.25 *Staphylococcus aureus*

Staphylococcus aureus is commonly associated with humans. It is a Gram-positive catalase-positive coccus. *Staphylococcus aureus* is the common cause of foodborne gastroenteritis known as staphylococcal food poisoning. Staphylococcal gastroenteritis is caused by the ingestion of food that contains one or more enterotoxin which are produced by some strains of *S. aureus*.

Although enterotoxin production is believed generally to be associated with coagulase and thermo nuclease producing *S. aureus* strains, many species of *Staphylococcus* that produce neither coagulase nor TNase are also known to produce enterotoxin.

The main reservoir of *S. aureus* is the nasal cavity of human beings from where they find their way to the skin and wounds. Mastitis in animals due to *S. aureus* is quite common and from the infected udder the organism finds its way to the milk.

The organism can grow well in NaCl concentrations of 7 to 10%. Though the optimum growth temperature of the organism is 37 °C , some strains can grow at a temperature as low as 6.7 °C. The organism can grow to water activity as low as 0.86.

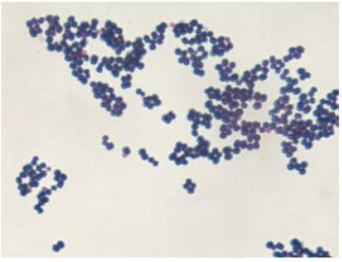

Fig-1.16: *Staphylococcus* spp.

1.2.26 *Shigella*

Bacillary dysentery, or shigellosis, is caused by *Shigella* species. *Shigella* is a member of the family *Enterobacteriaceae*. The growth temperature varies from 10 to 48 °C. *Shigella*$_2$S. *Shigella* does not usually survive well in low pH foods. *Shigella* is sensitive to ionizing radiations. species are non-motile, oxidase negative produce acid only from sugars; do not grow on citrate as sole carbon source, do not grow on KCN agar, and unlike Salmonellae do not produce H

Shigellosis is an important disease in developed and developing countries. Disease is caused by ingestion of contaminated foods, and in some instances it subsequently leads to rapid dissemination through contaminated feces from infected individuals. The infective dose may be as low as 100 cells. Contamination of foods usually does not occur at the processing plant but rather through an infected food handler. Humans are the natural reservoir of Shigella. The organism is spread through the fecal-oral route.

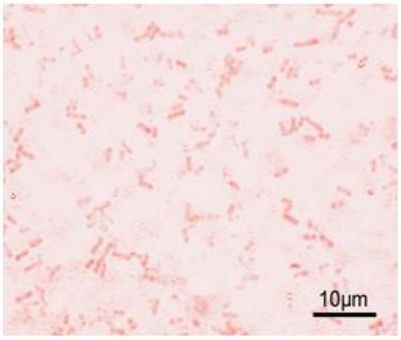

Fig. 1.17 *Shigella* spp.

1.2.27 *Vibrio*

Vibrio cholerae and *V. parahaemolyticus* are the two important species of the genus *Vibrio*. *Vibrio cholerae* O1 causes cholera, one of the few food borne illnesses with epidemic and pandemic potential. *Vibrio cholerae* are Gram-negative straight or curved rods and belong to the family *Vibrionaceae*. Important distinctions within the species are made on the basis of productions of cholera enterotoxin (CT) and serogroup.

Vibrio cholerae is part of the normal free living bacterial flora in estuarine areas. Amongst the many different enrichment broths described for the isolation of vibrios alkaline peptone water is the most commonly used. Though *V. parahaemolyticus* can grow in the presence of 1-8% NaCl, the best growth occurs in the salt concentration 2 to 4%.

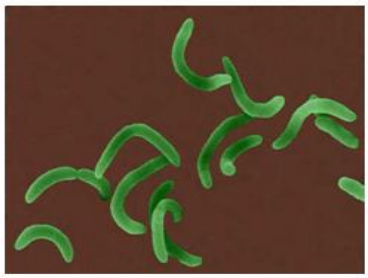

Fig. 1.18 *Vibrio* spp.

1.2.28 *Yersinia*

Yersinia enterocolitica and *Yersinia pestis* are the two important human pathogens while *Y. enterocolitica* causes food borne gastroenteritis, *Y. pestis* is an agent of human plague. *Y. enterocolitica* also known as newly emerging human pathogen is a heterogeneous species that is divisible into a large number of subgroups.

Y. enterocolitica is unusual because it can grow at temperatures below 4 °C. The generations time at the 28-30 °C (Optimum growth temperature) is almost 34 min. It also survives in frozen foods. It grows better in processed foods such as pasteurized milk, vacuum packed meat, boiled eggs, boiled fish, and cottage cheese.

Both the species can grow over a pH range of 4 to 10 (optimum pH is 7.6) and tolerate alkaline environment well. They can motile at a temperature < 30 °C. However, both these organisms are susceptible to pasteurization, ionizing and ultraviolet (UV) irradiation. The organism can also tolerate upto 5% NaCl.

Infections with *Yersinia* species are due to transmittance of the organism from animals to humans. The organism is frequently present in pork, lamb, poultry and dairy products.

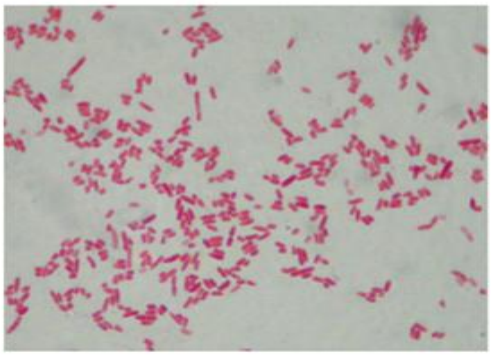

Fig-1.19: *Yersinia* spp.

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Lesson-2

Introduction to Food Microbiology: Part-II: Yeast, Mold and Virus

2.1. Food Borne Yeasts

Yeasts have been associated with foods since earliest times, both as beneficial agents and as major causes of spoilage and economic loss. Current losses to the food and dairy industry caused by yeast spoilage are estimated at several billion dollars. As new food ingredients and new food manufacturing technologies are introduced, novel food spoilage yeasts are emerging to present additional problems. To date over 70 biological species of yeasts have been described and thousands of different varieties have been shown to exist in all kinds of natural and artificial habitats.

Yeasts may be viewed as being unicellular fungi in contrast to the molds, which are multi-cellular. Yeasts can be differentiated from bacteria by their larger cell size and their oval, elongate, elliptical, or spherical cell shapes. Typical yeast cells range from 5 to 8 um in diameter, with some being even larger. Older yeast cultures tend to have smaller cells. Most of those of importance in foods divide by budding or fission.

Yeasts can grow in presence of various types of organic acids such as lactic, citric and tartaric acid etc and also over a wide range of acid pH and in up to 18% ethanol. Many grow in the presence of 55-60% sucrose. Many colours are produced by yeasts, ranging from creamy to pink to red. The asco- and arthrospores of some are quite heat resistant.

2.1.1. *Candida*

Members of the *Candida* genus form shining white colonies and cells contain no carotenoid pigments. *Candida tropicalis* is the most prevalent in foods in general Some members are involved in the fermentation of cocoa beans, as a component of kefir grains, and many other products, including beers, and fruit juices.

2.1.2. *Debaromyces*

Debaromyces is one of the most prevalent yeast genera in the dairy products. It can grow in 24% NaCl and at an a_w as low as 0.65.

2.1.3. *Kluyveromyces*

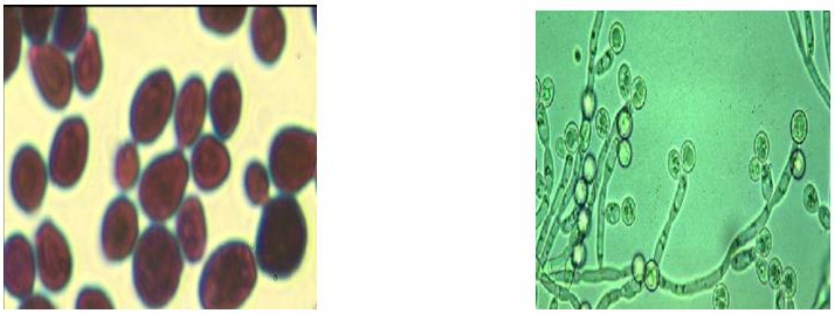
Kluyveromyces spp. produces β-galctosidase and are vigorous fermenters of sugars including lactose. *K. marxianus* is one of the two most prevalent yeasts in dairy products, kefir grain and causes cheese spoilage.

2.1.4 *Rhodotorula*

The genus *Rhodotorula* contains many psychrotrophic species that are found on fresh poultry, shrimp, fish and beef. Some grow on the surface of butter.

2.1.5 *Saccharomyces*

Saccharomyces are ascosporogenous yeasts that multiply by lateral budding and produce spherical spores in asci. They are diploid and do not ferment lactose. All bakers' brewers', wine and champagne yeasts are *S. cerevisiae*. They are found in Kefir grains and can be isolated from wide range of foods. *S. cerevisiae* rarely causes spoilage.



(a) (b)

Fig. 2.1 Food Borne Yeasts (a) *Saccharomyces* spp. (b) *Candida* spp.

2.1.6 *Torulaspora*

Torulaspora multiplies by lateral budding. They are strong fermenters of sugars. *Torula delbrueckii* is the most prevalent species.

2.2. Food-Borne Molds

Molds are filamentous fungi that grow in the form of tangled mass that spreads rapidly and may cover several inches of area in a very short period. It is also referred to as mycelial growth. Mycelium is composed of branches of filaments referred to as hyphae. The molds of great importance in foods multiply by ascospores or conidia. The ascospores of some of the mold genera are notable for their extreme degrees of heat resistance.

2.2.1. *Alternaria*

Alternaria spp. form septate mycelia with conidiophores and large brown conidia are produced. They cause brown to black rots of fruits, apples, and figs. Some species produce mycotoxins.

2.2.2 *Aspergillus*

The *Aspergillus* spp. appear yellow to green to black on a large number of foods. Some species cause spoilage of oils. *A. niger*produces β-galactosidase, glucoamylase, invertase, lipase and pectinase. *A. oryzae* produces a -amylase. Two species *A. flavus* and *A. parasiticus* produce aflatoxins, and others produce ochratoxin A and sterigmatocystin.

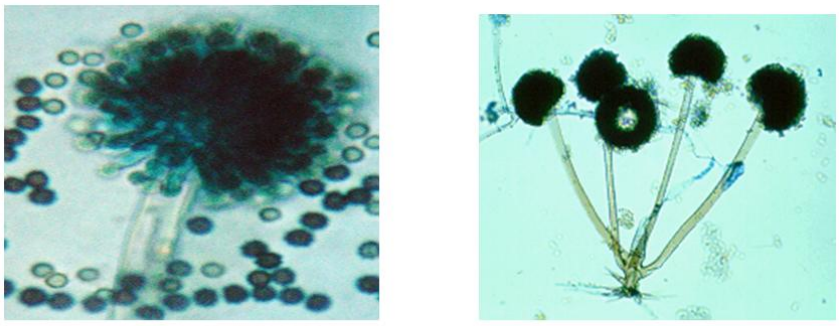
2.2.3 *Geotrichum*

The yeast like fungi, *Geotrichum* are also referred to as dairy mold.

2.2.4 *Mucor and Rhizopus*

Mucor species that produce non-septate hyphae are prominent food spoilers. Similarly, *Rhizopus* spp. also produce non septate hyphae but give rise to stolons and rhizoids. *R. stolonifer* is by far the most common species in foods and is also referred to as "bread mold".

Other important genera of molds related to spoilage of foods are *Neurospora, Thamnidium, Trichothecium, Penicillium* and*Cladosporium* etc.



(a) (b)

Fig. 2.2. Food Borne Molds (a) *Aspergillus* spp. and (b) *Rhizopus* spp.

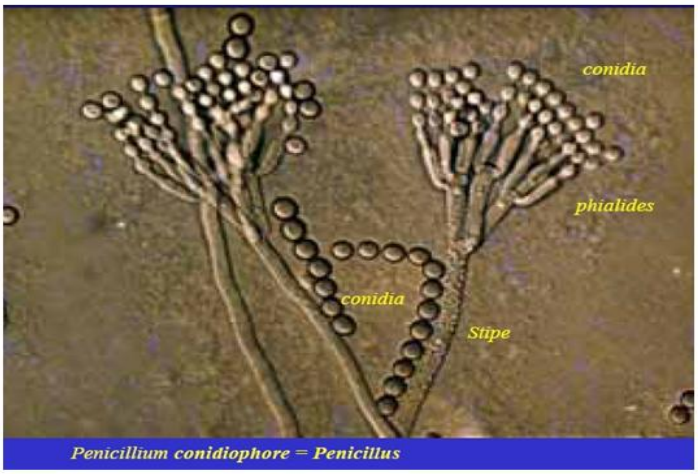


Fig. 2.3. Food Borne Molds (a) *Pencillium* spp.

2.3. Food borne viruses

Viruses are filterable, ultra microscopic particles and can be cultivated only on live tissues. Viruses consist of a core of nucleic acid (DNA or RNA) and a protein coat. It is commonly believed that some of the viruses are responsible for food borne diseases in humans, particularly some non bacterial gastroenteritis due to enteroviruses. Contaminated water and food are important carriers of hepatitis viruses. Foot and mouth disease (FMD) causing virus in cattle can be transmitted to human beings through foods. Similarly, viral diseases of poultry have also been source of ailments in humans.

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Lesson 3

Microbial Growth And Its Quantification

3.1 Introduction

Growth is an orderly increase in the quantity of cellular constituents. It depends upon the ability of the cell to form new protoplasm from nutrients available in the environment. In most bacteria, growth involves increase in cell mass and number of ribosomes, duplication of the bacterial chromosome, synthesis of new cell wall and plasma membrane, partitioning of the two chromosomes, septum formation, and cell division. This asexual process of reproduction is called **binary fission**. For unicellular organisms such as bacteria, growth can be measured in terms of two different parameters: changes in **cell mass** and changes in **cell numbers**.

3.2 Methods for Measurement of Cell Biomass

Methods for the measurement of the cell mass involve both direct and indirect techniques.

i. Direct **physical measurement** of dry weight, wet weight, or volume of cells after centrifugation.

ii. Direct **chemical measurement** of some chemical component of the cells such as total N, total protein, or total DNA contents.

iii. Indirect **measurement of chemical activity** such as rate of O_2 production or consumption, CO_2 production or consumption, etc.

iv. **Turbidity measurements** employ a variety of instruments to determine the amount of light scattered by a suspension of cells. Particulate objects such as bacteria scatter light in proportion to their numbers. The turbidity or **optical density** of a suspension of cells is directly related to cell mass or cell number. The method is simple and nondestructive, but the sensitivity is limited to about 10^7cells per ml for most bacteria.

3.3 Methods for Measurement of Cell Numbers

Measuring techniques involve direct counts, visually or instrumentally, and indirect viable cell counts.

3.3.1 Direct microscopic counts (DMC)

DMC are possible using special slides known as counting chambers. Dead cells cannot be distinguished from living ones. Only dense suspensions can be counted ($>10^7$ cells per ml), but samples can be concentrated by centrifugation or filtration to increase sensitivity.

A variation of the direct microscopic count has been used to observe and measure growth of bacteria in natural environments. In order to detect and prove that thermophilic bacteria were growing in boiling hot springs, T.D. Brock immersed microscope slides in the springs and withdrew them periodically for microscopic observation. The bacteria in the boiling water attached to the glass slides naturally and grew as micro-colonies on the surface.

3.3.2 Electronic counting chambers

This is done to measure size distribution of cells. F or cells size of the bacteria, the suspending medium must be very clean. Such electronic devices are more often used to count eucaryotic cells such as blood cells.

3.3.3 Indirect viable cell counts

This is also called **plate counts**, involve plating out (spreading) a sample of a culture on a nutrient agar surface. The sample or cell suspension can be diluted in a nontoxic diluent (e.g. water or saline) before plating. If plated on a suitable medium, each viable unit grows and forms a colony. Each colony that can be counted is called a **colony forming unit (cfu)** and the number of cfu's is related to the viable number of bacteria in the sample.

Advantages of the technique are its sensitivity (theoretically, a single cell can be detected), and it allows for inspection and positive identification of the organism counted. Disadvantages are (1) only living cells develop colonies that are counted; (2) clumps or chains of cells develop into a single colony; (3) colonies develop only from those organisms for which the cultural conditions are suitable for growth. The latter makes the technique virtually useless to characterize or count the **total number of bacteria** in complex microbial ecosystems such as soil or the animal rumen or gastrointestinal tract. Genetic probes can be used to demonstrate the diversity and relative abundance of procaryotes in such an environment, but many species identified by genetic techniques have so far proven unculturable.

3.4 The Bacterial Growth Curve

In the laboratory, under favorable conditions, a growing bacterial population doubles at regular intervals. Growth is by geometric progression: 1, 2, 4, 8, etc. or 2^0, 2^1, 2^2, 2^3.....2^n**exponential growth**. In reality, exponential growth is only part of the bacterial life cycle, and not representative of the normal pattern of growth of bacteria in nature. (where n = the number of generations). This is called

When a fresh medium is inoculated with a given number of cells, and the population growth is monitored over a period of time, plotting the data will yield a **typical bacterial growth curve** (Figure 3.1).

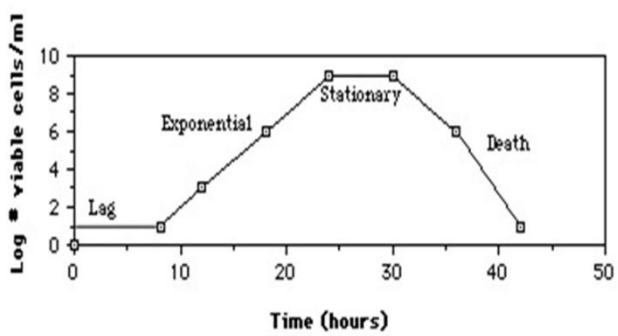


Fig. 3.1. The typical bacterial growth curve.

When bacteria are grown in a closed system (also called a batch culture), like a test tube, the population of cells almost always exhibits these growth dynamics: cells initially adjust to the new medium (lag phase) until they can start dividing regularly by the process of binary fission (exponential phase). When their growth becomes limited, the cells stop dividing (stationary phase), until eventually they show loss of viability (death phase). Note the parameters of the x and y axes. Growth is expressed as change in the number viable cells vs time. Generation times are calculated during the exponential phase of growth. Time measurements are in hours for bacteria with short generation times.

3.4.1 Four phases of the growth cycle

3.4.1.1 Lag phase

Immediately after inoculation of the cells into fresh medium, the population remains temporarily unchanged. Although there is no apparent cell division occurring, the cells may be growing in volume or mass, synthesizing enzymes, proteins, RNA, etc., and increasing in metabolic activity.

The length of the lag phase is apparently dependent on a wide variety of factors including the size of the inoculum; time necessary to recover from physical damage or shock in the transfer; time required for synthesis of essential coenzymes or division factors; and time required for synthesis of new (inducible) enzymes that are necessary to metabolize the substrates present in the medium.

3.4.1.2 Exponential (log) phase

The exponential phase of growth is a pattern of balanced growth wherein all the cells are dividing regularly by binary fission, and are growing by geometric progression. The cells divide at a constant rate depending upon the composition of the growth medium and the conditions of incubation. The rate of exponential growth of a bacterial culture is expressed as **generation time**, also the **doubling time** of the bacterial population. Generation time (G) is defined as the time (t) per generation (n = number of generations). Hence, G=t/n is the equation from which calculations of generation time derive.

3.4.1.3 Stationary phase

Exponential growth cannot be continued forever in a **batch culture** (e.g. a closed system such as a test tube or flask). Population growth is limited by one of the three factors *viz.*, 1. exhaustion of available nutrients; 2. accumulation of inhibitory metabolites or end products; 3. exhaustion of space, in this case called a lack of "biological space".

During the stationary phase, if viable cells are being counted, it cannot be determined whether some cells are dying and an equal number of cells are dividing, or the population of cells has simply stopped growing and dividing. The stationary phase, like the lag phase, is not necessarily a period of quiescence. Bacteria that produce **secondary metabolites**, such as antibiotics, do so during the stationary phase of the growth cycle (Secondary metabolites are defined as metabolites produced after the active stage of growth). It is during the stationary phase that spore-forming bacteria have to induce or unmask the activity of dozens of genes that may be involved in sporulation process.

3.4.1.4 Death phase

If incubation continues after the population reaches stationary phase, a death phase follows, in which the viable cell population declines. However, if counting is done by turbidimetric measurements or microscopic counts, the death phase cannot be observed. During the death phase, the number of viable cells decreases geometrically (exponentially), essentially the reverse of growth during the log phase.

3.5 Growth Rate and Generation Time

As mentioned above, bacterial growth rates during the phase of exponential growth, under standard nutritional conditions (culture medium, temperature, pH, etc.), define the bacterium's generation time. Generation times for bacteria vary from about 12 minutes to 24 hours or more. The generation time for *E. coli* in the laboratory is 15-20 minutes, but in the intestinal tract, the coliform's generation time is estimated to be 12-24 hours. For most known bacteria that can be cultured, generation times range from about 15 minutes to 1 hour. Symbionts such

as *Rhizobium* tend to have longer generation times. Many lithotrophs, such as the nitrifying bacteria, also have long generation times. Some bacteria that are pathogens, such as *Mycobacterium tuberculosis* and *Treponema pallidum*, have especially long generation times, and this is thought to be an advantage in their virulence. Generation times for a few bacteria are shown in Table 3.1.

Table 3.1 Generation times for some common bacteria under optimal conditions of growth.

Bacterium	Medium	Generation Time (minutes)
Escherichia coli	Glucose-salts	17
Bacillus megaterium	Sucrose-salts	25
Lactococcus lactis	Lactose broth	48
Staphylococcus aureus	Heart infusion broth	27-30
Lactobacillus acidophilus	MRS broth	66-87
Rhizobium japonicum	Mannitol-salts-yeast extract	344-461
Mycobacterium tuberculosis	Synthetic	792-932
Treponema pallidum	Rabbit testes	1980

3.5.1 Calculation of generation time

When growing exponentially by binary fission, the increase in a bacterial population is by geometric progression. If we start with one cell, when it divides, there are 2 cells in the first generation, 4 cells in the second generation, and 8 cells in the third generation, and so on. The **generation time** is the time interval required for the cells (or population) to divide.

G (generation time) = (time, in minutes or hours)/n(number of generations)

$G = t/n$

t = time interval in hours or minutes

B = number of bacteria at the beginning of a time interval

b = number of bacteria at the end of the time interval

n = number of generations (number of times the cell population doubles during the time interval)

$b = B \times 2^n$ (This equation is an expression of growth by binary fission)

Solve for n:

$\log b = \log B + n\log 2$

$$n = \frac{\log b - \log B}{\log 2}$$

$$n = \frac{\log b - \log B}{.301}$$

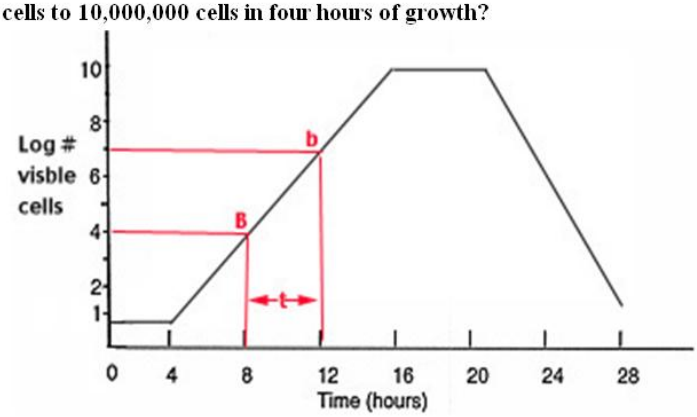
n = 3.3 logb / B

G = t/n

Solve for G

$$G = \frac{t}{3.3 \log b/B}$$

Example: What is the generation time of a bacterial population that increases from 10,000 cells to 10,000,000 cells in four hours of growth?



$$G = \frac{t}{3.3 \log b/B}$$

$$G = \frac{240 \text{ minutes}}{3.3 \log 10^7/10^4}$$

$$G = \frac{240 \text{ minutes}}{3.3 \times 3}$$

G = 24 minutes

3.6 Continuous Culture of Bacteria

The cultures so far discussed for growth of bacterial populations are called **batch cultures**. Since the nutrients are not renewed, exponential growth is limited to a few generations. Bacterial cultures can be maintained in a state of exponential growth over long periods of time using a system of **continuous culture** (Figure 2.2), designed to relieve the conditions that stop exponential growth in batch cultures. Continuous culture, in a device called a **chemostat or turbidostat** that can be used to maintain a bacterial population at a constant density, a situation that is, in many ways, more similar to bacterial growth in natural environments.

In a chemostat, the growth chamber is connected to a reservoir of sterile medium. Once growth is initiated, fresh medium is continuously supplied from the reservoir. The volume of fluid in the growth chamber is maintained at a constant level by some sort of overflow drain. Fresh medium is allowed to enter into the growth chamber at a rate that limits the growth of the bacteria. The bacteria grow (cells are formed) at the same rate that bacterial cells (and spent medium) are removed by the overflow. The rate of addition of the fresh medium determines the rate of growth because the fresh medium always contains a limiting amount of an essential nutrient. Thus, the chemostat relieves the insufficiency of nutrients, the accumulation of toxic substances, and the accumulation of excess cells in the culture, which are the parameters that initiate the stationary phase of the growth cycle. The bacterial culture can be grown and maintained at relatively constant conditions, depending on the flow rate of the nutrients.

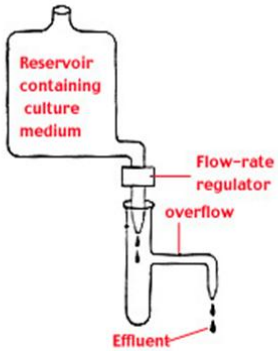


Figure 3.2. Schematic diagram of a chemostat, a device for the continuous culture of bacteria. The chemostat relieves the environmental conditions that restrict growth by continuously supplying nutrients to cells and removing waste substances and spent cells from the culture medium.

3.6.1 Synchronous Growth of Bacteria

Studying the growth of bacterial populations in batch or continuous cultures does not permit any conclusions about the growth behavior of individual cells, because the distribution of cell size (and hence cell age) among the members of the population is completely random. Information about the growth behavior of individual bacteria, however, is obtained by the study of **synchronous cultures**. Synchronized cultures must be composed of cells which are all at the same stage of the **bacterial cell cycle**. Measurements made on synchronized cultures are equivalent to measurements made on individual cells.

Synchronous growth of a population of bacterial cells is illustrated in Figure 2.3. Synchronous cultures rapidly lose synchrony because not all cells in the population divide at exactly the same size, age or time.

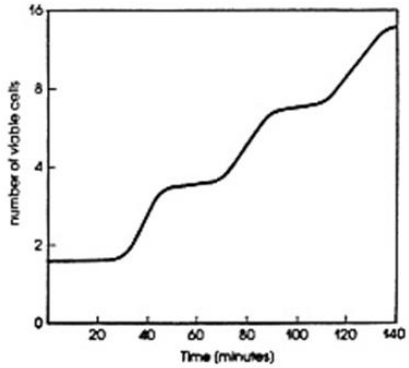


Figure 3.3. The synchronous growth of a bacterial population. By careful selection of cells that have just divided, a bacterial population can be synchronized in the bacterial cell division cycle. Synchrony can be maintained for only a few generations.

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Lesson 4

Factors Affecting Growth And Survival Of Microorganisms In Foods

4.1 Introduction

Food spoilage means the original nutritional value, texture, flavour, etc.,of the food are damaged, the food become harmful to people and unsuitable to eat. Food can deteriorate as a result of two main factors:

1) Growth of **micro-organisms** - usually from surface contamination - especially important in processed food

2) Action of **enzymes** - from within cells - part of normal life processes, (responsible for respiration, for instance). It is important to note that many plants - fresh vegetables and fruit - are still alive when bought and even when eaten raw, and meat from animals undergoes gradual chemical changes after slaughter.

The various sources through which microorganisms gain entry into the foods are shown in Table 3.1. Microorganisms involved in food spoilage (other than Canned Foods) with some examples of causative organisms are enlisted in Table 4.2

Table-4.1: Primary sources of microorganisms found in foods

Microflora present in soil and water
Microflora present in air
Microflora present on plant and plant products
Microflora present on food utensils and equipments
Microflora present in animal feeds
Microflora present on animal hides
Microflora present in intestinal tracts of humans and animals
Food handlers

Table 4.2 Microorganisms involved in food spoilage (Other than Canned Foods) with some examples of causative organisms

Food	Type of Spoilage	Microorganisms involved
Bread	Mouldy	*Rhizopus nigricans* *Penicillium* spp. *Aspergillus niger*
	Ropy	*Bacillus subtilis*
Maple sap and syrup	Ropy	*Enterobacter aerogenes*
	Yeasty	*Saccharomyces* *Zygosaccharomyces*
	Pink	*Micrococcus roseus*
	Mouldy	*Aspergillus* *Penicillium*
Fresh fruits and vegetables	Soft rot	*Rhizopus* *Erwinia*
	Gray mold rot	*Botrytis*
	Black mold rot	*A. niger*
Pickles, sauerkraut	Film yeasts, pink yeasts	*Rhodotorula*
Fresh meat	Putrefaction	*Alcaligenes* *Clostridium* *Proteus vulgaris* *Pseudomonas fluorescens*
Cured meat	Mouldy	*Aspergillus* *Rhizopus* *Penicillium*
	Greening, slime	*Lactobacillus* *Leuconostoc*
Fish	Discoloration	*Pseudomonas*
	Putrefaction	*Alcaligenes*
Eggs	Green rot	*P. fluorescens*
	Colorless rots	*Pseudomonas* *Alcaligenes*
	Black rots	*Proteus*
Concentrated orange juice	"Off" flavor	*Lactobacillus* *Leuconostoc* *Acetobacter*
Poultry	Slime, odor	*Pseudomonas* *Alcaligenes*

A variety of intrinsic and extrinsic factors determine whether microbial growth will preserve or spoil foods, as shown in Table 3.3. Intrinsic or food related parameters are those parameters of plants and animal tissues which

are inherent part of the tissue. e.g., pH, water activity (a_W), oxidation-reduction potential (Eh), nutrient content, antimicrobial constituents and biological structures. Extrinsic or environmental parameters are properties of storage environments which affect both foods as well as microorganisms and include temperature of storage, relative humidity of storage environment, and concentration of gases in environment.

Table 4.3 Factors affecting the development of microorganisms in foods

Intrinsic Factors	Extrinsic factors	Implicit factors	Processing factors
Nutrient content	Temperature	Synergism	Irradiation
pH	Relative humidity	Antagonism	Washing
Redox potential	Gaseous atmosphere	Commensalism	Slicing
Water activity	Growth rate		Pasteurization
Antimicrobial constituents & barriers			Packaging

4.2 Intrinsic Parameters

4.2.1 Nutrient content

Like all other living beings, microorganisms need water, a source of carbon, an energy source, a source of nitrogen, minerals, vitamins and growth factors in order to grow and function normally. Since foods are rich source of these compounds, they can be used by microorganisms also. It is because of these reasons that various food products like malt extracts, peptone, tryptone, tomato juice, sugar and starch are incorporated in microbial media. The inability to utilize a major component of the food material will limit its growth and put it at a competitive disadvantage compared to those that can. In general, molds have the lowest requirement, followed by yeasts, Gram-negative bacteria, and Gram-positive bacteria. Many food microorganisms have the ability to utilize sugars, alcohols, and amino acids as sources of energy. Few others are able to utilize complex carbohydrates such as starches and cellulose as sources of energy. Some microorganisms can also use fats as the source of energy, but their number is quite less. The primary nitrogen sources utilized by heterotrophic microorganisms are amino acids. Also, other nitrogenous compounds which can serve this function are proteins, peptides and nucleotides. In general, simple compounds such as amino acids are utilized first by a majority of microorganisms.

4.2.2 Water Activity (a_W)

Water is often the major constituent in foods. Even relatively ‘dry’ foods like bread and cheese usually contain more than 35% water. The state of water in a food can be most usefully described in terms of water activity.

Water activity of a food is the ratio between the vapour pressure of the food, when in a completely undisturbed balance with the surrounding air, and the vapour pressure of pure water under identical conditions. Water activity, in practice, is measured as Equilibrium Relative Humidity (ERH) and is given by the formula:

Water Activity (a_W) = ERH / 100

Water activity is an important property that can be used to predict food safety, stability and quality. The various applications of water activity includes maintaining the chemical stability of foods, minimizing non enzymatic browning reactions and spontaneous autocatalytic lipid oxidation reactions, prolonging the desired activity of enzymes and vitamins in foods, optimizing the physical properties of foods such as texture.

Water activity scale extends from 0 (bone dry) to 1.00 (pure water). But most foods have a water activity in the range of 0.2 for very dry foods to 0.99 for moist fresh foods. Based on regulations, if a food has a water activity value of 0.85 or below, it is generally considered as non-hazardous. This is because below a water activity of 0.91, most bacteria including the pathogens such as*Clostridium botulinum* cannot grow. But an exception is *Staphylococcus aureus* which can be inhibited by water activity value of 0.91 under anaerobic conditions but under aerobic conditions, it requires a minimum water activity value of 0.86. Most molds and yeasts can grow at a minimum water activity value of 0.80. Thus a dry food like bread is generally spoiled by molds and not bacteria. In general, the water activity requirement of microorganisms decreases in the following order: Bacteria > Yeast > Mold. Below 0.60, no microbiological growth is possible. Thus the dried foods like milk powder, cookies, biscuits etc are more shelf stable and safe as compared to moist or semi-moist foods.

Factors that affect water activity requirements of microorganisms include the following- kind of solute added, nutritive value of culture medium, temperature, oxygen supply, pH, inhibitors, etc. Each microorganism has a minimal water activity for growth as shown in Table 4.4.

Table 4.4 Minimum water activity values of spoilage microorganisms

Microbial group	Minimum a_w	Examples
Most bacteria	0.91	*Salmonella* spp.
		Clostridium botulinum
Most yeasts	0.88	*Torulopsis* spp.
Most molds	0.80	*Aspergillus flavus*
Halophilic bacteria	0.75	*Wallemia sebi*
Xerophilic molds	0.65	*Aspergillus echinulats*
Osmophilic yeasts	0.60	*Saccharomyces bisporus*

Water activity of some foods and susceptibility to spoilage by microorganisms is shown in Table 4.5. Water acts as an essential solvent that is needed for most biochemical reactions by the microorganisms. Water activity of the foods can be reduced by several methods: by the addition of solutes or hydrophilic colloids, cooking, drying and dehydration: (e.g., egg powder, pasta), or by concentration (e.g. condensed milk) which restrict microbial growth so as to make the food microbiologically stable and safe.

Table 4.5: Water activity of some foods and susceptibility to spoilage by microorganisms

Water activity	Microorganisms grow at this a_w and above	Food examples
1.00 – 0.95	*Pseudomonas, E. coli, Proteus, Shigella, Klebsiella, Bacillus, Clostridium perfringens* & some yeasts	Highly perishable fresh foods & canned fruits, vegetables, meat, fish, milk, eggs; foods containing up to 40% (w/w) sucrose or 7% NaCl.
0.95 – 0.91	*Salmonella, Vibrio parahemolyticus, Clostridium botulinum, Serratia, Lactobacillus, Pediococcus,* some molds and yeasts	Some cheeses (cheddar, Swiss), cured meats; some fruit juice concentrates; bread; high moisture prunes; foods containing 55% (w/w) sucrose or 12% NaCl
0.91 – 0.87	Many yeasts like *Candida, Torulopsis, Hansenula; Micrococcus*	Fermented sausages; sponge cakes; dry cheese; margarine; foods containing 65%(w/w) sucrose (saturated) or 15% NaCl
0.87 – 0.80	Most molds, *Staphylococcus aureus*, most *Saccharomyces spp, Debaromyces*	Most fruit juice concentrates, sweetened condensed milk, flour, rice, pulses containing 15-17% moisture, salami
0.80 – 0.75	Most halophilic bacteria, Mycotoxigenic *Aspergilli*	Jam, Marmalade, Soy sauce
0.75 - 0.65	Xerophilic molds, *Saccharomyces bisporus*	Rolled oats containing 10% moisture; Fudge; marshmallows; Jelly; Some dried fruits; Nuts, Peanut Butter
0.65 - 0.60	Osmophilic yeasts, few molds	Dried fruits containing 15-20% moisture; Honey
0.50	No microbial proliferation	Pasta containing 12% moisture; spices containing 10% moisture
0.40	No microbial proliferation	Whole egg powder containing 5% moisture
0.30	No microbial proliferation	Cookies, biscuits crackers, bread crusts etc. containing 3- 5% moisture
0.20	No microbial proliferation	Whole milk powder containing 2-3% moisture, Dried vegetables containing 5% moisture, Corn flakes containing 5% moisture, Instant coffee

A wide variety of foods are preserved by restricting their water activity. These include:

4.2.2.1 Dried or Low Moisture Foods

These contain less than 25% moisture and have a final water activity between 0.0 and 0.60. e.g., Dried egg powder, milk powder, crackers, and cereals. These products are stored at room temperature without any secondary method of preservation. These are shelf stable and do not spoil as long as moisture content is kept low.

4.2.2.2 Intermediate Moisture Foods

These foods contain between 15% and 50% moisture content and have a water activity between 0.60 and 0.85. These foods normally require added protection by secondary methods such as pasteurization, pH control, refrigeration, preservatives, but they can also be stored at room temperature. These include dried fruits, cakes, pastries, fruit cake, jams, syrups and some fermented sausages. These products are usually spoiled by surface mold growth.

4.2.3 pH and buffering capacity

The pH, or hydrogen ion concentration, [H+], of natural environments varies from about 0.5 in the most acidic soils to about 10.5 in the most alkaline lakes. Since the pH is measured on a logarithmic scale, the [H+] of natural environments varies over a billion-fold and some microorganisms are living at the extremes, as well as every point between the extremes. The range of pH over which an organism grows is defined by three cardinal points: the minimum pH, below which the organism cannot grow, the maximum pH, above which the organism cannot grow, and the optimum pH, at which the organism grows the best. Microorganisms which grow at an optimum pH well below neutrality (7.0) are called acidophiles. Those which grow best at neutral pH are called neutrophiles and those that grow best under alkaline conditions are called alkalophiles. In general, bacteria grow faster in the pH range of 6.0- 8.0, yeasts 4.5-6.5 and filamentous fungi 3.5-6.8, with the exception of lactobacilli and acetic acid bacteria with optima between pH 5.0 and 6.0 (Table 4.6). The approximate pH ranges of some common food commodities are shown in Table 4.7.

Table 4.6 Approximate pH ranges of different microbial groups

Microbe	Minimum	Optimum	Maximum
Most Bacteria	4.5	6.5 – 7.5	9.0
Yeasts	1.5 – 3.5	4.0 – 6.5	4.0 – 6.5
Molds	1.5 – 3.5	4.5 – 6.8	8.0 – 11.0

Table 4.7 Approximate pH ranges of some common food commodities

Product	pH
Citrus fruits	2.0-5.0
Soft drinks	2.5-4.0
Apples	2.9-3.3
Bananas	4.5-4.7
Beer	3.5-4.5
Meat	5.6-6.2
Vegetables	4.0-6.5
Fish (most spp)	6.6-6.8
Milk	6.5-6.8
Wheat flour	6.2-6.8
Egg white	8.5-9.5
Fermented shark	10.5-11.5

4.2.4 Redox potential (Eh)

Microorganisms display varying degrees of similarity to Oxidation-Reduction potential of their growth medium. The O/R potential is the measure of tendency of a revisable system to give or receive electrons. When an element or compound looses electrons, it is said to be oxidized, while a substrate that gains electrons becomes reduced. Thus a substance that readily gives up electrons is a good reducing agent, while one that readily gains electrons is a good oxidizing agent. When electrons are transferred from one compound to another, a potential difference is created between the two compounds and is expressed in as milk volts (mV). If a substance is more highly oxidized, the more positive will be its electrical potential and vice versa. The O/ R potential of a system is expressed as Eh. Aerobic microorganisms require positive Eh values for growth while anaerobic microorganisms require negative Eh values (reduced). The redox potential we measure in a food is the result of several factors: redox couples present, ratio of oxidant to reductant, pH, poising capacity, availability of oxygen and microbial activity. Some redox couples typically encountered in food material and their standard redox potential (Eh) values are shown in Table 4.8.

With the exception of oxygen, most of the couples present in foods, e.g, glutathione, cysteine, ascorbic acid and reducing sugars tend to establish reducing conditions. pH of the food has a bearing on the redox potential and for every unit decrease in the pH the Eh increases by 58 mV (Table 4.8). As redox conditions change there will be some resistance to change in food's Eh, and is known as poising and is similar to buffering of the medium. Poising is maximum when the two redox couples are present in equal amounts. Oxygen is the most powerful of redox couple present in food system and if the food is stored in the presence of air, high positive potential will result. Thus, increasing the exposure to oxygen in air by mincing, cutting, chopping, grinding of food will increase the Eh as evident by high positive Eh of minced meat as compared to raw meat (Table 4.9). Finally microbial growth in the food reduces the Eh due to oxygen depletion. The decrease in Eh due to microbial activity forms the basis of some tests used frequently in raw milk such as platform MBRT test.

Table 4.8 Some important redox couples and their standard redox potential (Eh) values

Couple	E_0 (mV)
½ O_2/H_2O	+820
Fe^{3+}/Fe^{2+}	+760
Dehydroascorbic acid / ascorbic acid	+80
Methylene blue ox/ red	+11
Pyruvate/ lactate	-190
NAD^+/NADH	-320

Table 4.9 Redox potential of some foods

Food	Eh(mV)	pH
Raw meat	-200	5.7
Minced meat	+225	5.9
Whole wheat	-350	6.0
Ground grain (Barley)	+225	7.0
Fruit juices	+300 to +	3.0-5.5
Grape	+410	3.8
Lemon	+380	2.2

4.2.5 Antimicrobial constituents and barriers

Some foods can resist the attack by microorganisms due to the presence of certain naturally occurring substances which possess antimicrobial activity such as essential oils in spices (eugenol in cloves and cinnamon, allicin in garlic, cinnamic aldehyde in cinnamon, thymol in sage); lactaferrin, lactoperoxidase and lysozyme in milk; and ovatransferrin, avidin, lysozyme and ovoflavoprotein in hen's egg albumin. Similarly, casein as well as free fatty acids found in milk also exhibit antimicrobial activity. The hydroxycinnamic acid derivatives (p-coumaric, feluric, caffeic and chlorogenic acids) found in fruits, vegetables, tea and other plants possess antibacterial and antifungal activity. Also natural covering of foods like shell of eggs and nuts, outer covering of fruits and testa of seeds, hide of animals provide protection against entry and subsequent spoilage by microorganisms.

4.3 Extrinsic Parameters

4.3.1 Temperature of storage

Microorganisms have been found growing in virtually all temperatures. A particular microorganism will exhibit a range of temperature over which it can grow, defined by three cardinal points in the same manner as pH. Considering the total span of temperature where liquid water exists, the prokaryotes may be subdivided into several subclasses on the basis of one or another of their cardinal points for growth. For example, organisms

with an optimum temperature near 37°C are called mesophiles. Organisms with an optimum temperature between about 45°C and 70°C are thermophiles e.g, *Bacillus*, *Clostridium* etc. Some archaebacteria with an optimum temperature of 80°C or higher and a maximum temperature as high as 115°C , are now referred to as extreme thermophiles or hyperthermophiles. The cold-loving organisms are psychrophiles defined by their ability to grow at 0°C. A variant of a psychrophile (which usually has an optimum temperature above 10°C) is a psychrotroph, which grows at below 7°C but displays an optimum temperature in the mesophile range, nearer room temperature. Psychrotrophs are the scourge of food storage in refrigerators since they are invariably brought in from their mesophilic habitats and continue to grow in the refrigerated environment where they spoil the food. Of course, they grow slower at 2°C than at 25°C. In food microbiology mesophilic and psychrotrophic organisms are of greatest importance.

4.3.2 Relative humidity of the storage environment

Relative humidity and water activity are interrelated. When foods with low a_W are stored in environment of high humidity, water will transfer from the gas phase to the food and thus increasing a_W of the food leading to spoilage by the viable flora. There is a relationship between temperature and humidity which should be kept in mind. In general, the higher the temperature, lower is the relative humidity and vice-versa. Foods that undergo surface spoilage from molds, yeasts, and some bacteria should be stored in conditions of low relative humidity to increase their shelf life. This can also be done by proper wrapping of the food material also. However, variations in storage temperature should be minimal to avoid surface condensation in packed foods.

4.3.3 Gaseous atmosphere

Oxygen is one of the most important gases which come in contact with food influence the redox potential and finally the microbial growth. The inhibitory effect of CO_2 on the growth of microorganisms is applied in modified atmosphere packaging of foods. The storage of foods in atmosphere containing 10% of CO_2 is referred to as “Controlled Atmosphere”. This type of treatment is applied more commonly in case of fruits such as apples and pears. With regards to the effect of CO_2 on microorganisms, molds and Gram-negative bacteria are the most sensitive, while the Gram-positive bacteria, particularly the lactobacilli tend to be more resistant. Some yeasts such as *Bretanomyces* spp. also show considerable tolerance of high CO_2 levels and dominates the spoilage microflora of carbonated beverages. Some microorganisms are killed by prolonged exposure to CO_2 but usually its effect is bacteriostatic. Also, the presence of CO_2 tends to decrease the pH of foods and thereby inhibiting the microorganisms present in it by adversely affecting the solute transport, inhibition of key enzymes involved in carboxylation/ decarboxylation reactions.

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Lesson 5

Role of Predictive Microbiology

5.1 Introduction

Every year thousands of new food products are added to the previous list world over. The initial microbial population of these products generally includes spoilage as well as pathogenic organisms which may grow to a higher number and may thus make the food unsafe as well as spoil it. In order to ensure their longer shelf life & safety it is important that adequate control measures are adopted to prevent the growth of microorganisms in the entire food chain i.e. from production to consumption. It is very challenging to produce these new products that are healthy (low fat, low salt), minimally processed fresh to taste, do not contain chemical preservatives and are safe.

To produce new types of foods with desirable safety, stability and acceptance quality we need to determine the influence of several factors on microbial growth such as, concentration of different ingredients, processing and storage conditions, water activity, pH under the intrinsic and extrinsic factors. For determining the safety and stability of the processed foods it may not be always possible to do the microbiological analysis of each product by the traditional methods. Thus there is a need to develop mathematical models and put into application the computers for determining the influence of a vast array of different parameters that affect the microbial growth.

Microbiologists and hygienists would like to improve their approach by using new tools for modeling and simulation. Some models have been proposed and used with the aim to describe the effect of temperature & heat treatments on microbial destruction but it is only at the end of 1980s that the first mathematical tools have been built to stimulate the complete behavior of microflora in food products under processed conditions. However, predictive microbiology started as a purely empirical (though quantitative) science. In 1922 Esty and Meyer described the thermal death of *Clostridium botulinum* type A spores by a log-linear model, which is still used to estimate the necessary heat processing of low-acid canned foods. This model simply says that, at a given temperature, the *relative* (or*specific*) death rate of the bacteria is constant with time. In other words, the percentage of the cell population inactivated in a unit time is constant.

5.2 General Principle

As food safety is a growing concern in modern society, the scientific discipline of predictive food microbiology gains more and more interest worldwide. The term 'Predictive Microbiology' describes the scientific discipline of predicting microbiological growth or decline as a function of environmental factors. Such methods may not allow us to arrive at a very definite and accurate conclusion but certainly they can be an effective tool in obtaining hostile information quite rapidly. They can be a forerunner for conducting traditional studies that are relatively feasible experimentally as well as economically.

Predictive Modeling is the detailed knowledge of the behavior (growth, survival, and inactivation) of microorganisms in food products condensed into a mathematical model that enables an objective evaluation of the microbiological safety and quality of foods.

Food processing may be described as a succession of steps, from the input of raw materials to the distribution and consumption of the processed food. Different steps can be identified as critical from the microbiological point of view. At these steps microbial contamination or multiplication can occur.. If we consider that a

contamination occurs at a given initial level during one of these critical steps, predictive microbiology consists of simulation of the behavior of these contaminations, from the starting point to a given time, taking into account variation in processing conditions. This approach allows the microbial hazard to be predicted and therefore, helps to prevent and control the risk, allows quantification of the risk, optimize experimental designs and thus reduces delay and cost. Further this approach is supportive of debate, and thus helps to improve communication between the experts under managers.

The objective of the predictive microbiology is to be a good forward microbial dynamics using mathematical models. But before that, it is important to consider the classical behavior of the microbial population.

i. **The lag phase** : This is considered as an adaptation phase between an initial physiological state and growth state wherein the cell number remains constant. This phase may get longer under unfavorable conditions.

ii. **Logarithmic growth phase** : This is characterized by a linear portion if the logarithm of the variable (biomass, number or concentration of cells) is represented versus time.

iii. **The stationary phase** : This is linked to lack of nutrients, acidity etc. that leads to stable number of cells in the growth medium.

iv. **Death phase** : The decrease in cell number takes place when the medium and the conditions become too unfavorable. This phase is usually close to an exponential decrease of the cell density.

5.3 Conventional Methods for predicting shelf life of food products

5.3.1 Spiking studies

In this method a food is inoculated with the microorganisms that are expected to be the major causes of loss of shelf life or safety under storage conditions. A definite but realistic number of microbial cells or spores is used as inoculum and stored under conditions at which the food would normally be placed. Afterwards it is examined for microbial growth or toxin production. However a large number of variables (extrinsic and intrinsic factors) need to be studied. This is quite time consuming, costly and cumbersome.

5.3.2 Storage studies

A food product is stored under normal storage conditions and microbiological analysis is done at regular intervals and extent of spoilage and the growth of pathogenic organisms/ production of toxin is assessed. The data thus generated can be useful for predicting the expected shelf life and safety of the product. However, there are certain limitations to the use of this method as it would not take into consideration the temperature of use for a short time and other analytical problems.

5.3.3 Accelerated tests

In this procedure the product is held at relatively higher temperature (near ambient) so as to increase the rate of growth of the organism and thereby accelerate the spoilage. This process is used particularly for foods that have relatively longer shelf life. However, there are also limitations to arriving at a logical conclusion as different organisms in the mixed flora of the food behave differently at different temperatures.

5.4 Predictive Modeling

A number of mathematical models have been developed to predict the growth of pathogenic and spoilage microorganisms in foods from the data generated by studying growth rate at different pH, water activity,

temperature and preservative conditions in the laboratory media, with suitable computers help in the rapid analysis of the huge data. Two kinetic based models that take into consideration the effect of culture parameters on the growth rate of microorganisms are:

5.4.1 Square root model

This model is based on the linear relationship between the square root of the growth rate and temperature. This model is quite effective when one or two parameters are used. The effectivity of this model decreases if several parameters in combination are used to control the microbial growth.

5.4.2 Sigmoidal model

This model has been developed by the US department of Agriculture (USDA) to predict microbial growth in a food system that is controlled by several parameters. It has been tested in the laboratory media to determine the growth rate of several pathogenic microorganisms under different physical and chemical parameters. This model is extensively used because of its simplicity and effectiveness.

Predictive microbiology is quite interesting and important. With the advent of computers and subsequent developments, the processing of data has become easier and quicker. Most of the studies so far on this aspect have been carried out in laboratory media and a very limited number of studies have been carried out in food systems. Therefore, there is a limit to the effectiveness of this modeling system. The information obtained through this modeling system, therefore, needs to be used with caution.

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Module 3. Microbiology of food commodities

Lesson 6

Overview of Spoilage

6.1 Introduction

Spoilage of food can be defined as any visible or invisible change which can makes food or product derived from food unacceptable for human consumption. Major causes of food spoilage are enlisted in Table 6.1.

Spoilage of food not only causes health hazard to the consumer but also cause large economic losses. Spoilage not only leads to loss of nutrients from food but also cause change in original flavor and texture. It is estimated that about 25% of total food produced is spoilt due to microbial activities only despite range of preservation methods available. Thus the spoilage of food is not only a health hazard but also carry lot of economic significance too.

In total, the food spoilage is considered a complex phenomena whereby a combination of microbial and biochemical activities take place. Due to such activities, various types of metabolites are formed which aid in spoilage. The detection of these metabolites help in detection of spoilage.

The ease with which foods are spoiled depend upon factors described. The foods are thus divided into different classes as:

6.1.1 Perishable foods

These foods are readily spoilt and require special preservation and storage conditions for use. The foods of this class are mostly used daily such as milk, fruits, vegetables, fish etc.

6.1.2 Semi-perishable foods

This class of foods if properly stored can be used for a long duration e.g. potatoes.

6.1.3 Non-perishable foods

These foods remain in good form for long duration unless handled improperly. It include sugar, flour etc.

Table 6.1 Major causes of spoilage

Biological factors	Non Biological factors
Insects	Purely chemical reactions
Enzyme action of food (plant/animal)	Physical changes
Activity of microorganisms	

6.2 Factors Affecting Microbial Spoilage of Foods

While the spoilage by physical and chemical modes play important role, the microbial spoilage has most significant role. Combination of all these factors is ultimately responsible for overall decay of food. The spoilage of food can occur at different stages of production, processing. Various causes of spoilage at different stages are depicted in (fig. 6.1)

The spoilage of foods due to microbial activity initiates when undesirable microorganism colonize the food. Once colonization is established, the microbial community grow on the food constituents, thereby utilizing them for their metabolism. During the course of such microbial activity, the food become unsuitable for human consumption. Various parameters affect the proliferation of microbes. These include intrinsic to food such as water activity, acidity, oxidation-reduction potential, presence of antimicrobial compounds in food and food structure. Extrinsic parameters such as temperature, humidity and other storage condition aid in spoilage.

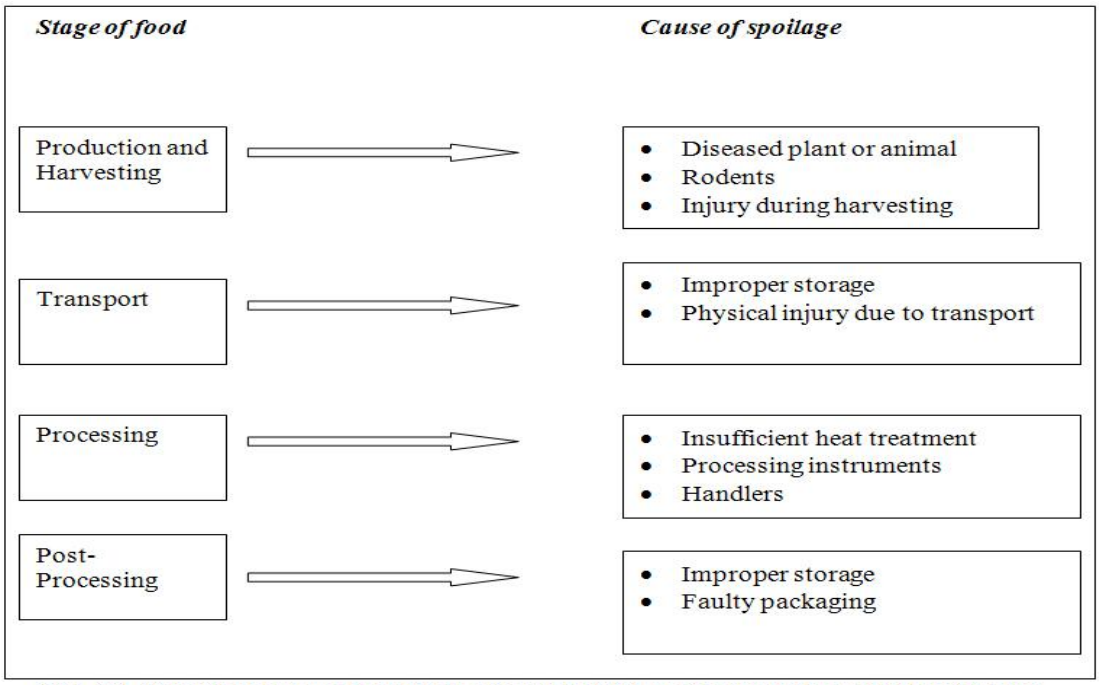

Fig. 6.1 Possible causes of spoilage and microbial contamination at different stages of food production

6.3 Microorganisms in Food

Microorganisms by virtue of their ubiquity and diversity in metabolism are most significant cause of food spoilage. Bacteria and fungi (including yeasts and moulds) are major cause of food spoilage.

Bacteria are round; rod or spiral shaped microorganisms and can grow under a wide variety of conditions. There are many types of bacteria that cause spoilage. Food spoiling bacteria are primarily divided two groups viz. *spore-forming* and *nonspore-forming*. Bacteria generally grow in low acid foods like vegetables and meat.

Yeasts growth causes fermentation which is the result of yeast metabolism. There are two types of yeasts *true* yeast and *false* yeast.*True yeast* metabolizes sugar producing alcohol and carbon dioxide gas. This is known as fermentation. *False yeast* grows as a dry film on a food surface, such as on pickle brine. False yeast occurs in foods that have a high sugar or high acid environment.

Molds grow in filaments forming a tough mass which is visible as `mold growth'. Molds form spores which, when dry, float through the air to find suitable conditions where they can start the growth cycle again.

Mold can cause illness, especially if the person is allergic to molds. Usually though, the main symptoms from eating moldy food will be nausea or vomiting from the bad taste and smell of the moldy food.

Both yeasts and molds can easily grow in high acid foods like fruit, tomatoes, jams, jellies and pickles. Both are easily destroyed by heat. Processing high acid foods at a temperature of 100°C (212°F) in boiling water in can for the appropriate length of time destroys yeasts and moulds.

a) Rod shaped Bacteria

b) Mould

c) Yeast

6.4 Changes in foods due to Microorganisms

As microorganisms grow in food, by virtue of their diversity in metabolism they utilize components of foods and convert them into variety of chemical compounds. An overview of these changes is given in **Figure 6.2**

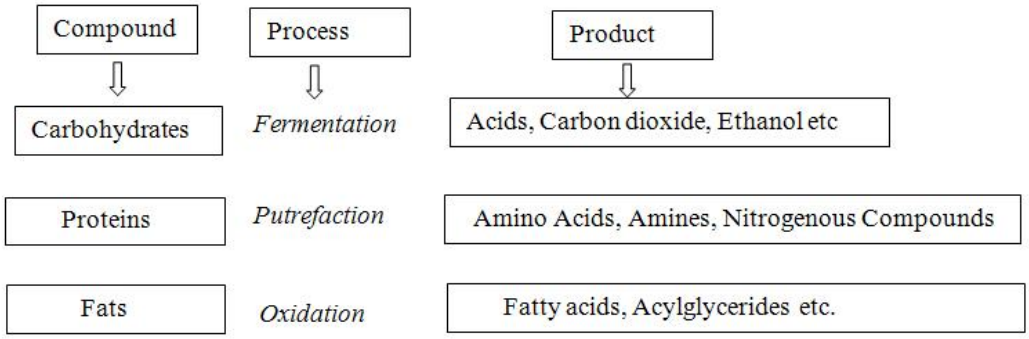

Fig. 6.2 Changes in major food components during spoilage

6.5 Change in Carbohydrates

Carbohydrates are used to obtain energy. While monosaccharide are preferred over complex carbohydrates, microorganisms have ability to convert polysaccharides to simpler forms before obtaining energy. The utilization of simple sugar such as glucose vary under aerobic and anaerobic conditions. In aerobic conditions it is converted into carbon dioxide and water through glycolysis and other related pathways. In absence of oxygen, the process yields a number of compounds in different organisms. This process is known as fermentation. These compounds include:

6.5.1 Alcoholic fermentation

It occurs due to yeasts and carbon dioxide and ethanol are the major end products.

6.5.2 Lactic fermentation

It is of two types viz. homolactic fermentation where primarily lactic acid is the end product and heterolactic fermentation where along with lactic acid, acetic acid, ethanol, glycerol, carbon dioxide are produced.

6.5.3 Coliform type fermentation

This type of fermentation occur in coliform bacteria. In this process acids such as lactic, acetic, formic. Ethanol, glycerol etc. are produced.

6.5.4 Propionic fermentation

It occurs in propionic bacteria and in it along with propionic acid, succinic acid and carbon dioxide are produced.

6.6 Change in Nitrogenous Compounds

Proteins are the major source of nitrogenous compounds in foods. Thus degradation of proteins, include hydrolysis by enzymatic reactions. The source of enzymes can be either microbes or foods own enzymes. Complex proteins are converted into polypeptides, simpler peptides and amino acids. The enzymes involved in conversion of proteins into polypeptide are termed as proteinase while those catalyzing conversion of polypeptides to amino acids are called peptidases. The decomposition of proteins can be aerobic or anaerobic. Usually the anaerobic decomposition of proteins results in obnoxious odors. This process is known as Putrefaction. Along with nitrogenous compounds, other compounds responsible for such smells also include sulfur compounds.

The microbial activity on amino acids cause either deamination (removal of amine group) or decarboxylation (removal of carboxyl group). Major organisms involved in conversion of nitrogenous compounds include *Pseudomonas, E. coli, Clostridium, Desulfotomaculum* etc.

6.7 Changes in Lipids

The hydrolysis of lipids is accomplished by lipase enzymes produced by different microorganisms. The major end products include glycerol and fatty acids, which are further used by microorganisms for their metabolism. The process of conversion is known as oxidation. The oxidation of fats is also done by enzymes of food itself. High fat containing foods are prone to such processes.

6.8. Public Health Aspect

Worldwide, foodborne pathogens cause numerous sufferings and deaths. In Africa, Asia, and Latin America, there are about 1,000 million cases of gastroenteritis per year in children under the age of 5, which leads to 5 million deaths. In Mexico and Thailand, half of the children aged 0–4 years suffer from the *Campylobacter* induced enteritis. In Europe, 50,000 cases/ million population suffer from acute gastroenteritis. In the Netherlands about 300,000 cases/million population occur yearly. In Northern Ireland and the Republic Ireland, about 3.2 million episodes of gastroenteritis are reported each year. In Australia, 5.4 million cases of foodborne gastroenteritis occur each year. In England 20% of population, i.e., 9.4 million people suffer from acute gastroenteritis each year and the primary contributing microorganisms are identified as Norovirus, *Campylobacter* species, rotavirus, and nontyphoidal *Salmonella* species. In the US, there are an estimated 6 million cases with 350,000 hospitalizations and 9,000 deaths associated with foodborne infections each year. Foodborne pathogen statistics show declines in incidences from 1996–1998 to 2005 for some pathogens but increased for others: the incidence of *Shigella* decreased by 43%, *Yersinia* species by 49%, *Listeria monocytogenes* by 32%, *Campylobacter*species by 30%, EHEC O157:H7 by 29%, and *Salmonella Typhimurium* by 42%; however, the incidence of *Salmonella Enteritidis*and *S. HeidelbergS. javiana* by 82%. Interestingly, the number of outbreaks and product recalls continued to increase thus placing a huge economic burden on producers and processors. *Mycobacterium paratuberculosis* is the causative organism

of Johne's disease in dairy cows. However it has also been linked to Crohn's disease in human beings. The organism may survive conventional pasteurization treatment of milk increased, each by 25%, and

Mycotoxins are fungal metabolites that get formed in foods due to the growth of some strains of *Pencillium, Aspergillus* and some other molds. They are secondary metabolites and are of low molecular weight. They are highly thermo stable and withstand the conventional processing temperatures. They are highly toxic to a large number of animals and also to human beings. The food borne disease resulting from the ingestion of toxin in mold contaminated food is called mycotoxicosis. They affect kidney, liver, cause skin irritation, birth defect and death. They have also been found to be carcinogenic in nature.

Table 6.2. Mycotoxins produced by Fungi in foods

Sr. No.	Mycotoxins	Organism
1	Aflatoxins	*A. flavus* *A. parasiticus*
2	Ochra toxin	*Aspergillus ochraceus* *Pencillium* spp.
3	Tricothecenes	*Fucarium, Tricoderma,* *Tricothecium*
4	Patulin	*P. expansum,* *P. patulum*
5	Pencillic acid	*P. puberulum, P. cyclopium* *Aspergillus ochraceus*
6	Citrinin	*P. citrinum* *P. viridicatum*

Aflatoxins are produced by some strains of *Aspergillus flavus* and *A. parasiticus* and they occur in different chemical forms like B_1, B_2, G_1 and G_2. Aflatoxin M_1 and M_2 are the hydroxylated derivatives of B_1 and B_2. Milch animals fed with aflatoxin B_1 and B_2, excret aflatoxin M_1 and M_2, in milk, urine and feces. Aflatoxin B_1 is the most toxic among all aflatoxins. However M_1 and M_2, are relatively less toxic as compared to the parent compounds B_1 and B_2. Aflatoxins are found in nuts, spices and figs and they are produced under hot and humid conditions during storage.

Viruses are obligate intracellular parasites. They are host specific. Several viruses have been implicated in food borne outbreaks. There are four acute gastro enteritis namely calici virus, rota virus, astro virus and adeno virus. The infectious hepatitis A virus enters a person through the contaminated food or water and it causes gastro enteritis. Viruses responsible for poultry diseases have also been implicated in human aliments.

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Lesson-7

Microbial spoilage of fruits and fruit juices

7.1 Introduction

Fruits are natural sources of minerals, vitamins besides carbohydrates and other essential substances. Naturally fresh fruits and juices made out of them contain high amount of water thereby making them highly prone to attack by microorganisms. While most of the fruits are naturally provided with coatings and coverings in the form of skins, but these are fragile enough to be easily disturbed by various biological and mechanical factors. Like vegetables, fruits being produce of plants get contaminated through different sources by a variety of microorganisms which may play significant role in their spoilage. These are soil, water, diseased plant, harvesting and processing equipments, handlers, packaging and packing material and contact with spoiled fruits.

7.2 Microorganisms Associated With Spoilage in Fruits and Juices

The microorganisms associated with fruits depend on the structure of fruit. The fruits contain different organic acids in varying amounts.The types of acids which are predominately found are citric acid, malic acid and tartaric acid. The low pH of fruits restricts the proliferation of various types of organisms. The pH and type of acids found in different fruits is given in Table 7.1.

Due to the low pH, a large number of microorganisms are restricted to grow on fruits. Fungi are most dominating organisms to grow on fruits because of the ability of yeasts and molds to grow under acidic conditions. A small number of bacteria which are aciduric (ability to resist acidic conditions) also grow. Also the dry conditions prevailing on the skin and surface do not allow the growth of certain microorganisms. Besides these plants also produce certain antimicrobial components too.

Table 7.1 Type of acid associated with fruits and their pH

Fruit	Type of Acid	pH
Apple	Malic, citric, lactic	3.3–4.1
Watermelon	Citric, malic	5.8–6.0
Banana	Citric, malic, tartaric	4.5–5.2
Grape	Tartaric, malic	3.0–4.5
Plum	Malic, quinic	2.8–4.6
Pineapple	Citric, malic	3.2–4.0
Guava	Citric, malic, lactic	3.0–3.2
Lemon	Citric	2.2–2.4
Mango	Citric, tartaric	3.3–3.7
Orange	Citric, malic	3.0–4.0
Papaya	Citric, malic, ketoglutaric	4.5–6.0

Despite the high water activity of most fruits, the low pH leads to their spoilage being dominated by fungi, both yeasts and molds but especially the latter.

7.2.1 Yeasts

Yeasts are unicellular fungi which normally reproduce by budding. Of the 215 species important in foods, about 32 genera are associated with fruits and fruit products . Only a few species of yeasts are pathogenic for man and other animals. None of the pathogenic species are common contaminants of fruits and fruit products. Fruit that has been damaged by birds, insects, or pathogenic fungi usually contain very high yeast populations. The yeasts are introduced into the exposed tissue, often via insects, and are able to use the sugars and other nutrients to support their growth. Types of yeasts growing in fruits depend upon the nature of the fruit and the strain of yeast. Growth of a strongly fermentative type such as certain strains *of Saccharomyces cerevisiae* may produce sufficient CO_2 (90 lb/in. or more) to burst the container,. Growth of some species in a clear fruit juice may produce only slight haze and sediment. While carbon dioxide and ethanol are the predominant metabolic products of yeasts, other products such as glycerol, acetaldehyde, pyruvic acid, and a -ketoglutaric acid are also formed. Oxidative yeasts such as species of *Brettanomyces* produce acetic acid in wines and other fruit products. Although yeasts produce hydrolytic enzymes which degrade pectins, starch, and certain proteins, enzymatic activity is usually much less than that exhibited by other aciduric microorganisms, molds in particular.

7.2.2 Molds

These are filamentous fungi which are important group of microflora of fruit products due to following reasons

1. Some of the members are xerophilic, thereby having potential to spoil foods of low water activity such as dried fruits and fruit juice concentrates.
2. Some of the species have heat resistant spores such as ascospores which can survive the commercial pasteurization treatments that are given to most fruit products.
3. Growth of molds on processing equipment such as wooden tanks can result in the generation of off-flavors in wines, juices, and other fruit products.
4. Mold-infected raw fruit may become soft after processing because pectinases were not inactivated by the thermal treatment.
5. The metabolic products of many molds are toxic to humans. Of these toxins, mycotoxins are important components.

Molds are aerobic microorganisms, but many of them are very efficient scavengers of oxygen. Due to this property of molds, processed fruits, including those hermetically sealed in cans or glass, are susceptible to spoilage. In case of limited vegetative growth, evidence of spoilage may be the changes produced by fungal enzymes such as the breakdown of starch or pectins while in case of heavy growth, colonies develop in the headspace or as strands throughout a beverage or similar product. Some types of spoilage by fungi are shown in the Figure 7.1 to 7.10.

Penicillium italicum (blue mold) and *Penicillium digitatum* (green mold) seen in oranges, lemons and citrus fruits (Figure 7.1).

Fig. 7.1: Green and blue mold by *Pencillium* growth on oranges and lemons

Fig. 7.2. Soft rot of apples- *Penicillium expansum* (blue mold)

Fig. 7.3. Grey mold *Botrytis cinerea*

Fig.7.4. Another mold in fruits- *Botryotinia fuckeliana*

Fig. 7.5. *Rhizopus* soft *Rot*

- *R. stolonifer* cause soft and mushy food ,cottony growth of mold.
- Anthracnose -*Colletotrichum lindemuthianum,* cause spotting of leaves and fruits and seedpods.

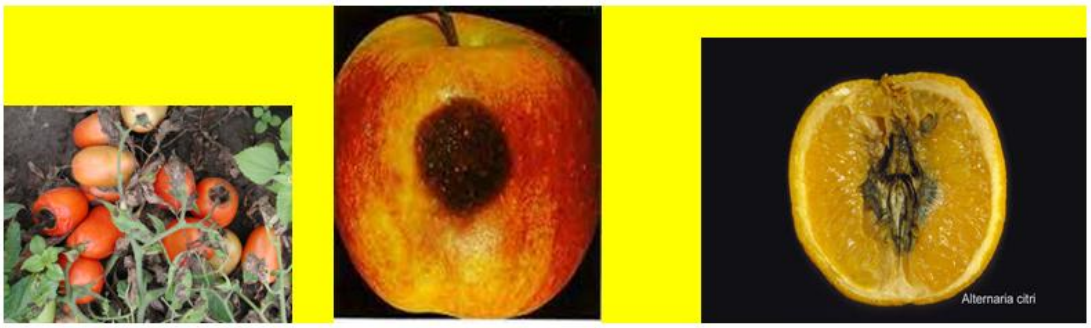

Fig. 7.6. Alternaria rot– *Alternaria tenuis,* Area becomes greenish brown and then black spots

Fig. 7.7. Blue mold rot– *Penicillium digitatum,* bluish green color

Downy mildew:Initially, the lesions tend tobe small and confined to the upper surface of wrapper leaves. As the areas enlarge, they turn from light green or yellowish to brown and become soft. It is caused by *Phytophthora*

Fig. 7.8. Downy mildew - *Phytophthora* :wooly masses

Watery soft rot. This rot occurs on the lower part of heads. The tissue is water soaked and light or pinkish brown.A white, cottony mold spreads over the decayed tissue , and the head eventually becomes a watery mass .

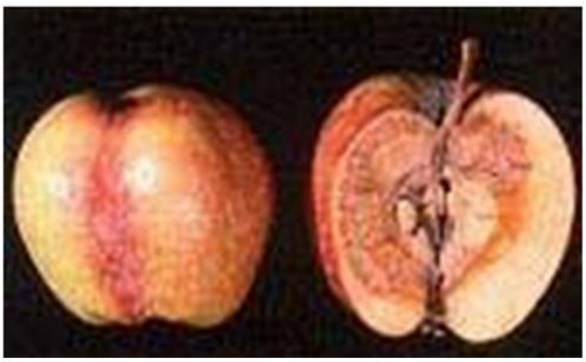

Fig. 7.9. Watery soft rot *Sclerotina sclerotiorum*

Fig. 7.10. Black rot – *Alternaria* mainly,*Physalospora* and others

7.2.3 Bacteria

Various groups of bacteria have ability to grow on fruits and its juices. These bacteria by virtue of their diversity in metabolism grow on fruits and produce different types of compounds. The major group of bacteria which are involved are:

- Lactic acid bacteria
- Acetic acid bacteria
- Spore formers

7.2.3.1 Lactic acid bacteria

The lactic acid bacteria are Gram-positive, catalase negative organisms which can grow under anaerobic conditions. These are rod-shaped (lactobacilli), or cocci (pediococci and leuconostocs) . The homofermentative species produce mainly lactic acid from hexose sugars; the heterofermenters produce one molecule of lactic acid, one molecule of carbon dioxide, and a two-carbon compound, which is usually acetic acid or ethanol or a combination of the two.

Growth of lactic acid bacteria in juices and other fruit products cause the formation of haze, gas, acid, and a number of other changes. Certain heterofermentative lactobacilli lead to slime in cider. The lactobacilli and leuconostocs that are present in citrus juices generate acetylmethylcarbinol and diacetyl, compounds that give the juices an undesirable, buttermilk-like flavor. Some strains, being extremely tolerant to ethanol grow in wines. *Lactobacillus fructivorans* can grow in appetizer and dessert wines containing as much as 20% ethanol. Lactic acid bacteria have the ability to decarboxylate malic acid to lactic acid. This malolactic fermentation is often desirable in high-acid wines because the acidity is reduced and desirable flavors are produced. *Oenococcus oenos* is the most acid and alcohol-tolerant species and often is isolated from wines that are undergoing a malo-lactic fermentation.

7.2.3.2 Acetic acid bacteria

These are Gram negative, aerobic rods having two genera, viz. *Acetobacter* and *Gluconobacter* . Both of these species oxidize ethanol to acetic acid under acidic condition, *Acetobacter* species can oxidize acetic acid to carbon dioxide thus, the genus is called as over oxidizer. Because the bacteria are obligate aerobes, juices, wines, and cider are most susceptible to spoilage while held in tanks prior to bottling. Some strains of *Acetobacter pasteurianus* and *Gluconobacter oxydans* produce microfibrils composed of cellulose, which leads to formation of flocs in different fruit juice beverages.

7.2.3.3 Spore formers

Spores are heat resistant, so role of organisms producing spores is important in heat treated juices and beverages. Variuos spore formers such as *Bacillus coagulans, B. subtilis, B. macerans, B. pumilis, B. sphaericus* , and *B. pantothenticus* have been found to grow in different types of wines. Some of these organisms have also been involved in canned fruits. Spore-forming bacilli that actually prefer a low pH have been responsible for spoilage of apple juice and a blend of fruit juices.

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Lesson-8

Microbial Spoilage of Vegetables

8.1 Introduction

Vegetables form an integral part of diet due to their role in providing various types of vital nutrients such as carbohydrates, minerals, vitamins, roughage etc. Vegetables being a part of fresh produce, contain high moisture which makes them highly perishable foods and hence more prone to spoilage. Microorganisms gain entry into vegetables from various sources. These sources include:

- Soil
- Water
- Diseased plant
- Harvesting and processing equipments
- Handlers
- Packaging and packing material
- Contact with spoiled vegetables

The conditions in which vegetables are stored and transported after harvesting also contribute to rate of spoilage. Other than microbial, sources, the spoilage of vegetables can also occur due to the activity of native enzymes.

8.2 Types of Spoilage in Vegetables

The microbial spoilage of vegetables is predominately of following types

8.2.1 Spoilage due to pathogens

The plant pathogens which infect stem, leaves, roots, flowers and other parts or the fruit itself.

8.2.2 Spoilage due to saprophytes

Vegetables have general microflora inhabiting them. These organisms under certain conditions can grow on these vegetables and spoil them. The list of these organisms is given in Table 8.1. There are certain secondary invaders which may enter the healthy food or grow after growth of pathogens.

It is well known that plant diseases are mostly caused by fungi. Thus most of the spoilage causing pathogens in vegetables are fungi. Fungi have specific characteristics when spoiling food as it leads to mushy areas which may be water soaked. The fungi produce characteristic spores which may be pigmented. The pigmentation helps in identification of the type of spoilage by fungi. The bacterial diseases too cause spoilage of vegetables but to a lesser extent. Figure 8.1 represent bacterial and fungal diseases of tomato.

Table 8.1 Normal microflora of vegetables

Bacteria	Fungi
Alcaligens	Alternaria
Bacillus	Aureobasidium
Erwinia	Botrytis
Micrococci	Fusarium
Pseudomonas	Penicillin
Lactic acid bacteria	Rhizopus
Xanthonomas	

Table 8.2 The major types of spoilages by pathogens in vegetables

Type of Spoilage	Causative organisms	Symptoms
Bacterial soft rot	*Erwinia carotovora* *Pseudomonas marginalis* *Clostridium*	Water soaked appearance, soft-mushy, bad odor
Alternaria rot	*Alternaria tenuis*	Greenish brown to black brown spots
Rhizopus soft rot	*Rhizopus sp*	Cottony mold growth with small black dots
Blue mold rot	*Penicillim digitatum*	Bluish green color
Downy mildew	*Phytophthora, Bremia*	White woolly mass
Black mold rot	*Aspergillus niger*	Brown to black mass, referred as smut
Fusarium rot	*Fusarium*	
Sliminess or souring	*Saprophytic bacteria*	
Stem end rots	*Diplodia* *Alternaria* *Phomopsis*	Involve stem ends
Watery soft rot	*Sclerotenia sclerotinium*	

Spoilage in vegetables is largely affected by composition of vegetable. The non acidic foods are thus spoiled by bacterial rot while acidic foods with dry surfaces are more prone to mold spoilage. The product on which organism grows and types of organisms growing largely determine the character of spoilage.

Bacterial Soft Rot

q Caused by *Erwinia carotovora* and *Pseudomonas* such as *P. marginalis*. *Bacillus* and *Clostridium* spp. are also implicated.

q Breaks down pectin, giving rise to a soft, mushy consistency, sometimes a bad odour and water-soaked apperance.

q Vegetables affected- onions, garlic, beans, carrot, beets, lettuce, spinach, potatoes, cabbage, cauliflower, radishes, tomatoes, cucumbers, watermelons.

Some types of spoilage in vegetables by bacteria are shown in the Figure 8.1 to 8.7

Fig. 8.1 Bacterial and fungal disease of tomato

Soft rot in tomato caused by *Erwinia carotovora* (Left)

Blue mould rot in tomato caused by *Penicillium* spp (right)

Fig. 8.2. Bacterial soft rot in Tomato, Capsicum and Tomato

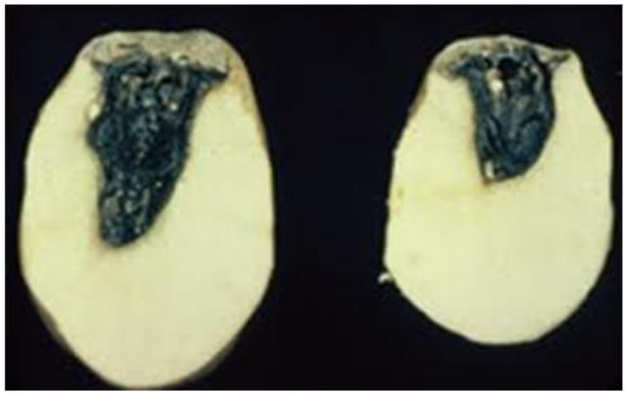

Fig. 8.3. Black leg of potatoes- *Erwinia carotovora* var. *atroseptica*

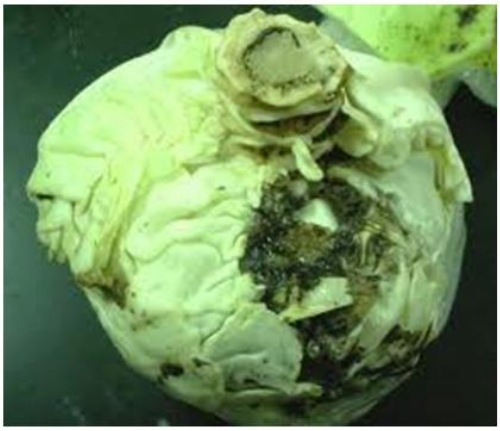

Fig. 8.4. Black rot of cabbage and cauliflower- *Xanthomonas campestris*

Fig.8.5. Bacterial wilt of beans- ***Corynebacterium flaccumfaciens***

Fig. 8.6. Slime of lettuce- ***Pseudomonas marginalis***

Fig. 8.7. Sliminess/souring - bacteria

8.2.3. Fungal spoilage of vegetables

Penicillium, Cladosporium, Rhizopus, Aspergillus spp. are responsible for various defects in vegetables.

Some types of spoilage in vegetables by fungi are shown in the Figure 8.8 to 8.13.

Gray mold rot – caused by *Botrytis cinera* in vegetables. Favoured by high humidity and warm temperature

Fig. 8.8. Gray mold rot in peas and Tomato

8.2.3. Fungal spoilage of vegetables

Penicillium, Cladosporium, Rhizopus, Aspergillus spp. are responsible for various defects in vegetables.

Some types of spoilage in vegetables by fungi are shown in the Figure 8.8 to 8.13.

Gray mold rot – caused by *Botrytis cinera* in vegetables. Favoured by high humidity and warm temperature

Fig. 8.9. Black mold rot – *Aspergillus niger*, dark brown to black masses

Fig. 8.10. Pink mold Rot – *Trichothecium roseum*

Fig. 8.11. Fusarium Rot – *Fusarium* spp.

Fig. 8.12. Green Mold Rot – *Cladosporium* and *Trichoderma*

Fig. 8.13. Brown Rot – Sclerotinia spp

Table 8.2 Examples of fungal spoilage of vegetables

Examples of Commodities Most Affected	**Genus**	**Type of Spoilage**
Most vegetables especially carrot, lettuce, celery, cabbage	*Botrytis*	Grey mould rot
Most vegetables. Especially carrot, lettuce, legumes, *Brassica* spp.	*Sclerotinia*	Watery soft rot
Legumes, carrot, *Brassica* spp.	*Rhizopus*	Soft rot
Tomato, cucumber, asparagus, potato	*Fusarium*	Dry rots
Tomato, potato, carrot	*Phytophthora*	Brown rots (blight)
Tomato, potato, beetroot Cucumber, legumes	*Phoma* *Pythium*	Dry brown, black rots Cottony leak

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Lesson 9

Microbial Spoilage Of Cereals And Bakery Foods

9.1 Introduction

Cereals are important foods which provide bulk of our dietary requirements. They are also source of carbohydrates which are metabolized by body for energy generation. Besides cereals also provide minerals, proteins and vitamins. India produces a large variety of cereals such paddy, wheat, maize, barley millets like, jowar, bajra, ragi. Various types of products are prepared from cereals. Cereal products can be broadly classified into the following groups:

- Whole cereals where only the husk of the grain is removed, e.g. rice, wheat, gram, lentils, etc.
- Milled grain products are made by removing the bran and usually the germ of the seed and then crushing the kernel into various sized pieces. These include wheat flour, maida, semolina (rawa), etc.
- Processed cereals like weaning food, breakfast cereals, etc.
- Ready mixes like cake mix, idli mix, vada mix etc.

The country is self sufficient in grain production and is the second largest rice producer in the world with a 20% share. But due to constantly increasing population there is still a shortfall in cereals. A large amount of these cereals are spoilt every year due to various factors.

9.2 Spoilage Factors

The grains are low moisture commodities due to which they are less susceptible to spoilage and have greater shelf-life. The spoilage mainly occurs due to moisture absorption during storage leading to fungal growth at high temperature and humidity. Before bulk packaging and storage, the whole grains are fumigated to reduce microbial load and increase storage period. The factors influencing the quality of cereals are:

9.2.1 Physical

Physical losses are caused by spillages, which occur due to use of faulty packaging materials.

9.2.2 Physiological

Physiological losses include respiration and heating in grains, temperature, humidity and oxygen.

9.2.3 Biological

Biological losses occurs due to micro-organisms, insects, rodents, etc.

The sources of contamination in cereals are:

- Soil
- Air
- Insects
- Natural microflora of harvested grains

9.3 Cereal Grains and Flours

At initial stages, the grains are contaminated by *Pseudomonas*, Micrococci, *Lactobacillus* and *Bacillus*. The initial bacterial population may vary from 10^3 to 10^6 per gram while mold population may be more than 10^4 spores per gram.

Due to low moisture content grains and flours usually have long shelf life if these are properly harvested or stored under proper conditions as microbial growth is not supported. If due to any reason they attain moisture, the microbial growth may occur with molds growing at initial stages of moisture while yeasts and bacteria may grow with increasing moisture.

Spoilage of stored grains by molds is attributed to the following factors:

- Type and number of microorganisms
- Moisture content of more than 12-13%
- Storage temperature
- Physical damage

Most common species of molds are *Aspergillus, Rhizopus, Mucor, Fusarium*. A significant aspect of spoilage of molds is production of mycotoxins, which may pose danger to health.

The process of flour making such as washing, milling reduce the microbial content. Moisture content of less than 15% does not allow growth of molds. Most molds and bacteria in flours can grow only above 17% moisture, thus moistening of flours is essential for spoilage by microbes (Fig 9.1).

Fig-9.1. Stem rot and head blight of wheat and barley- *Fusarium culmorum* & *Fusarium graminearum*.

Storage fungi- *Penicillium, Aspergillus* and *Fusarium* when grain stored under moist conditions.

9.4 Spoilage of Bread

Bread is a major product prepared using flours. Dough is prepared from flours which undergo fermentation for which desirable microorganisms must grow. If this fermentation exceeds the required limits, it causes souring. Excessive growth of proteolytic bacteria reduces the gas holding capacity which is otherwise required for dough rising. Spoilage of bread is usually of two types viz. moldiness and ropiness.

During bread making, it is baked at very high temperature, thereby there are less chances of survival of microorganisms. Thus the contamination usually occurs when cooling is done as well as during packing, handling and from the environment. The molds which are prevalent are *Rhizopus stolonifer* (referred as bread mold), *Penicillium expansum, Aspergillus niger*. *Mucor* and *Geotrichum*also develop.

Ropiness in bread is usually due to bacterial growth and is considered more prevalent in home made breads. The chief causative organism is *Bacillus subtilis* or *B. licheniformis.* These are spore forming bacteria with their spores surviving baking temperatures. These spores can germinate into vegetative cells, once they get suitable conditions as heat treatment activates them. In ropiness, the hydrolysis of bread flour protein (gluten) takes place by proteinases. Starch is also hydrolysed by amylases, which encourage ropiness. The manifestation of ropiness is development of yellow to brown color and soft and sticky surface. It is also accompanied by odor.

Another type of spoilage of bread is chalky bread which is caused by growth of yeast like fungi *Endomycosis fibuligera* and*Trichosporon variable.* This spoilage is characterized by development of white chalk like spots.

An unusual spoilage of bread is Red or Bloody bread, which is due to the growth of bacteria *Serratia marcescens*. This organism produces brilliant red color on starchy foods giving blood like appearance. *Neurospora* and *Geotrichum* may also be involved in imparting pigmentation during spoilage of bread.

Some spoilage of bread are shown in Figure 9.2 to 9.4.

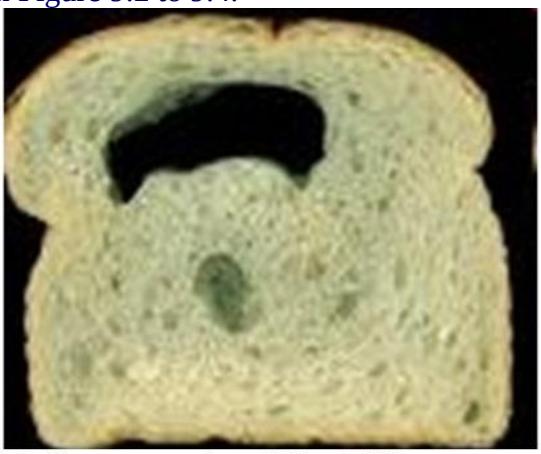

Fig.9.2. Green spored mold-*Penicillium expansum*

- Bread mold- *Rhizopus stolonifer*.
- White cottony mycelium and black spots

Fig. 9.3. Red bread mold- *Neurospora sitophila*

- Ropiness of home-made breads- *Bacillus subtilis (Bacillus mesentericus).*
- *Ropyness due to hydrolysis of flour protein by proteinase of the bacillus and capsulation of bacillus*

Fig. 9.4. Chalky bread

- Chalky bread—chalk like white spots due to yeast like fungi ----*Endomycopsis fibuligera* and *Trichonospora variable*

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Lesson-10

Microbiology of Meat, Poultry and Sea Foods

10.1. INTRODUCTION

The microbiological profile of meat products presented to the consumers is the sum total of the slaughtered animal health, conditions under which it was reared, quality of slaughtering, processing, packaging and conditions under which the meat was stored. Meat pathogens can cause self-limiting human enteric diseases or systemic and fatal infections of the immunocompromised, the elderly and the young. Meat can act as an ideal substrate for microbial proliferation. Major meat associated pathogenic bacteria include*Clostridium perfringens, Staphylococcus aureus, Salmonella spp,* pathogenic strains *of Escherichia coli, Campylobacter spp, Yersinia enterocolitica, Listeria monocytogenes* and *Aeromonas hydrophila*

10.2. MICROORGANISMS ASSOCIATED WITH MEAT DURING PROCESSING

Meat spoilages indicate (a) color changes (b) textural changes and (c) development of off-flavour or off-odor or slime as a result of microbial growth. *Salmonella* is the primary microbial challenge for poultry. The primary microbial to the beef industry is *Escherichia coli* O157: H7. *Listeria*, which is an adulterant with zero tolerance, is the major problem for ready to eat meat products. Treatment with organic acids, hot water steam carcass pasteurization and steam carcass vacuuming, trisodium phosphate, acidified sodium chlorite, chlorine dioxide, lactoferrins, peroxyacetic acid, sodium lactate, sodium acetate and sodium diacetate, ozone and radiation have been used as microbial decontaminants during meat processing operations. Carcass washing with hot water of 80°C for 10 seconds can reduce microbial loads by 2 logs. Current regulatory policies and inspection in the meat industry include the HACCP (Hazard Analysis Critical Control Point) food safety system with an objective to provide safe food for consumption and prevent chemical, physical and biological hazards.

10.2.1. Gram-negative bacteria (Aerobes): *Neisseriaceae*: *Psychrobacter immobilis, P. phenylpyruvica, Acinetobacter* spp., *A. twoffii, A. Johnsonii,*

Pseudomonadaceae: *Pseudomonas fluorescens, P. lundensis, P. fragi, P. putida*

Gram-positive bacteria: *Brochothrix thermosphacta, Kurthia zophii, Staphylococcus spp., Clostridium estertheticum, Clostridium frigidicarnis, Clostridium casigenes, Clostridium algidixylanolyticum* sp. *nov.*

10.2.2. Spoilage

Fresh Meat

In contrast to fruits and vegetables, meats are composed mainly of protein and fats rather than carbohydrates. Water content is 71–76% and therefore moisture is not an issue except for spoilage microbes on cured meats. Muscles of healthy animals do not contain any bacteria or fungi but as soon as animals are slaughtered, meat is exposed to contaminants and good sanitation practices are essential to produce high quality meats. The number of spoilage organisms on meat just after slaughter is a critical factor in determining shelf life. The surface of beef carcasses may contain anywhere from 10^1 to 10^7 cfu/cm2, most of which are psychrotrophic bacteria. Chopping and grinding of meats can increase the microbial load as more surface area is exposed and more water and nutrients become available. A large variety of microbes are commonly found on fresh meat, but different

microbes become dominant during spoilage of different meats depending on pH, composition and texture of processed meats, temperature and packaging atmosphere. *Pseudomonas* spp. is the predominant spoilage bacteria in aerobically stored raw meat and poultry. Once the initial low levels of glucose are depleted by various microbes, *Pseudomonas* has an advantage because it can catabolize gluconates and amino acids more readily than other microbes. Break down of these compounds results in production of malodorous sulfides, ammonia, and amines, including the biogenic amines putrescine and cadaverine. Dark, firm and dry meat with a relatively high pH of 6.0 spoils more rapidly because deamination of amino acids starts earlier. *Shewanella putrefaciens* does not grow on meat at pH<6.0 but can produce sulfides and ammonia even when glucose is still available. These sulfides not only smell bad but also cause color changes in meat, and therefore Shewanella has a high spoilage potential on fresh, high pH meats stored aerobically even when it is not a dominant microbe.*Brochothrix thermosphacta* is often a significant spoilage organism on fresh meat stored aerobically at refrigeration temperatures.*Enterobacteriaceae*, particularly species of *Serratia*, *Enterobacter*, and *Hafnia*, are major causes of spoilage in vacuum-packed, high pH fresh meats. These organisms are facultative anaerobes that produce organic acids, hydrogen sulfide and greening of meats.

Lactic acid bacteria (LAB) grow on meat and poultry packaged under vacuum and modified atmospheres, producing organic acids from glucose by fermentation. This gives rise to aciduric off-odors which may be accompanied by gas and slime formation and greening of meat. However, LAB are weakly proteolytic and so do not produce large amounts of amines or sulfides, and spoilage of meat by LAB is not as offensive. Psychrophilic, anaerobic *Clostridium* spp. are associated with spoilage of vacuum-packaged meats. "Blown pack" meat spoilage is characterized by excessive gas formation with off odors due to formation of butyric acid, butanol and sulfurous compounds. Yeasts and molds grow relatively slowly on fresh meat and do not compete well with bacteria. Therefore, they are a minor component of spoilage flora.

Processed Meat

Addition of sodium chloride, nitrites and/or nitrates, along with various other seasonings, emulsifiers and preservatives to ground or whole muscle meats changes the environment significantly and also the spoilage flora of processed meats. Dried and dry-fermented meats generally do not support microbial growth although process deviations may allow growth of some organisms. Spoilage organisms can grow on fresh and cooked cured meats, so they are best stored chilled, under a vacuum or modified atmosphere.*Pseudomonas* spp. are not usually important causes of spoilage in processed meats because of their sensitivity to curing salts and heat pasteurization and their inability to grow well in meats packed with a vacuum or high carbon dioxide atmosphere. However, when packages have been opened and there has been insufficient curing, these bacteria may spoil refrigerated processed meats. Some cold- and salt tolerant *Enterobacteriaceae* have been found to cause spoilage in some specific processed meats, such as ham or bacon.

Lactic acid bacteria (LAB) is the group of bacteria primarily associated with spoilage of processed meats. They produce sour off-flavors, gas, slime, and greening, and this spoilage may be more severe than in fresh meat because of the presence of added carbohydrates. Competitive ability of different LAB strains is related to pH and water activity of the meat, cooking and storage temperatures and oxygen and carbon dioxide levels. Sporeformers (*Clostridium* and *Bacillus*) are usually not a spoilage problem in processed meats because of the presence of nitrite and other curing salts. However, faulty cooking/cooling procedures, including long cooling periods and temperature abuse, has allowed growth of these organisms in some cases. Spores of these organisms may be introduced with spices or other ingredients. Yeasts cause some spoilage in processed meats but are generally only important when sulfite is used as a preservative or when meats have been irradiated or are stored aerobically in the cold. Slime may be produced along with vinegary or malty off-odors in some sausages.

Spoilage under aerobic condition

1.) Surface slime, caused by *Pseudomonas acinetobacter, Moraxella alcaligenes Streptococcus, Leuconostuoc, Bacillus* and*Micrococcus.*

2.) Change in colour of meat pigment. The red colour of meat may be changed to shades of green, brown or grey by *Lactobacillus*and *Leconostocs* spp.

3.) Changes in fat. The unsaturated fat in meat gets oxidized by lypolitic bacteria which produce off odours due to hydrolysis of fats and production of aldehydes and acids. This type of spoilage is caused by lypolitic *Pseudomonas*, Achromobacter and yeast.

4.) Surface color change. The red pigment producing bacteria, *Serratia marcescens*, caused red spots on meat. Blue color surface is caused by *Pseudomonas syncyanea* and yellow color is caused by *Micrococcus* species.

5.) Off odor and off taste. Volatile acid like formic, acetic, butyric and propionic acid produce sour odor and *Actinomycetes* produce musty or earthy flavor. Yeast also cause sliminess discoloration and off odor and taste defects.

6.) Aerobic mold also cause spoilage in meat. These are stickiness, whiskers, black-spot, white-spot, green patches off odor and off taste.

7.) Spoialage under anerobic condition.

i) Souring is caused by production of formic, acetic, butyric, lactic, succinic and propionic acid.

ii) Putrefaction. It is caused by decomposition of proteins under anaerobic condition by *Clostridium* species. The foul smell is due to production of hydrogen sulphide, mercaptans, indol, scatol, ammonia and amines.

Photographs of spoilage of meat is shown in Figure 10.1

Fig. 10.1. Spoilage of meat

10.3. EGG

Freshly laid eggs are generally sterile particularly the inner contents. However the shells get contaminated from the environmental sources such as fecal matter of the bird, beddings, by the handlers and wash water and also the packaging materials in which the eggs are packed. There are several extrinsic and intrinsic mechanisms through which the egg protects itself from the microbial invasion. Waxy shell membrane retards the entry of microorganisms. Further, the shell also prevents the entry of microorganisms. The membranes inside the shell behave as mechanical barriers to the entry of microorganisms. Further lysozymes present in the egg white is effective against Gram positive bacteria and avidin in the egg white forms a complex with biotin, thus making it

unavailable for the microorganisms. Also high pH (pH 9-10) of albumin inhibits the microbial growth. Binding of riboflavin by the apo protein and chelation of iron by conalbumin further helps in hindering the growth of microorganisms that might have gained entry inside the egg.

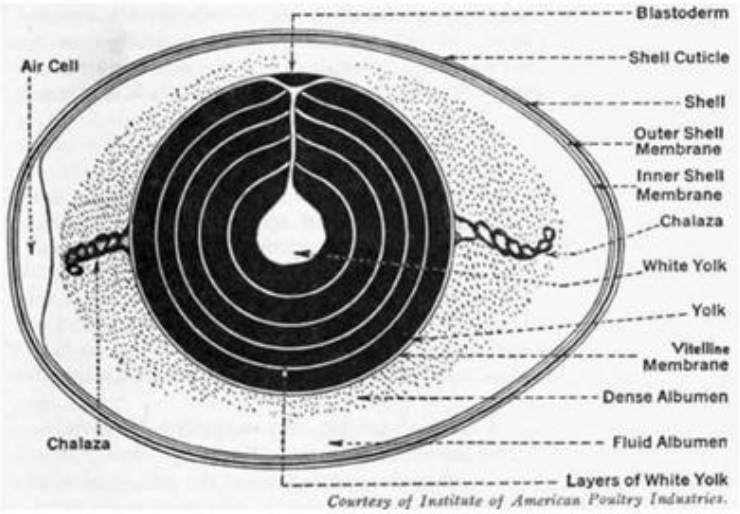

Fig. 10.2. Structure of egg

10.3.1. Spoilage of egg

Breaks or cracks in egg shell taking place due to transportation or mechanical damage may allow microorganisms to enter in to the egg yolk and cause spoilage on storage. Eggs on storage may lose moisture and, therefore, weight. The white of the egg becomes thinner and more watery on storage. The major changes in the egg take place due to spoilage organisms. In general the spoilage of eggs is caused by bacteria as compared to molds and can be described as green rot due to the growth of *Pseudomonas fluorescens,* colourless rot due to the growth of *Pseudomonas, Acinetobacter* and other species; black rots due to *P roteus, Pseudomonas;* red rots due to*Serratia* spp. and custrad rots due to *Proteus vulgaris* and Pseudomonas *intermedium.* Growth of *Aeromonas* in the egg yolk turns it to black colour and also there is strong putrid odour due to the formation of hydrogen sulphide (H_2S). Storage of eggs in high humid atmosphere may help in growth of several molds on the surface of the egg shell. Molds causing spoilage of eggs include species *of Pencillium, Mmucor, Alterneria* , etc.

10.4. POULTRY MEAT

Poultry meat like meat of other animals is also susceptible to contamination by various sources. Contamination of skin and lining of the body cavity take place during various processing operations. The organisms of great importance in poultry are *Salmonella* spp. and*Campylobacter jejuni.* Several Gram negative psychrotropic bacteria *viz., Pseudomonas, Acenitobacter* and *Flavobacterium* have also been isolated from poultry carasses. Ground turkey also may carry fecal streptococci. It is important to freeze the poultry fast in order to keep it in good condition for several months. Freezing further reduces the number of microorganisms in the poultry meat provided the temperature is maintained quite low (-18 ° C or below).

10.5. FISH SPOILAGE

Fish is a very perishable, high-protein food that typically contains a high level of free amino acids. The lipid content of the fish is up to 25%. It has very low content of connective tissue, i.e. approximately 3% of the total weight as compared with around 15% in meat. Fish flesh generally contains 15-20% protein and less than 1%

carbohydrate. Non-fatty fish such as teleosts cod, haddock and whiting, the fat levels are only about 0.5%, while in fatty fish such as mackerel and herring, levels can vary between 3 and 25%.

Composition of a fish

Water 65 – 80 %

Fat 1 – 20 %

Protein 14 – 20 %

Microbes metabolize these amino acids, producing ammonia, biogenic amines such as putrescine, histamine, and cadaverine, organic acids, ketones, and sulfur compounds. Degradation of lipids in fatty fish produces rancid odors . In addition, marine fish and some freshwater fish contain trimethylamine oxide that is degraded by several spoilage bacteria to trimethylamine (TMA), the compound responsible for fishy off odors. Iron is a limiting nutrient in fish, and this favors growth of bacteria such as *pseudomonas* that produce siderophores that bind iron. Spoilage bacteria differ somewhat for freshwater and marine fish and for temperate and tropical water fish. Storage and processing conditions also affect microbial growth. *Pseudomonas* and *Shewanella* are the predominant species on chilled fresh fish under aerobic conditions. Packing under carbon dioxide and addition of low concentrations of sodium chloride favor growth of lactic acid bacteria and *Photobacterium phosphoreum*. Heavily wet-salted fish support growth of yeasts while dried and salted fish are spoiled by molds. Addition of organic acid select for lactic acid bacteria and yeasts. Pasteurization kills vegetative bacteria but spores of Clostridium and Bacillus survive and may grow, particularly in unsalted fish.

Spoilage of fish and sea foods : Halophilic bacteria like *Serratia*, *Micrococcus, Bacillus, Alcaligenes* and *Pseudomonas* cause spoilage of salt fish. Shell fish are spoiled by *Acenetobacter, Moraxella* and *Vibrio*. Crab meat is spoiled by*PseudomonasAcinetobacter* and *Moraxella* at low temperature and by *Proteus* at high temperature.

Microbial loads in shrimps, oysters, and clams depend on the quality of the water from which they are harvested. If the sewage is drained to water bodies, the microbial quality deteriorates. During handling, fecal coliforms, fecal streptococci, and *S. aureus* may be incorporated into the product. *Salmonella* also is found in oysters possibly due to contaminated water. Seafood also is the source for*Pseudomonas* spp., *C. perfringens, L. monocytogenes, Vibrio parahemolyticus, Salmonella enterica* serovar *enteritidis* and*typhimurium*, *Campylobacter jejuni, Yersinia enterocolitica*, and Enteroviruses (Hepatitis A). Smoked salmon and shrimps also are found to carry pathogenic *L. monocytogenes*.

10.6. MEAT BORNE DISEASES

Food borne microbial hazards have a devastating impact on human suffering. Microbial pathogens of current concern that need to be controlled in the fresh meat include *Salmonella, Campylobacter*, enterohaemorrhagic *Escherichia colli* including serotype O157:H7 and other enteric pathogens. Infection due to *Listeria monocytogenes* following consumption of ready to eat meat and poultry products is a major problem in the recent years. Also there are food borne infections caused by *Yersinia enterocolitica,Staphylococcus aureus, Clostridium botulinum, Clostridium perfringens* and *Bacillus cereus*. Prevalence of some food borne pathogens recognized since 1970's include *Vibrio cholerae, Vibrio vulnificus*, Noro virus,, *Enterobacter sakazakii*, prions and resistant bacteria. In recent years the food borne pathogens associate with animal health pandemics include Avian Influenza (AI) and Foot and Mouth Disease (FMD) viruses. Avian influenza is not of concern to poultry meat safety, because it is inactivated by proper cooking with a temperature of 70°C and more. Also the oral route of transmission is less important than the non food borne route. Presently there is continuous adaptation and development of resistance by pathogenic microorganisms to antibiotics and

potentially to traditional food preservation barriers, like low pH, heat, cold temperatures, dryness, low water activity and chemical additives. Development of antibiotic resistance in food borne pathogens is very important from public health view point in present days and in the future.

10.6.1. Control of meat borne pathogens

Effective control of meat borne pathogens and enhancement of meat safety can be achieved by control of latent infections among livestock, animal welfare and humane treatment, application of antimicrobial interventions at the farm, during harvesting, dressing and product processing, improvement in process food hygiene and potential application of new or novel processing and preservation technologies. Animal stress can damage meat quality and lead to more contamination and increased pathogen shedding. Antimicrobial intervention technologies can be used effectively to improve the microbiological quality of meats. These technologies include reduction of contamination on the raw product, minimization of microbial access to the products, reduction of contamination that has gained access to the product, inactivation of the microbes on the product without cross contamination and prevention and control of the growth of non-inactivated microbes, which have gained access to the meat. An effective pathogen control at pre-harvest, postharvest, processing, storage, distribution, merchandizing, preparation, food-service and consumption of meat include activities employed during pre-harvest or in the field, during post harvest or processing in the plant, at retail and food service and at home. Pre-harvest pathogen control interventions include diet manipulation, use of food additives, antibiotic, bacteriophage therapy, and immunization of the animals, complete exclusion, probiotics and proper animal management practices like pen management, clean feed, clean and chlorinated water, and clean and unstressful transportation. Antimicrobial intervention activities during harvest and post harvesting should be designed to minimize introduction of microbial contaminants and reduce existing contaminant levels through implementation of decontamination and sanitization interventions, processing treatments for partial or complete destruction of contaminants and antimicrobial procedures for inhibition or retardation of microbial growth. Certain inspection regulations should be followed in meat and poultry plants, such as establishment of sanitation standard operating system, operations under the HACCP system and performing HACCP verifications to meet microbiological standards, establishment of good manufacturing practices (GMP) and good hygiene practices (GHP). Antimicrobial interventions used to control pathogens in further processed meat products include physical hurdles (low and high temperature, non thermal process like irradiation and high pressure and packaging treatments), physiochemical interventions (low pH, reduced water activity, modification of oxidation reduction potential through packaging and application of antimicrobial additives), and biological interventions (microbial competitors, such as lactic acid bacteria and antimicrobial products, such as bacteriocins). Events of the most food borne illness happen due to mishandling of foods in various ways. So, there should be provisions to educate the food handlers and consumers particularly on culinary tips.

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Lesson-11

Microbial Spoilage of Canned Foods

11.1 INTRODUCTION

Canning is one of important method of packaging food for long term storage. Normally food is stored in metallic containers along with heat treatment. The heat treatment varies depending upon type of food. There is always a chance that microorganisms may survive if the heat treatment is not proper thereby leading to spoilage of food. Usually the incidences of food spoilage in cans are low. The spoilage of can could be due to biological or chemical reasons or combination of both. The biological spoilage is primarily due to microbial growth while chemical spoilage is due to hydrogen produced due to reaction of acid in food and iron on can. The degree of swelling can also be increased by high summer temperature and high altitudes. Certain other factors such as overfilling, buckling, denting or closing the can while cool may also be responsible for spoilage of foods in cans.

11.2. CAUSES OF SPOILAGE IN CANS

11.2.1. Chemical spoilage

The chemical spoilage in most cases is due to production of hydrogen gas produced in can because of action of acid of food on iron of can. This spoilage is termed as Hydrogen swell. It occurs due to following factors:

a) Increased storage temperature.

b) Increased acidity of food

c) Improper exhaust

d) Presence of soluble sulfur and phosphorous compounds

e) Improper timing and lacquering of can at internal surfaces

11.2.2. Biological spoilage

The cause of biological spoilage is microbial activity. In heat treated cans, the growth of microorganisms occur due to:

11.2.2.1. Leakage of can

It occurs because of manufacturing defects, punctures or rough handling. Bacteria are introduced into can by either in holes or improper seams. In this type, the microorganisms are not usually heat resistant and wide array of organisms had been found to cause spoilage as it is post processing contamination. Microbes may also get entry into can due to cold water, used to cool cans after heat treatment. Leakage may also be responsible for release of vacuum, which can favor the growth of microorganisms. Presence of low heat resistance organisms usually indicates leakage of can.

11.2.2.2. Under processing

It includes sub-optimal heat treatment, faulty retort operations, excessive microbial load and contamination in product, change in consistency of the product.

11.3. STAGES IN APPEARANCE OF CAN

A can undergo different transformations from being a normal can to completely spoilt can as it depend upon various factors. All these stages are described based on appearance of can from outside (Table 11.1).

Table- 11.1: Various stages of spoilage in can

Appearance of Can	Description
Flat	This is normal stage of can where ends of can are slightly concave. At this stage partial vacuum exist in can. It maintains the condition even if dropped on solid surface.
Flipper	This appears flat but on dropping it on solid surface, either end can become convex. On applying pressure the can regains its shape.
Springer	It has both ends bulged but if pushed inside, one end remain concave but other flips out. This appearance describes slight pressure in the cans usually caused by poor exhaust, overfilling, denting or variation in temperature. Usually no gas production occurs.
Soft swell	The ends are bulged due to presence of gas in can but the ends can be pushed inside with light pressure.
Hard swell	This is stage where high gas pressure accumulates inside can and bulged ends cannot be pushed inside manually. Due to pressure the ends or side seam may buckle or distort. A very high pressure may lead to bursting of can through seam or ends.
Breather	This is a type of can which has minute leak allowing air movement into the can. It may not allow microbial entry into can.

11.4. MICROBIAL SPOILAGE OF CANNED FOODS

The microbial spoilage of canned food is classified as caused by thermophilic bacteria and mesophilic organisms. Most common spoilages of microbial origin are known as flat sour spoilage, Thermophilic anaerobic (TA) spoilage and putrefaction. These different types are briefly described here.

11.4.1. Spoilage by thermophilic spore forming bacteria

Spoilage by these types of bacteria is most prevalent in under processed heat treated canned foods. Their spores survive the heat treatment and undergo vegetative cell formation and subsequent growth in canned conditions. Major spoilages by these organisms are:

11.4.2. Flat sour spoilage

This is caused by souring bacteria. One characteristic of this spoilage is that ends of can remain flat during souring. Because of this condition, the detection of spoilage from outside is not possible thereby culturing of

contents become necessary to detect the type of organisms. Main organisms involved are *Bacillus,* while it occurs more frequently in low acid foods. *Bacillus* spp. has ability to produce acid without gas formation.

11.4.3. TA spoilage

This type of spoilage is caused by thermophilic anaerobe not producing hydrogen sulfide. *Clostridium thermosaccharolyticum* is the main organism involved. It produces acid and gas in foods. Spoiled food produces sour or cheesy smell.

11.4.4. Sulfur stinker spoilage

This type of spoilage occurs in low acid foods and primarily *Desulfotomaculum nigricans* is involved. The spores of these organisms are destroyed at optimal heat treatment, thus presence of this organism usually indicates under processing in terms of heat treatment. It produces hydrogen sulfide which produce typical odour.

11.5 SPOILAGE BY MESOPHILIC SPORE FORMERS

Bacillus and Clostridium are involved in this type of spoilage which is usually indicative of under spoilage.

Table-11.2: Characteristic of Mesophilic spore former spoilage in can

Spoilage type	Characteristic
Mesophilic *Clostridium* type	• Sugar fermenting species producing butyric acid involved e.g. *C. butyricum, C. pasteurinum* • Swelling of container due to CO_2 and Hydrogen • Putrefactive species such as *C. sporogenes, C. putrefaciens, C. botulinum* too play role • Decompose proteins and produce ammonia, indole, H_2S, skatole • Spoilage prevalent in foods processed at 100°C
Mesophilic *Bacillus* type	• *Bacillus* is most involved • Problem more prominent in poorly evacuated cans • Sea foods, meat, evaporated milk most affected • *B. polymyxa* and *B. macerans* involved • Entry of organism through leakage of cans

11.6 Spoilage by Non-Spore Formers

Presence of non spore formers in cans indicate post processing contamination. The organisms whose vegetative cells are heat resistant are more readily found. Following organisms are more prominent:

Enterococcus	*Streptococcus thermophilus*
Micrococcus	*Lactobacillus*
Leuconostoc	*Microbacterium*

Presence of these organisms indicates leakage of container. Cooling water is one of the important source of contamination, thus coilforms also gain entry into the can through leakage.

11.7 Spoilage by Fungi

11.7.1 Yeasts

Yeasts and their spores are not thermo tolerant, thus they are not found in suitably heat treated cans. Their presence indicates under processing or post pasteurization contamination through leakage. Fermentative yeasts are more prominent and they produce carbon dioxide, thus causing swelling of cans. Film yeasts too can grow on the surface of the food products.

11.7.2 Molds

Among molds, *Aspergilus* and *Penicillium* are most spoiling organisms. These can grow at high sugar concentration. Acidification is considered method of preventing growth of molds. Some of the molds are resistant to heat. Molds are more common in home canned foods where heating as well as sealing is not under total aseptic conditions.

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Module 4. Microbiology of food preservation

Lesson-12

Physical Methods-Thermal Processing

12.1. INTRODUCTION

Heat kills microorganisms by changing the physical and chemical properties of their proteins. When **heat** is used to preserve foods, the number of microorganisms present, the **microbial load** , is an important consideration. Various types of microorganisms must also be considered because different levels of resistance exist. For example, bacterial spores are much more difficult to kill than vegetative bacilli. In addition, increasing acidity enhances the killing process in food preservation.

12.2 HIGH TEMPERATURE

Three basic heat treatments are used in food preservation: **pasteurization** , in which foods are treated at about 62°C for 30 minutes or 72°C for 15 to 17 s; **hot filling** , in which liquid foods and juices are boiled before being placed into containers; and **steam treatment**under pressure, such as used in the canning method. The heat resistance of microorganisms is usually expressed as the **thermal death time** , the time necessary at a certain temperature to kill a stated number of particular microorganisms under specified conditions.

12.2. Pasteurization

It is the process of heating a food-usually a liquid-to or below its boiling point for a defined period of time. The purpose is to destroy all pathogens, reduce the number of bacteria, inactivate enzymes and extend the shelf life of a food product. Pasteurization treatment is able to kill most heat resistant non spore forming organisms like *Mycobacterium tuberculosis* and *Coxiella burnetti*. Foods with a pH of less than 12.6, such as milk and spaghetti sauce, can be pasteurized. Permanent stabilitythat is, shelf life of about two years is obtained with foods that can withstand prolonged heating, such as bottled juices. There is a greater loss of flavour from foods that are exposed to a longer time-temperature relationship. Therefore, temporary stability (that is, limited shelf life) is only obtained with some foods where prolonged heating would destroy its quality. These foods, such as milk, usually require subsequent refrigeration. "High Temperature Short Time" (HTST) and "Ultra High Temperature" (UHT) processes have been developed to retain a food's texture and flavour quality parameters. Pasteurization is not intended to kill all microorganisms in the food. Instead pasteurization aims to reduce the number of viable pathogens so that they are unlikely to cause disease. Pasteurization involves a comparatively low order of heat treatment, generally at temperature below the boiling point of water. eating may be done by means of steam , hot water, dry heat or electric currents . Products are immediately cooled. Desired pasteurization can be achieved by a combination of time and temperature such as heating food to a low temperature and maintain for a long time i.e. LTLT -62.8 ^{0}C for 30 minute (Figure 12.1), or by heating food to a high temp and maintain for a short time: HTST-71.7 ^{0}C for 15 second(Figure 12.2).

Pasteurization is used when more rigorous heat treatment might harm the quality of the food product, as the market milk and for the main spoilage organisms which are not heat resistant, such as yeast in fruit juice. It also kills the pathogens .

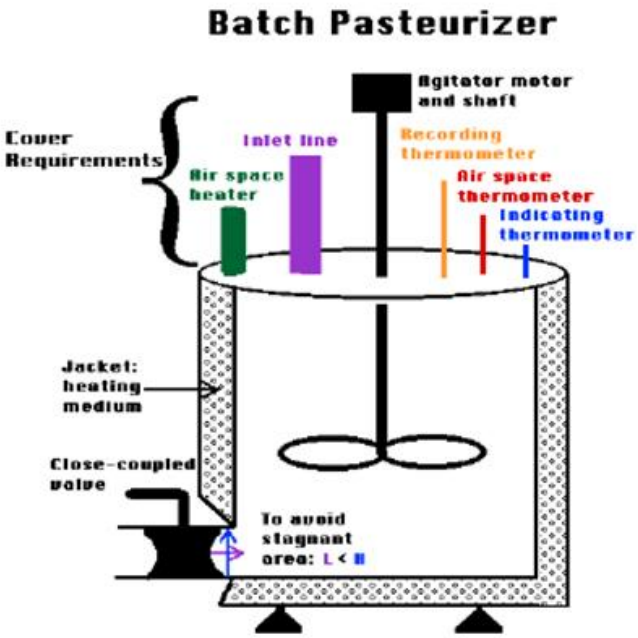


Fig. 12.1 Batch pasteurizer

12.2.1. Ultra heat pasteurization

In this process milk is heated to 120-138 ^{0}C for 2-4 seconds and followed by rapid cooling. This treatment kills all the spoilage microorganisms. UHT pasteurized milk is packaged aseptically resulting in a shelf stable product that does not require refrigeration until opened.

12.3. Heat resistance of microorganisms and their spores

It is expressed in terms of their thermal death time (TDT).

12.3.1. Thermal Death Time (TDT)

It is the time taken to kill a given number of microorganisms or spores at a certain temperature under specified conditions.

12.3.2. Thermal death point

It is the temperature necessary to kill all the organisms in ten minutes.

Heat resistance of different microorganisms is different. Microorganisms are more heat resistant than their spores. Heat resistance of vegetative yeast is 50-58 ° C in 10-15 min and the ascospores is 60 ° C for 10-15 min. However, yeast and spores are killed by pasteurization.

12.4. Heat resistance of microorganisms

Heat resistance of mold is 60^0 C in 5 to 10 min and asexual spores are more heat resistance than the ordinary mycelium and require a temperature 5-10°C higher for their destruction. *Aspergillus, Muco, ,Penicillium* are more resistant than yeast. Heat resistance of bacteria and bacterial spores is different. Cells high in lipid content and capsule containing bacteria are harder to kill. Higher the optimal and maximal temperature for growth , the greater the resistance to killing.

12.5. Heat resistance of enzymes

Most of the food and microbial enzymes are destroyed at 79.4 °C . Some hydrolases will retain a substantial levels of activity after an ultra high temperature treatment. Bovine phosphatase, if present, in processed milk indicates that the milk was not properly pasteurized.

12.6. D value

It is the decimal reduction time, or the time required to destroy 90% of the organisms. Mathmatically, it is equal to reciprocal of the slop of the survivor curve and is a measure of the death rate of a microorganisms. When D is determined at 250 ^{0} F, it is expressed as D_r.

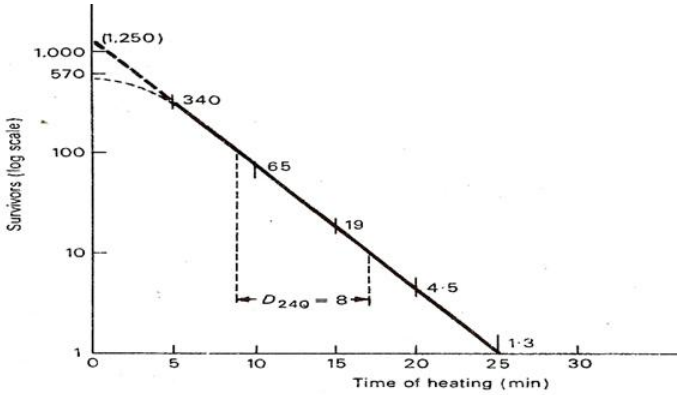


Fig.12.2: D value of organisms

12.7. z value

It refers to the degree F required to reduce TDT ten fold. Mathematically, this value is equal to the reciprocal of the slope of the TDT curve.

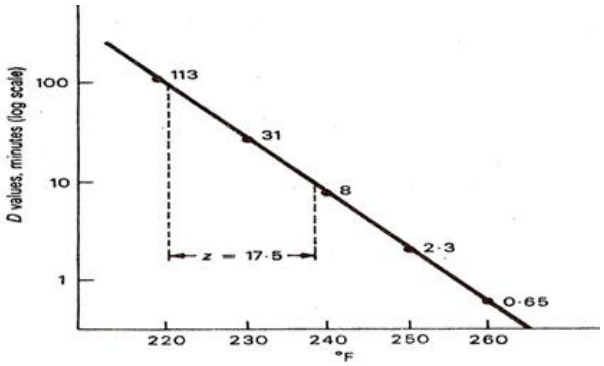


Fig. 12.3: z value of organisms

12.8. F value

This value is the equivalent time, in min at 250 ° F, of all heat considered, with respect to its capacity to destroy spores or vegetative cells of a particular organisms or F is the time in minute required to destroy the microorganisms in a specified medium at 250 ° F.

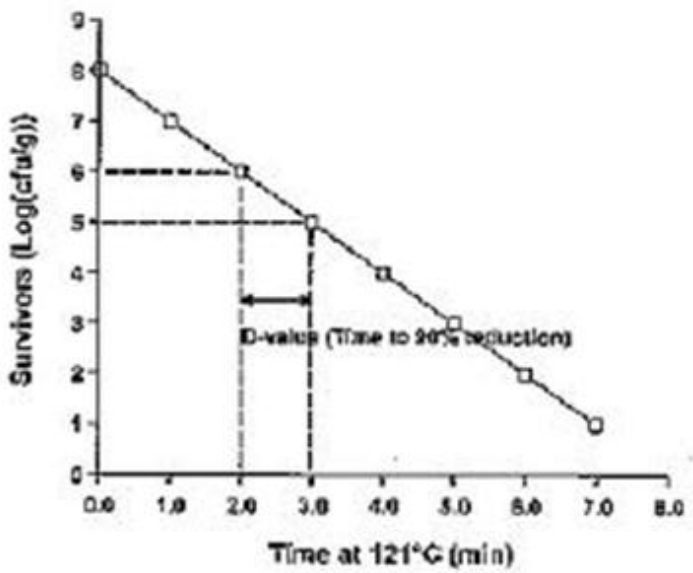
12.2.2.9 Thermal death time curve

Mean viable counts determined at intervals of 5 minute are as follows-

Time Mean viable count 5 3120.0 10 65.0

15 19.0

Time of heating in min is plotted on semi-log paper along the linear axis and the number of survivors is plotted along the log scale to produce the TDT curve .



12-D concept: It is the time temperature process that will reduce the most heat resistant *Cl. botulinum* spores by 12 log cycles. Processing of food for 2.52 min at 250°C reduces *Cl. botulinum* spores to 1 spore in 10^{12} containers

12.2.3 Effect of pasteurization

The positive effects of pasteurization are the destruction of pathogenic microorganisms to increase the safety of market milk for human consumption, improved keeping qualityandinactivation of certain naturally occurring enzymes.

The negative effects are: certain preformed products of microbial origin are not inactivated during pasteurization, e.g. Staphylococcal toxins and aflatoxins. There is small loss of native aroma particularly in case of fruit juices. In case of milk, it destroys the natural microbicidal property of milk by inactivating different natural occuring antimicrobial substances and the rennet coagulation time also increases.

Table 12.1 Pasteurization Treatment for Different Food

Food	Main purpose	Subsidiary purpose	Minimum processing condition
Fruit juice	Enzyme inactivation	Destruction of spoilage causing microorganisms	65°C or 30 min 77°C for 1 min 88°C for 15 min
Beer	Destruction of spoilage organisms		65-68°C or 20 min 72-75°C for 1-4 min
Milk	Destruction of pathogens	Destruction of spoilage causing microorganisms and enzymes	68.2°C or 30 min 71.7°C for 15 sec
Liquid	Destruction of pathogens	Destruction of spoilage causing microorganisms.	64.4°C or 2.5 min 60°C for 3.5 min
Ice cream	Destruction of pathogens	Destruction of spoilage causing microorganisms	71°C for 10 min 80°C or 15 min

12.2.4 Blanching

It is a kind of pasteurization generally applied to fruits and vegetables , primarily to inactive natural food enzymes. It is a common practice when such food products are to be frozen, since frozen storage itself would not completely arrest enzyme activity. Peroxidase and catalase are the most heat resistant enzymes; the activity of these enzymes is used to evaluate the effectiveness of a blanching treatment. If both are inactivated then it can be assumed that other significant enzymes also are inactivated. The heating time depends on the type of fruit or vegetable, method of heating, the size of fruits or vegetable or the temperature of the heating medium.

For commercial blanching typical times at 212 ^{0}F are given in the Table 12.2.

Table 12.2 Blanching time for different foods at 212°F

Vegetables	Blanching time(min) in water at 212°F
Beans	1-3
Beets	3
Corn	2-3
Peas	1-2
Spinach	1-2
Asparagus	2-12

Rapid changes in colour, flavor and nutritive value occur as a result of enzyme activity. Blanching is a slight heat treatment, using hot water or steam, that is applied mostly to vegetables before canning or freezing. The main objectives of blanching are to inactivate enzymes, to remove the tissue gases, to clean the tissue, to increase the temperature of the food. Blanching is also used before canning for different reasons, because enzymes will inevitably be destroyed during canning. Blanching induces a vacuum in canned goods, and it is also used to control the fill into containers (for example, spinach).

12.2.5 Sterilization (Retorting)

Sterilization destroys all pathogenic and spoilage microorganisms in foods and inactivates enzymes by heating. All canned foods are sterilized in a retort (a large pressure cooker) and called commercial sterilization which indicates that no viable organisms are present. This process enables food to have a shelf life of more than two years. Foods that have a pH of more than 4.6, such as meat and most vegetables must undergo severe heating conditions to destroy all pathogens. These foods are heated under pressure to 121°C for varying times. Severe conditions are applied primarly to ensure that *Clostridium botulinum* spores are destroyed during processing. These spores produce the deadly botulinum toxin under anaerobic conditions (that is, where there's no oxygen). The spores are destroyed by heat or are inhibited at pH values of less than 4.6 Therefore, a food with a pH of less than 4.6 that is packaged anaerobically, such as spaghetti sauce, doesn't need to undergo such a severe heat treatment. The destruction of vegetative and sporeforming organism and pathogens is secondary objective of commercially sterilized foods.

Nicolas Appert , a Parisian confectioner by trade, established the heat processing of foods as an industry in 1810. The food product is washed, sorted, and graded and then subjected to steam for three to five minutes. This last process called blanching, destroys many enzymes in the food product and prevents further cellular metabolism. The food is then peeled and cored, and diseased portions are removed. For canning, containers are evacuated and placed in a pressurised steam steriliser, similar to an autoclave at 121°C. This removes especially *Bacillus* and *Clostridium* spores. If canning is defective, foods may become contaminated by anaerobic, bacteria which produce gas. These are species of *Clostridium*, and coliform bacteria (a group of Gram-negative non spore-forming rods which ferment lactose to acid and gas at 32°C in 48 hours).

Canning cooking fruits or vegetables, sealing them in sterile cans or jars, and boiling the containers to kill or weaken any remaining bacteria as a form of pasteurization. High-acid fruits like strawberries require no preservatives to can and holding for only a short boiling cycle, whereas marginal fruits such as tomatoes require longer boiling and addition of other acidic elements. Many vegetables require pressure canning. Food preserved by canning or bottling is at immediate risk of spoilage once the can or bottle has been opened. Lack of quality control in the canning process may allow ingress of water or micro-organisms. *Clostridium botulinum*produces an acute toxin within the food and may lead to severe illness or death. This organism produces no gas or obvious taste and remains undetected by taste or smell. Food contaminated in this way include Corn, beef and Tuna.

In canning process heat is applied to food that is sealed in a jar in order to destroy any microorganisms that can cause food spoilage. Proper canning techniques stop this spoilage by heating the food for a specific period of time and killing these unwanted microorganisms. During the canning process, air is driven from the jar and a vacuum is formed as the jar cools and seals.

Water-bath canning and pressure canning are two approved methods of canning.

12.2.5.1 Water-bath canning

This method sometimes referred to as *hot water canning,* uses a large kettle of boiling water (Figure 12.4). Filled jars are submerged in the water and heated to an internal temperature of 212°F for a specific period of

time. This method is used for processing high-acid foods, such as fruit, items made from fruit, pickles, pickled food, and tomatoes.

Fig. 12.4 : Water Bath Canning Kettle

12.2.5.2 Pressure canning

Pressure canning uses a large kettle that produces steam in a locked compartment (Figure 12.5). The filled jars in the kettle reach an internal temperature of -240 °C under a specific pressure (stated in pounds) that is measured with a dial gauge or weighted gauge on the pressure-canner cover. A pressure canner should be used for processing vegetables and other low-acid foods, such as meat, poultry and fish.

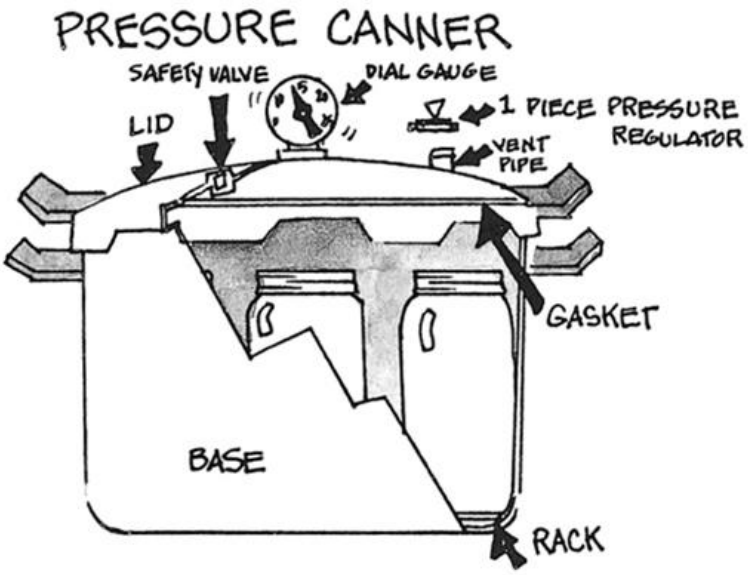


Fig. 12.5 Pressure canner

12.2.6. DRYING

One of the oldest methods of food preservation is by drying, which reduces water activity sufficiently to delay or prevent bacterial growth. Drying is done to produce concentrated form of foods, inhibits microbial growth and autolytic enzymes, retains most nutrients. Drying can cause loss of some nutrients, particularly thiamine and vitamin C. Sulphur dioxide is sometimes added to dried fruits to retain vitamin C, but some individuals are sensitive to this substance.

Most types of meat can be dried. This is especially valuable in the case of pig meat, since it is difficult to keep without preservation. Many fruits can also be dried; for example, the process is often applied to apples, pears, bananas, mangos, papaya, and coconut and grapes . Drying is also the normal means of preservation for cereal grains such as wheat, maize, oats, barley, rice, millet and rye. Drying is an excellent way of preserving several of the seasonal fruits for use during the off season. There are several types of dryers which are used. These include: drum dryer, cabinet dryer, tunnel dryer, rotary dryer, spray dryer and solar dryer. The basic methods of drying involves ai r and contac t dr ying under atmospheric pressure . In this cas e the heat is transferred through the food either from heated air or heated surfaces, and the resulting water vapour is removed with the air current . Solar drying, sun drying, drum and spray drying all use this technique.

Advantages of drying are many

i) Long Shelf Life – Since most microorganisms responsible for food spoilage are unable to grow and multiply in the absence of moisture, spoilage due to microbial degradation is limited in dried foods. Furthermore, enzymes which catalyse undesirable changes in foods need moisture to be effective.

ii) Reduced Weight – This results in reduced transportation, storage and shipping costs.

iii) Convenience – The production of convenience items with novelty appeal for niche markets makes drying an attractive option.

iv) Concentration of nutrients – The removal of most of the water from a food results in a highly concentrated source of nutrients.

v) No refrigeration is required for dried products – Savings in energy and storage costs together with the long shelf life provide a lucrative processing alternative for tropical countries.

Disadvantages of Drying

Disadvantages of Drying are few and mainly relate to oxidation, which usually accompanies drying. This results in losses of micronutrients such as carotene and ascorbic acid and minimal loss in protein as a result of browning reactions. Reduced consumer appeal is often linked with the latter. There might also be changes in flavour and texture if drying is not properly controlled, particularly with regard to maximum temperatures.

12.2.7 Microwave sterilization

Microwave sterilization is a thermal process. A microwave oven (Figure 12.6) works by passing non ionizing microwave radiation, usually at a frequency of 2.125 GHz (a wavelength of 12.212 cm), through the food. Microwave radiation is between common radio and infrared frequencies. Microwave heating takes place due to the polarization effect of electromagnetic radiation at frequencies between 300 MHz and 300 GHz. It delivers energy to the food package under pressure and controlled temperature to achieve inactivation of bacteria harmful for humans. Most processed foods today are heat treated to kill bacteria. Prolong exposure to high heat often diminishes product quality. Microwaves interact with polar water molecules and charged ions. The

friction resulting from molecules aligning in rapidly alternating electromagnetic field generates the heat within food. Since the heat is produced directly in the food, the thermal processing time is sharply reduced. The colour, texture and other sensory attributes of foods processed by microwave sterilization are often better compared with those of conventionally retorted foods while meeting microbial safety requirements. US Federal Communication Commission (FCC) allocates 915 MHz and 21250 MHz bands for industrial and domestic microwave heating applications. The microwave sterilization technology using the combination of 915 MHz microwave and conventional heating to improve heating uniformity. Microwave ovens use electromagnetic radiation to excite water molecules in food. The actual waves penetrate only about 10 inches from the source of the radiation. Within the food, the waves only penetrate 3/12 to 1 inch on all sides. As a result, the actual ovens must be limited in size. Heat is produced within the food by the friction of water molecules, which spreads to the centre of the food by conduction. Small portions are cooked rapidly in microwave ovens. As the quantity of food increases, however, the efficiency is lost.

Microwave heating has also found applications in the food industry, including tempering of frozen foods for further processing, pre-cooking of bacon for institutional use and final drying of pasta products. In those applications, microwave heating demonstrates significant advantages over conventional methods in reducing process time and improving food quality.

The shelf life of a product is determined by its microbiological safety and sensory attributes. In general, microwave sterilization can achieve the same reduction of bacterial population as conventional retorting. Products intended for microwave sterilization are usually packaged in plastic trays or pouches. The ability of plastics to withstand oxygen permeation will affect the organoleptic or sensory acceptance of the product during storage. Normal shelf life expectancy of microwave-sterilized products pre-packaged in plastic containers or pouches is 2-3 years or longer. With innovative plastic technologies coming to the market, the new generations of plastics may increase the expected shelf life even longer.

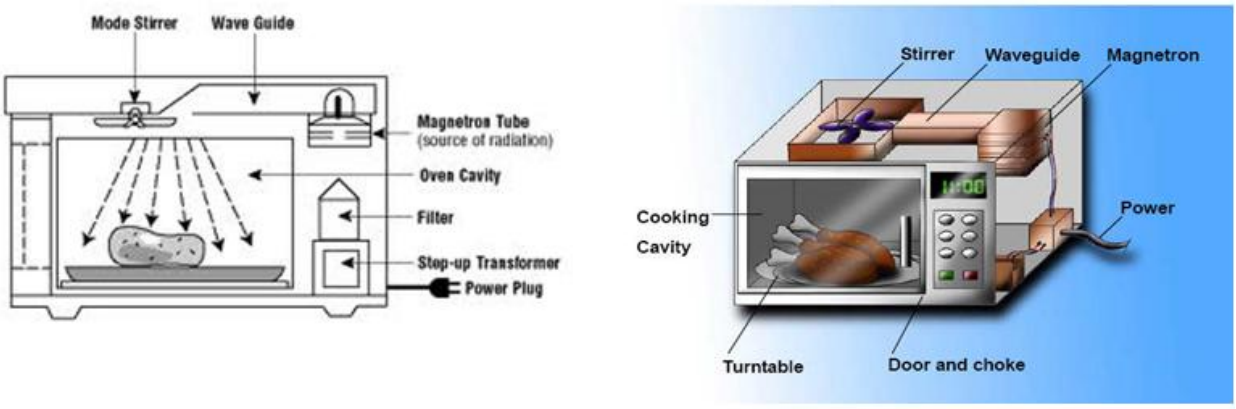


Fig 12.6 Microwave sterilization

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Lesson-13
Physical Methods: Part-II: Non-Thermal processing

13.1. INTRODUCTION

The application of extreme heat treatments used for food preservation affect the nutritional and organoleptic properties of food. In recent years, the consumers demand for fresher, higher quality and safe food has increased. Therefore, nonthermal methods of food preservation for the inactivation of microorganisms and enzymes as an alternative to thermal processes are being used. However, the high resistance of certain enzymes and microorganisms to nonthermal processes, especially bacterial spores, limit their application. During nonthermal processing, the temperature of foods is held below the temperature normally used in thermal processing; therefore, a minimal degradation of food quality is expected. Nonthermal process of food preservation improves food quality and enhances safety levels. Overall, most nonthermal preservation techniques are highly effective in inactivating vegetative cells of bacteria, yeast, and molds. Bacterial spores and most enzymes are however, difficult to inactivate with these procedures. Thus their use is limited to foods where enzymatic reactions do not affect food quality or where spore germination is inhibited by other prevailing conditions, such as low pH.

13.2. Low Temperature Preservation

Storage at low temperatures prolongs the shelf life of many foods. In general, low temperatures reduce the growth rates of microorganisms and slow many of the physical and chemical reactions that occur in foods. Low temperatures are used to preserve food by lowering microbial activity through the reduction of microbial enzyme activity. However, psychrophic bacteria are known to grow even at commercial **refrigeration** temperatures (7^0C). These bacteria include members of the genera *Pseudomonas, Alcaligenes, Micrococcus* and *Flavobacterium*. Some of the fungi also grow at refrigeration temperatures. Slow freezing and quick freezing are used for long-term preservation. **Freezing** reduces the number of microorganisms in foods but does not kill all of them . In microorganisms, cell proteins undergo denaturation due to increasing concentrations of solutes in the unfrozen water in foods, and damage is caused by ice crystals.

13.2.1. Refrigeration

Refrigeration slows down the biological, chemical, and physical reactions that shorten the shelf life of food. Exposure of microorganisms to low temperatures reduces their rates of growth and reproduction. This principle is used in refrigeration and freezing. Microbes are not killed at refrigeration temperature for a considerable period of time. In refrigerators at 5°C, foods remain unspoiled but in a freezer at -5°C the crystals formed tear and shred microorganisms. It may kill many of the microbes. However, some are able to survive. *Salmonella* spp. and *Streptococcus spp*. Survive freezing. For these types of microorganisms rapid thawing and cooking is necessary.

Refrigerators should be set to below 12°C to control the growth of micro-organisms in foods. This lowered temperature also reduces the respiration rate of fruits and vegetables, which retards reactions that promote spoilage. All perishable foods should be refrigerated as soon as possible, preferably during transport, to prevent bacteria from multiplying. Refrigeration is generally used to: i) reduce spoilage during distribution of perishable foods, ii) increase the holding period between harvesting and processing; and iii) extend the storage life of commercially processed foods. All foods are not benefited from cold temperatures. For example, bananas turn black and bread goes stale when refrigerated.

13.2.2. Freezing

Freezing is also one of the most commonly used processes commercially and domestically for preserving a very wide range of food stuffs including prepared food stuffs which would not have required freezing in their unprepared state. For example, potato waffles are stored in the freezer, but potatoes themselves require only a cool dark place to ensure many months storage. Freezing makes water unavailable to microorganisms. The chemical and physical reactions leading to deterioration are slowed by freezing. White or grayish patches on frozen food caused by water evaporating into the packages air spaces called freezer burn occurs which causes deterioration of taste and appearance. This occurs in fruits, vegetables, meat, poultry and fish. While many home freezers are held at -10°C, commercial freezers are under -18°C. At this temperature, the growth of micro-organisms is almost stopped. Deteriorative microbial reactions will still occur, but over a much longer time. In addition, deteriorative enzymatic reactions will still take place during frozen storage. Uncooked fruits and vegetables must be blanched before freezing to prevent these reactions. During freezing, the water in food forms ice crystals. The rate of this phenomenon has a big impact on the quality of frozen foods:

Slow freezing

Slow freezing (e.g. home freezer) forms large ice crystals which puncture cell walls and cellular fluid is released and also results in shrunken appearance of thawed food. In this process the freezing is done for 3-72 h. This method is used in home freezer and temperature is lowered to -15 to -29°C.

Rapid freezing

During rapid freezing small, numerous ice crystals are formed and cell structure is not changed. In this process the temperature of food is lowered to about -20°C within 30 min. This process blocks or suppresses the metabolism.

The shelf life of frozen foods is largely dependent on storage conditions. Under ideal conditions, frozen foods can have a shelf life of one year. However, if foods are continuously exposed to warmer temperatures, such as the opening and closing of freezer doors, then heat shock occurs. Heat shock is when ice melts and re-forms into larger ice crystals. The best example is ice cream, which has a gritty texture if large ice crystals have developed.

Advantages of freezing are generally good retention of nutrients and prevention of microbial growth by low temperature and unavailability of water. However, disadvantages of freezing are loss of some B-Group vitamins and vitamin C due to blanching of vegetables prior to freezing and unintended thawing can reduce product quality.

13.2.3. Preservation by Freeze Drying

The process of freeze drying or lyophilization is commonly used these days for preservation. The food is deep frozen, after which the water is drawn off by a vacuum pump in a machine. The dry product is then sealed in foil and is reconstituted with water. This method is very useful for storing, transporting and preserving bacterial cultures. Drying or dehydration involves the removal of water from the food by controlled processes. This may be done by evaporation due to heating of the product, e.g., drying of fruits, osmotic dehydration, e.g. brining of fish and sublimation, or freeze drying e.g. in the drying of coffee.

There are two distinct stages in this technology. In the first stage, the removal of surface water depends solely on the state of the air surrounding the food, such as its temperature, relative humidity and speed. In the second phase of drying, the moisture within the food moves to the surface. As the air is heated, its relative humidity decreases, resulting in more absorption of water. Here the rate of drying is dependent on the time the moisture

takes to get to the surface. The heating of the air around a food product can, therefore, cause it to dry more quickly. The principle of sublimation is used in freeze drying and lyophilization. This is the process in which a solid changes directly to a vapor without passing through the liquid phase.

13.3. IRRADIATION

Generally alpha, beta and gamma radiation particles are used for the preservation of food. These radiations are of high frequency with a high energy content and they have the power to break molecules into oppositely charged units termed as ions. These radiations, are, therefore, called ionizing radiations. The treatment has a range of effects, including killing bacteria, molds and insect pests, reducing the ripening and spoiling of fruits and at higher doses inducing sterility. The technology may be compared to pasteurization. It is sometimes called 'cold pasteurization', as the product is not heated. Irradiation is useful only for foods of high initial quality. A spoiled food cannot be reverted to un-spoiled state. Irradiation is not effective against viruses and prions. It cannot eliminate toxins already formed by microorganisms.

The radiation process is unrelated to nuclear energy, but it may use the radiations emitted from radioactive nuclides produced in nuclear reactors. Irradiated food does not become radioactive. National and International expert bodies have declared food irradiation as 'wholesome'. The food is exposed to controlled levels of ionizing radiation in the form of gamma radiation, X-rays and electron beams to kill harmful bacteria, pests, or parasites, or to preserve its freshness. The particle sources are readily available in the form of radio-isotope Cobalt 60. This is the most suitable gamma-particle emitter. The penetration power of different radiation particles is different. Alpha particles are stopped by a sheet of paper, beta particles can penetrate through 1-2 cm thick sheet and gamma particles can penetrate through 30-120 cm thick sheet. UV-radiation are also used as an alternative means of treatment of foods but penetration power is very low. **Ultraviolet radiation** is valuable for reducing surface contamination on several foods. This short-wavelength light has been used in the cold storage units of meat processing plants.

A Roentgen (r) is the quantity of gamma or X-rays which produces one electrostatic unit of electric charge of either sign in a cubic centimeter of air under standard conditions. An electrovolt (eV) is the energy gained by an electron in moving through a potential difference of 1 volt. A meV is 1 million electrovolts.

13.3.2 Mechanism of microbial inactivationby radiation

Radiations causes disruption of internal metabolism of cells by destruction of chemical bonds, DNA cleavage results in loss of cells ability to reproduce . Free radicals are formed upon contact with water containing foods. These free radicals react with cellular DNA causing radiation damage. DNA is considered “radiation sensitive” portion of cells. Shorter wavelengths have enough energy to “knock off” an electron to form a “free radical” but not high enough to “split” an atom and cause target to become “radioactive”. Interaction between free radicals and DNA is responsible for “killing effect” of ionizing radiation (IR). The ionizing radiations can cause chemical reaction and alterations of chemicals in tissues and which can be toxic or fatal to humans in high dose. Much of the reactivity of ionizing radiations in organism is with water and produces superoxide radicals (O_2^-), hydroxradicals (HO^-), hydropyroxyl radicals (HOO^-) and hydrogen peroxide. These are produced during high energy collisions of gamma rays and heavy elements (i.e. Tungsten)

Alpha Particles are positively charged particle ejected spontaneously from the nuclei of some radioactive elements and have low penetrating power and short range. The most energetic alpha particles will generally fail to penetrate the dead layer of cell covering the skin. Alpha particles are hazardous when an alpha–emmitting isotope is inside the body.

Gamma Radiations are the most widely used type of ionizing radiation as they have good penetrating effect upto 20 cm in food depending on exposure time and emitted in all directions continuously. These are produced

by exposure of natural Cobalt-59 to neutrons in a reactor where reaction between the two species produces Cobalt-60. Cobalt-60 is specifically manufactured, for radiotherapy, medical device sterilization and food irradiation and is not a waste product of nuclear reactors.

Beta Particle is a charged particle emitted from a nucleus during radioactive decay. It has mass equal to 1/1837 that of a proton. A negatively charged beta particle is identical to an electron and a positively charged beta particle is called a positron. Large amount of beta radiations may cause skin burns and beta emitters are harmful if they enter in the body. Beta particle may be stopped by thin sheets of metal or plastics. The lethal doze for vegetative bacteria is 0.50-10 Kgy, bactrial spores is10-50 Kgy, human beings and animals is 0.005-0.01 Kgy and for insects is 0.1-1.0 Kgy. There are many benefits of irradiations. These are to reduce or eliminate harmful food borne pathogens e.g. *E.coli* O157:H7, *Camplyobacter*, *Salmonella*, *Trichinella*, *Listeria* and many others, delays ripening of fruits and vegetables, eliminates insects in fruits and vegetables, inhibits sprouting in onions, potatoes, etc (Figure 13.1). These irradiations also replace the need for chemical fumigation. This method is cheaper than freezing and refrigeration.

Fig.13.1: Sprout inhibition in bulbs and tubers (0.03 - 0.15 kGy)

Irradiation dose reduced microbial spoilage (1.5 - 3 kGy) and eliminated pathogenic microbes (3-7 kGy) to improve shelf-life of meat, poultry and sea foods under refrigeration. It also reduced number of microorganisms in spices to improve hygienic quality (10 kGy)

13.3.3 Food Irradiation Processes

Radurization: (0.75-2.5 Kgy)

This method mimics pasteurization. It inhibits sprouting and delays ripening, kills insects and it is used for shelf life extension,

Radicidation (2.5-10KGy)

This method will eliminate spoilage microorganisms and non spore forming pathogens. Food will not get spoiled but still may contain some pathogens

Radapperization (10-50 kGy)

This method is also called **radiation sterilization**. Here reduction of microorganisms occurs to the point of sterility.

Effect of Radiation on Microorganisms : Gram negative bacteria are generally more sensitive than Gram positive forms, bacterial spores are strongly resistant, yeasts tends to be rather more resistant than molds and smallest viruses required doses of >200 kGy to achieve a million-fold reduction in their numbers. The principal

targets of irradiation are nucleic acids and membrane lipids. Approximate Killing Doses of Ionizing Radiations in Kilorays (kGy for various organisms are shown in Table 13.1

Table-13.1: Approximate Killing Doses of Ionizing Radiations in Kilorays (kGy)

Organism	Approximate lethal dose (kGy)
Viruses	10 to 40
Yeasts (fermentative)	4 to 9
Yeasts (film)	3.7 to 18
Molds (with spores)	1.3 to 11
Bacteria	2-25
Mycobacterium tuberculosis	1.4
Staphylococcus aureus	1.4 to 7.0
Bacillus stearothermophilus (Spore)	10 to 17

13.4. pH CONTROL

Almost every food, with the exception of egg whites and soda crackers, has a pH value of less than 7. Foods can be broadly categorized on the basis of their pH as high acid, acid, medium acid or low acid. Examples of each category include:

· **High acid (pH: 3.7)** : apples, lemons, raspberries

· **Acid (pH: 3.7 to 4.6)** : oranges, olives, tomatoes (some)

· **Medium acid (pH: 4.6 to 5.3)** : bread, cheese, carrots

· **Low acid (pH: over 5.3)** : meat, fish and most vegetables

Most micro-organisms grow best in the pH range of 6.5 to 7.5. Yeasts and moulds are capable of growing over a much broader pH range than bacteria. Most spoilage yeast and molds grow at pH value greater than 2.0. Few pathogens will grow below pH 4.0. This information is important, because it will help in determining food stability with respect to microbial spoilage.

13.5. Osmotic pressure

The principle of osmosis is applied. Foods are preserved by adding salts and sugars to them. These chemicals remove the water out of microbial cells causing them to shrink in hypertonic environment, thus stopping their metabolism. Jams, jellies, fruit syrups, honey etc. are preserved by high sugar concentration. Fish, meat beef and vegetable products are preserved with salt.

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Lesson-14
Chemical Methods

14.1. INTRODUCTION

Preservatives are substances that limit, slow down or stop the growth of microorganisms (bacteria, yeasts, molds) or present within the food and thus prevent the deterioration of the goods or food poisoning. They are used in cooked food, wine, cheese, fruit juices and margarines. Chemical preservatives prevent microbial growth without loss of nutrient but some people are sensitive to some chemical preservatives. Chemical food preservatives are the best and the most effective for a longer shelf life and are generally fool proof for the preservation purpose. Chemical substances are the artificial preservatives that stop or delay the growth of bacteria, spoilage and its discoloration. These artificial preservatives can be added to the food or sprayed on the food. Several kinds of chemicals can be used for food preservation including **propionic acid, sorbic acid, benzoic acid** , and **sulfur dioxide.** These acids are acceptable because they can be metabolized by the human body. Acids such as citric acid, acetic acid (vinegar) and ascorbic acid are also known to confer protection against product deterioration. In these cases, the pH of the product is shifted to being low, that is, more acidic, where very few moulds, yeast and bacteria are able to grow and multiply. Food additives such as benzoate and sorbate are quite commonly used in the fruit drink industry to protect against microbial spoilage, while nitrites are used in meat processing. The most commonly used preservatives are the acids such as sorbic acid, benzoic acid and propionic acid. These check mainly the growth of yeasts and molds. Sorbic acid is used for preservation of syrups, salads jellies and some cakes. Benzoic acid is used for beverages, margarine, apple cider etc. Propionic acid is an ingredient of bread and bakery products. Sulphur dioxide, as gas or liquid is also used for dried fruits, molasses and juice concentrates. Ethylene oxide is used for spices, nuts and dried fruits. Some **antibiotics** can also be used, depending upon local laws and ordinances. Tetracycline, for example, is often used to preserve meats. Storage and cooking normally eliminates the last remnants of antibiotic. Antioxidants are also the chemical food preservatives that act as free radical scavengers. In this category of preservatives in food comes the vitamin C, BHA (butylated hydroxyanisole). Unlike natural food preservatives some of the chemical food preservatives are harmful. Sulfur dioxide and nitrites are the examples. Sulfur dioxide causes irritation in bronchial tubes and nitrites are carcinogenic. All of these chemicals act as either antimicrobials or antioxidants or both. They either inhibit the activity of or kill the bacteria, molds, insects and other microorganisms. Antimicrobials prevent the growth of molds, yeasts and bacteria and antioxidants keep foods from becoming rancid or developing black spots. They suppress the reaction when foods come in contact with oxygen, heat and some metals. They also prevent the loss of some essential amino acids and some vitamins. These substance works on the principle of controlling water activity, pH and osmotic pressure of food.

14.2 WATER ACTIVITY

Water is the most important factor in controlling the rate of deterioration of a food. It is the availability of water for microbial, enzymatic, or chemical activity that determines the shelf life of foods. This water availability is measured as water activity (a_w). a_w of milk is 0.97, honey is 0.5-0.7 and dried fruits is 0.5-0.6. Food spoilage micro-organisms, in general, are inhibited in food where the water activity is below 0.6. However, if the pH of the food is less than 13.6, micro-organisms are inhibited when the water activity is below 0.85. The water activity (a_w) values for inhibition of some of the microorganism are *Cl.botulinum 0.97* , Ps. *fluorescence* 0.97,*E.coli* 0.95, *B.cereus* 0.93 and *S.aureus* 0.86. Generally, Intermediate moisture food (IMFs) possesses water activities that range from 0.6 to 0.85. This enables the food to be stable at room temperature, because the growth of most micro-organisms is inhibited at these levels. Binding the water that's present preserves intermediate moisture foods, for example, cookies, cake and bread. This reduces the availability of the water for deteriorative reactions. Water is immobilized by adding permissible humectant additives such as

glycerol, glycols, sorbitol, sugars and salts. The addition of some chemicals inhibits microbial growth in foods. These chemicals include not only those classified as preservatives . Salt, sugars, wood smoke and some spices also inhibit the growth of micro-organisms.

14.3. TYPES OF ARTIFICIAL/CHEMICAL FOOD PRESERVATIVES

These are generally grouped as :

1. Antimicrobial agents
2. Antioxidants
3. Chelating agent

In antimicrobial the benzoates, sodium benzoate, sorbates and nitrites are generally used. Antioxidants include the Sulfites, Vitamin E, Vitamin C and Butylated hydroxytoluene (BHT) and the c helating agent like the disodium ethylenediaminetetraacetic acid (EDTA), polyphosphates and citric acid.

14.3.1. Some common chemical preservatives

a) **Nitrates and nitrites:** Nitrates are converted to nitric acids which form stable red colour in meat. Nitrites have inhibitory action against *Clostridium botulinum* in meat products but forms carcinogenic nitrosamine. It is also used to preserve meats such as sausage, ham, bacon, beef, etc. The side effects are: allergy, asthma, nausea, vomiting and headaches and sodium nitrite can be converted to nitrous acid in the body and cause cancer

b) Sulfites (sulfur dioxide and metabisulfite): Sulfites form sulphurous acid which is active antimicrobial compound and can kill the microbial cell by reduction of sulphide linkage, formation of carbonyl compounds and inhibiting the respiratory mechanism. These are used to prevent fungal spoilage and browning of peeled fruits and vegetables.The commonly used level of sulphites is 0.005 -0.2% . The side effects are allergy , asthma, nausea, vomiting, joint pain, palpitation, and headaches.

c) **Sodium benzoate or benzoic acid:** They are more effective against yeast and molds and are used at a concentration of upto 0.2%. The action of benzoates against microorganisms are by inhibiting enzymes necessary for oxidative phosphorylation, inhibiting membrane protein function and also by destroying membrane potential. They are added to carbonated drinks, margarine, flour, pickles, fruit purees, and fruit juices. The side effects are severe allergic reaction and cancer.

d) **Propionates:** Calcium and sodium propionate are effective against mold and bacteria at 0.1-0.2% but not effective against yeast at this concentration. The inhibitory action is due to cytoplasm acidification and destabilization of membrane proton gradient.

e) **Sorbate:** Sodium, calcium or potassium salt is used at the concentration of 0.05-0.2%. It is more effective against yeast and mold than bacteria. The activity of sorbic acid increases as the pH decreases. Sorbic acid and its salts are tasteless and odourless when used at levels below 0.3%. It is used in non-alcoholic drinks, alcoholic drinks, processed vegetables and fruits and dairy desserts.

f) **Butylated Hydroxytoluene (BHT) and Butylated Hydroxyanisole (BHA):** They serve as antioxidants that prevent the oxidation of fats (rancidity). They are used in preserving fresh meat, pork, sausages, potato chips &crackers, beer, baked goods, drink powder, dry cereals, and frozen pizza. These compounds have several side effects and they can cause cancer and liver disease.

g) **Mono-glycerides and Diglycerides:** They are used as preservatives for cookies, cakes, pies, bread, peanut butter, roasted nuts, shortening, and margarine. They may cause cancer and birth defects.

Salt and sugar: Salt and sugar have long been used as effective means of extending shelf life of various products as these solutes bind water, leaving less water available for the growth of microorganisms. Essentially the water activity (a_w) of the product is reduced. Since most microorganisms require a high water activity, they are unable to survive.

Table-14.1: Examples of commonly used preservatives in different foods

Chemical Preservatives	Foods in Which they are used
Sorbic acid and sorbates	Cheeses, wines, dried fruits, fruit purees, trimmings
Benzoic acid and benzoates	Pickled vegetables, jams and jellies low in carbohydrates, fruits, semi-preserved fish products, sauces
Sulfur dioxide and sulphites	Inhibit bacterial growth in the dried fruit, canned fruit and wine. They also have antioxidant properties
Natamycin	Surface treatment of cheese and sausages
Nitrites and nitrates	Employees in the preparations of meat (ham, sausages etc.) To prevent the growth of *Clostridium botulinum*.

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Lesson-15

Natural Antimicrobial Compounds

15.1 INTRODUCTION

Natural foods preservatives have been used and recognized to mankind since long time. Apart from the preservatives, in all-natural preservation, freezing, pickling, deep frying, salting and smoking also come. Organic substances such as salt, sugar, vinegar and alcohol. These are utilised in both raw and cooked food stuff to increase the shelf value of food so that aroma, taste as well as the foods itself may be stored for a longer period of time. Some preservatives targets enzymes in fruits and vegetables that continue to metabolize after they are cut. For instance, citric and ascorbic acids from lemon or other citrus juice can inhibit the action with the enzyme phenolase which turns surfaces of cut apples and potatoes brown.

Natural Food Preservatives have many advantages. They do not alter the color of the food and gives the required flavour. Artificial preservatives are responsible for causing a lot of health trouble pertaining to respiratory tract, heart, blood and other. There is no main health concern associated with the use of natural food preservatives. These compounds naturally do the process of osmosis and are completely safe for consumption.

15.2.1 Activated lactoferrin (ALF, Activin)

Lactoferrin is an antimicrobial protein present as normal component of fresh milk. It has antibacterial, antifungal and antiviral activity.It also occurs in saliva, tears, and some other body fluids. Activin is activated lactoferrin and is a more potent antimicrobial than plain lactoferrin. It has been accorded GRAS status by the U.S. Food and Drug Administration. It has antimicrobial activity due to its capacity to chelate Fe^{2+} along with HCO^{-3}. It binds to cell surfaces and has a high affinity for the outer membrane proteins (OMP) of Gram-negative bacteria. It also inhibits growth and neutralizes endotoxins. It has been approved at a level of 65.2 ppm for beef carcasses, and may be applied either as a mist or by spraying. It is not acidal agent but acts primarily by preventing pathogens from establishing a niche on meat surfaces.

15.2.2 Ozone (O 3)

This gaseous compound possesses antimicrobial activity. Like chlorine, it is the most powerful oxidant available for conventional water treatment and is highly reactive. It is 1.5 times more potent than chlorine. It is effective in solution and in its gaseous form as it is unstable it must be generated on site and used. Because it is more effective in killing *Cryptosporidium parvum* than chlorine, its use in water treatment systems is increasing. It is normally supplied from ozone generators. The cell target for O_3 is the membrane where it disrupts permeability functions. Ozone is GRAS for bottled water use, and for use on a variety of fresh foods, but its strong oxidizing power does not recommend its use for red meats. A typical concentration used is 0.1-0.5 ppm, which is effective against Gram-positive and Gram-negative bacteria as well as viruses and protozoa. Ozone treatment can be used in vegetables, fruits, beef etc. to destroy pathogens like *E.coli* O157:H7, *S. typhimurium, Giardia lamblia,* etc.

15.2.3 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is a strong oxidizing agent and it is formed to some extent by all aerobic organisms, and it is enzymatically degraded by the enzyme catalase :

$2H_2O_2 \rightarrow 2H_2O + O_2$

It is used as a sterilant for food-contact surfaces of olefin polymers and polyethylene in aseptic packaging systems. Hydrogen peroxide vapors have microbiocidal properties. The antimicrobial effect of hydrogen peroxide attributes to a strong oxidizing effect on the bacterial cells and to the destruction of basic molecular structure of cellular proteins. H_2O_2 also prevent spores of *Bacillus cereus* from swelling properly during the germination process. It is used in the treatment of vegetables, fruits and fruit juice in the form of vapors.

15.2.4 Sodium chloride and sugars

These compounds are grouped together because of the similarity in their modes of action in preserving foods. NaCl has been employed as a food preservative since ancient times. The early food uses of salt were for the purpose of preserving meats. This use is based on the fact that at high concentrations, salt exerts a drying effect on both food and microorganisms. Salt (saline) in water at concentrations of 0.85–0.90% produces an isotonic condition for non-marine microorganisms. Because the amounts of NaCl and water are equal on both sides of the cell membrane, water moves across the cell membranes equally in both directions. When microbial cells are suspended in high salt concentration such as 5% saline solution, the concentration of water is greater inside the cells than outside. In diffusion, water moves from its area of high concentration to its area of low concentration. In this case, water passes out of the cells at a greater rate than it enters. The result to the cell is plasmolysis, which results in growth inhibition and possibly death. This is essentially what is achieved when high concentrations of salt are added to fresh meats for the purpose of preservation. Both the microbial cells and those of the meat undergo plasmolysis (shrinkage), resulting in the drying of the meat, as well as inhibition or death of microbial cells. Enough salt must be used to effect hypertonic conditions. The higher the concentration, the greater are the preservative and drying effects. In the absence of refrigeration, fish and other meats may be effectively preserved by salting. The inhibitory effects of salt are not dependent on pH, as are some other chemical preservatives. Most nonmarine bacteria can be inhibited by 20% or less NaCl, whereas some molds generally tolerate higher levels. Organisms that can grow in the presence of and require high concentrations of salt are referred to as halophiles; those that can withstand but not grow in high concentrations are referred to as halodurics. Sugars are involved in the preservation of food. Sugars, such as sucrose, exert their preserving effect in essentially the same manner as salt. One of the main differences lies in relative concentrations. It generally requires about six times more sucrose than NaCl to affect the same degree of inhibition. The most common uses of sugars as preserving agents are in the making of fruit preserves, candies, jams, jellies, fruit juices, condensed milk etc. The shelf stability of certain pies, cakes, and other such products is due in large part to the preserving effect of high concentrations of sugar, which, like salt, makes water unavailable to microorganisms. Microorganisms differ in their response to hypertonic concentrations of sugars, with yeasts and molds being less susceptible than bacteria. Some yeasts and molds can grow in the presence of as much as 60% sucrose, whereas most bacteria are inhibited by much lower levels. Organisms that are able to grow in high concentrations of sugars are designated osmophiles; osmoduric microorganisms are those that are unable to grow but are able to withstand high levels of sugars. Some osmophilic yeasts such as *Zygosaccharomyces rouxii* can grow the presence of extremely high concentrations of sugars.

15.2.5 Flavoring agents

Many flavoring agents possessing definite antimicrobial effects are used in foods. Flavor compounds generally have more antifungal activity than antibacterial. The non-lactic, Gram-positive bacteria are the most sensitive, and the lactic acid bacteria are rather resistant. The minimal inhibitory concentrations (MIC) of many flavoring compounds are 1,000 ppm or less against either bacteria or fungi. All were pH sensitive, with inhibition increasing as pH and temperature of incubation decreased. Diacetyl is one of the most effective flavoring agents, which is produced by *lactobacillus leuconostoc* and *strectococus*. It is somewhat unique in being more effective against Gram-negative bacteria and fungi than against Gram positive bacteria diacetyl reacts with the arginine bingding proteins of gram negative bacteria. 2, 3-pentanedione is inhibitory to a limited number of

Gram-positive bacteria and fungi at 500 ppm or less. Menthol, which imparts a peppermint like aroma inhibits *S. aureus* at 32 ppm, and *E. coli* and *C. albicans* at 500 ppm. Vanillin and ethyl vanillin are inhibitory, especially to fungi at levels <1,000 ppm.

15.2.6 Spices and essential oils

Many spices possess significant antimicrobial activity. The antimicrobial activity is due to specific chemicals or essential oils, Such as eugenol and Allicin. Antimicrobial substances vary in content from the allicin of garlic (with a range of 0.3–0.5%) to eugenol in cloves (16-18%). Cinnamon and clove oils are also highly effective against *Aspergillus parasiticus* aflatoxin production. Plant EOs such as cumin, caraway and coriander have inhibitory effects on organisms such as *Aeromonas hydrophila*, *Pseudomonas fluorescens* and*Staphylococcus aureus*. Basil has high activity against *B. cereus*, *Enterobacter aerogenes*, *Escherichia coli*, and *Salmonella*. Lemon balm and sage EOs have adequate activity against *L.monocytogenes* and *S. aureus.* Oregano and thyme EOs had comparatively high activity against enterobacteria. Minimum inhibitory concentration (MIC) of oregano and thyme at a range of 190 ppm and 440 ppm, respectively for *E. cloacae* and for lactic acid bacteria MIC of oregano and thyme at a range of 5 ppm and 440 ppm, respectively,

15.2.7 Phosphates

These salts are commonly added to certain processed meats to increase their water holding capacity. They also contribute to flavor and antioxidative activity. Food-grade phosphates range from one phosphate (e.g. trisodium phosphate) to at least 13 (sodium polyphosphate). They possess antibotulinal activity, especially when combined with nitrites. Combination of 140 ppm $NaNO_2$, 0.26% potassium sorbate, and 0.14% sodium acid pyrophosphate (SAPP) delay *C. botulinum* neurotoxin production .

15.2.8 Fatty acids and esters

Acetic, propionic, and sorbic acids are short-chain fatty acids used as preservatives have antimicrobial activity. The fatty acids and esters have a narrow range of effectiveness and GRAS substances such as EDTA, citrate, and phenolic antioxidants also have limitations as antimicrobial agents when used alone. Although EDTA possesses little antimicrobial activity by itself, it renders Gram-negative bacteria more susceptible by rupturing the outer membrane and thus potentiating the effect of fatty acids or fatty acid esters.

15.2.9 Acetic and lactic acids

The organic acids are commonly used as preservatives. These acids are present in the fermented foods such as pickles, sauerkraut, and fermented milks due to their production within the food by lactic acid bacteria. lactic acid bacteria, produce acetic, lactic, and propeonic acids during fermentation. These acids posses' antimicrobial effect which is due to both the depression of pH below the growth range and specific toxicity by the undissociated acid molecules. Lactic acid function as a permeabilizer of the outer membrane of Gram-negative bacteria and thus possibly acts as a potentiator of other antimicrobials. Organic acids are employed to wash and sanitize animal carcasses after slaughter to reduce their carriage of pathogens and to increase product shelf life.

15.2.10 Antibiotics

Antibiotics are secondary metabolites produced by microorganisms that inhibit or kill a wide spectrum of other microorganisms. These antibiotics are produced by molds and bacteria of the genus *Streptomyces*, penicillium and a few by *Bacillus* . S ubtilin and tylosin have been used as heat adjuncts for canned foods, Chlortetracycline and oxytetracycline at 7ppm concentration has been applied to poultry and natamycin is used as a food fungistat. Tetracycline has been permitted for fresh and other see foods. The antibiotics may be applied as a dip or an ice.

Antimicrobial peptides

Antimicrobial peptides were first isolated from natural sources in the 1950s when nisin was isolated from lactic acid bacteria for potential application as a food preservative. Subsequently, antimicrobial peptides were isolated from other natural sources, such as plants, insects, amphibians, crustaceans, and marine organisms. Antimicrobial peptides (AMPs) are widely distributed in nature and are used by many if not all life forms as essential components of nonspecific host defence systems. Antimicrobial peptides present a promising solution to the problem of antibiotic resistance because, unlike traditional antimicrobial agents, specific molecular sites are not targeted, and their characteristic rapid destruction of membranes does not allow sufficient time for even fast-growing bacteria to mutate. Lactoferrin bovine and activated lactoferrin (ALF), an iron binding glycoprotein present in milk, has antimicrobial activity against a wide range of Gram-positive and negative bacteria, fungi, and parasites. Lactoferrin has been applied in meat products and approved for their application in preservation of beef in USA.

Biopreservation is the use of natural or controlled microbiota or antimicrobials as a way of preserving food and extending it's shelf life . Beneficial bacteria or the fermentation products produced by these bacteria are used in biopreservation to control spoilage and render pathogenic inactive in food. It is a benign ecological approach which is gaining increasing attention and lactic acid bacteria (LAB) are the important among them. Lactic acid bacteria have antagonistic properties which make them particularly useful as biopreservatives. When LABs compete for nutrients, their metabolites often include active antimicrobials such as lactic and acetic acid, hydrogen peroxide, and peptide bacteriocin. Some LABs produce the antimicrobial nisin which is a particularly effective preservative. These days LAB bacteriocins are used as an integral part of hurdle technology. Using them in combination with other preservative techniques can effectively control spoilage bacteria and other pathogens, and can inhibiting the activities of a wide spectrum of organisms, including inherently resistant Gram-negative bacteria.

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Lesson-16

Emerging Methods of Food Preservation

16.1 INTRODUCTION

The need for enhancing microbial food safety and quality, without compromising the nutritional, functional and sensory characteristics of foods, has created an increasing world-wide interest in low-temperature innovative processes for food preservation. In contrast, to the traditional thermal processes, these emerging technologies are predominantly reliant on physical processes, including high hydrostatic pressures, pulsed electric fields and low-temperature plasmas, high hydrostatic pressure, oscillating magnetic field, ultra sounds that inactivate microorganisms at ambient or moderately elevated temperatures and short treatment times. The relatively slow commercial implementation of ionizing radiation (gamma and high energy electron beams) to meet the needs of the international food industry for food preservation has, by and large, been associated with concerns about consumer responses to use of these technologies. Inherent disadvantage of thermal processing like loss of original flavor taste, color and nutritional quality of foods have embraced the uses of high pressure, high power ultrasonics, pulsed ultraviolet light, pulsed electric fields and more recently low-temperature plasma of gases, these techniques render food free of pathogens and spoilage organisms and improve shelf life and texture of foods.

16.2 HIGH PRESSURE PROCESSING

High Pressure Processing (HPP) is an emerging food treatment that makes food safer and extends its shelf life, while allowing the food to retain many of its original qualities and healthy attributes. High pressure processing is a non thermal processing. It was first commercialized in Japan in the early 1990s for pasteurization of acid foods for chilled storage. HHP subjects liquid or solid foods with or without packaging to pressure between 40 and 1000MPa for 1-20 min. The mechanism of action of HPP to breakdown the non covalent bonds and puncturing or the permeabilization of cell membrane i.e. vegetative cells at 300 MPa at room temperature (Figure 16.1). Spore formers >600 MPa at 60-70^{0}C, some enzymes at 300 MPa but effect is less at below 40% food moisture. Hydrostatic pressure is applied to food products through a water bath that surrounds the product. The hydrostatic pressure is transmitted to food products equally from all sides. This equal distribution of pressure is the reason why foods are not crushed during treatment.

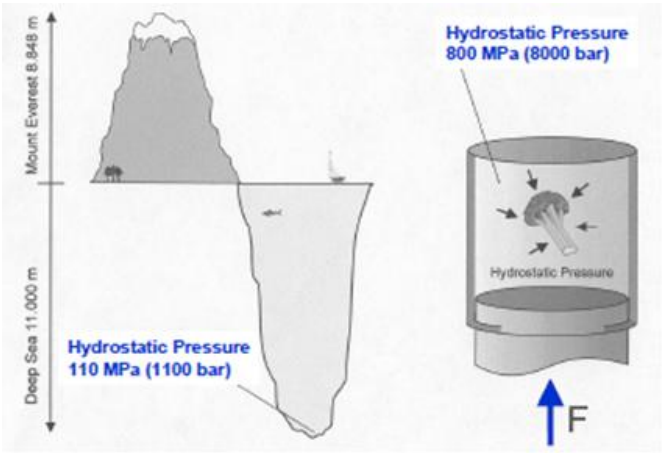

Fig. 16.1 High pressure process

High Pressure Processing (HPP) has been used with hundreds of products, and can reliably: Inactivate food borne pathogens, Inactivate spoilage organisms, Activate or inactivate enzymes, Germinate or inactivate some bacterial spores, Marinate meats, Shuck oysters, Extend shelf life, Reduce the potential for food borne illness, Pressure-shift freezing or thawing, Promote ripening of cheeses, Minimize oxidative browning

HPP products currently being marketed worldwide such as abalone, apple cider, apple juice, apple sauce (single serving packs), avocado (halves, pulp), beef, chicken, cod (both dried and salted), fruit purees, fruit smoothies, guacamole, jams/jellies, limeade, mussels, onions (chopped), orange Juice etc. The cell morphology is not significantly affected at the lower pressures, although membrane integrity is damaged. At elevated pressures, (500 - 700 MPa) progressive morphology changes become evident. Orange juice processed at 438M Pa for 60 seconds. 7log reduction of pathogens (E.Coli, Salmonala) can be obtained. Similarly, in case of meat process at 600M Pa, 3-4 log reduction of L.monocytogenes can be done.

16.3 PULSED ELECTRIC FIELD (PEF) PROCESSING

PEF utilizes high intensity electric field pulses to inactivate microorganisms mainly in liquid foods at relatively low or moderate temperatures (<60^0C), whilst preserving the fresh flavour, color and integrity of heat sensitive components. A typical PEF food processing unit comprises of a high voltage pulse generator, a treatment chamber, a fluid handling system and control and monitoring devices (Figure 16.2). Depending on the particular PEF systems used, typical PEF treatment parameters include pulsed field intensity of 15-50 kV cm^{-1}, pulse width of 1-5 ms, and pulse frequency of 200-400 Hz (pulses/s). PEF treatment at an electric field intensity greater than a critical threshold of trans-membrane potential of 1 V across the target cells causes irreversible pore formation and destruction of the semi-permeable barrier of the cell membrane and structural changes in enzymes. PEF treated bacterial cells substantially damaged at the cellular level. PEF treatment at up to 25 kV cm^{-1} and 35^0C for 400 ms caused less than 1 log reduction in *E. coli* O157:H7 in apple juice. Examples of pulse field processed foods are apple juice, milk, orange juices, green pea soup etc. PEF processing is restricted to foods that do not contain air bubbles and have low electrical conductivity. PEF is not suitable solid foods can not be pumped. PEF and thermal processing in combination lower the temperature of pasteurization and improve the quality of food

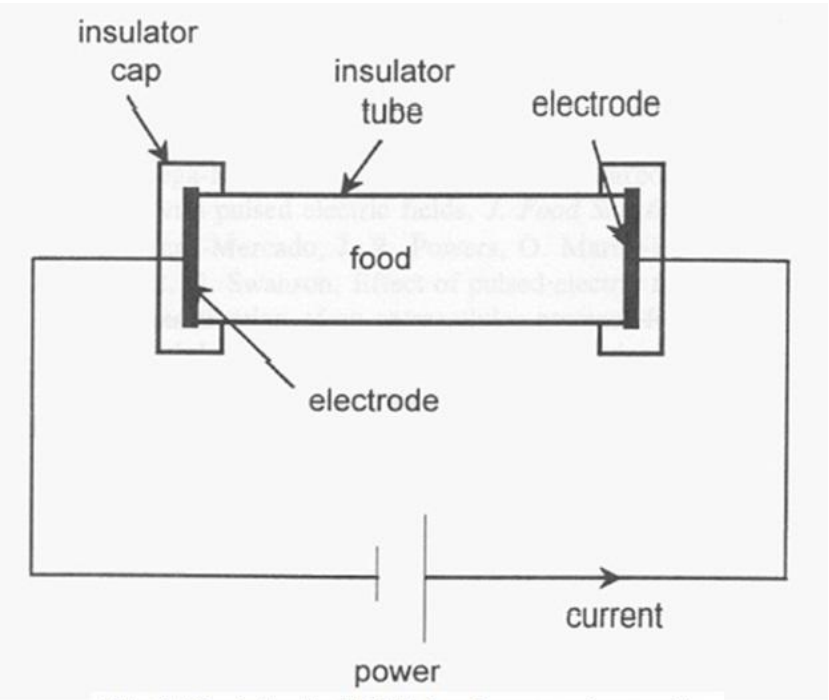

Fig.16.2: A typical PEF food processing unit

16.4 ULTRASONIC FOOD PROCESSING

Ultrasound is an efficient non-thermal alternative. Ultrasonic cavitation creates shear forces that break cell walls mechanically and improve material transfer. Generally, ultrasound equipment uses frequencies from 20kHz to 10MHz. Higher-power ultrasound at lower frequencies (20–100kHz), which is referred to as ‘‘power ultrasound’’, has the ability to cause cavitation that could be used in food processing to inactivate microorganisms. Low frequency ultrasound refers to pressure waves with a frequency of 20 kHz or more. Ultrasonic waves generate gas bubbles in liquid media, which produce a high temperature and pressure increase when they immediately burst. When the bubbles produced during ultrasonic treatment collapse, the compression/expansion cycles generated are thought to be responsible for cell disruption, microbial and enzyme inactivation in preservation of fruit juices and sauce. Ultrasound has potential to destruction of food borne pathogens like *E.coli, Salmonella, Giargia, poliovirus* etc. This method has application in the preservation of jam, marmalade or toppings e.g. for ice cream, fruit juices and sauces, meat products and dairy.

16.5 OHMIC HEATING OF FOODS

Ohmic heating (sometimes also referred to as Joule heating, electrical resistance heating, direct electrical resistance heating, electro heating, and electro conductive heating) is defined as a process wherein (primarily alternating) electric currents are passed through foods or other materials with the primary purpose of heating them. The heating occurs in the form of internal energy generation within the material. Ohmic heating is an advanced thermal processing method wherein the food material, which serves as an electrical resistor, is heated by passing electricity through it. Electrical energy is dissipated into heat, which results in rapid and uniform heating. Ohmic heating is also called electrical resistance heating, Joule heating, or electro-heating, and may be used for a variety of applications in the food industry. Ohmic heating can be used for heating liquid foods containing large particulates, such as soups, stews, and fruit slices in syrups and sauces, and heat sensitive liquids. The technology is useful for the treatment of proteinaceous foods, which tend to denature and coagulate when thermally processed. At low-frequency (50–60 Hz), electrical charges can build up and form pores across microbial cells and causes death of those microbial cells.

16.6 INTENSE PULSE LIGHT

Pulsed light (PL) is a technique to decontaminate surfaces by killing microorganisms using pulses of an intense broad spectrum, rich in UV-C to near IR (180-1100nm) which is produced using xenon discharge lamp light (Figure 16.3). One pulse is 1-20 flashes/seen for duration of 1µs to 0.15, energy density is 0.01- 50 J/cm^2. PL kills microorganisms using short time high frequency pulses of an intense broad spectrum, rich UV-C light. The germicidal effect of UV light on bacteria is primarily due to the formation of pyrimidine dimers, mainly thymine dimers. The dimer inhibits the formation of new DNA chains in the process of cell replication, thus resulting in the inactivation (inability to replicate, called clonogenic death) of affected microorganisms by UV. On bacterial spores, UV-C treatment results mainly in the formation of the ‘‘spore photoproduct’’ 5-thyminyl-5,6-dihydrothymine, and in single-strand breaks, double-strand breaks and cyclobutane pyrimidine dimers. PL has been used to successfully inactivate *Escherichia coli* O157:H7 on alfalfa seeds and *Aspergillus niger* spores on corn meal. Surface microorganisms are controlled in various foods. Such as meat products, cheese, baked goods, fish, shrimp, reduction of Pseudomonas on dry cottage cheese, inactivation of spoilage and pathogens in milk.

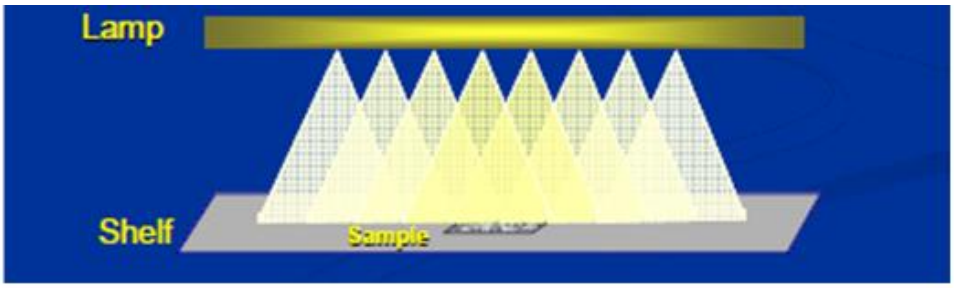

Fig. 16.3. Pulse light technology

16.7 Plasma Light

Plasma is defined as a neutral ionised gas. It is constituted by particles in permanent interaction. The particles include photons, electrons, positive and negative ions, atoms, free radicals and excited or non-excited molecules (Figure 16.4). Electrons and photons are usually designed as "light" species in contrast to the other constituents defined as "heavy" species. Consequently, the term "plasma" is considered to describe a state of matter in which the heavy species are neutral or ionised particles which result from an energetic transfer to a gas. Non-thermal plasma technology relies on electric discharge into air or liquid to produce energetic atoms, highly reactive radicals, ozone, etc., that can kill microbes in contact. In non-thermal plasma, electric energy is mostly used to generate non-thermal plasma species instead of heat. Therefore, this technology is energy-efficient and will cause minimal heat-induced damages to food products. Potential applications include pasteurization of liquid food products, produce wash, disinfection of processing equipment, plant floors, and packaging materials, city water and wastewater treatment and air pollution control. The primary advantages of plasma processing as a potential tool in the inactivation of microorganisms are (1) minimal thermal denaturation of nutritional and sensory properties, (2) reduced energy requirement for adequate processing, and (3) potential treatment of foods inside a flexible-film package. Non-thermal plasma was capable of killing *Escherichia coli* and *Salmonellae* in liquid foods. At the flow rate of 1000 mL/min, the 5 logs reduction in the bacteria counts has been achieved. This suggests that non-thermal plasma pasteurization can kill the food borne pathogens such as *Escherichia coli* and *Salmonellae* in liquid foods with minimal damage to active ingredients in foods.

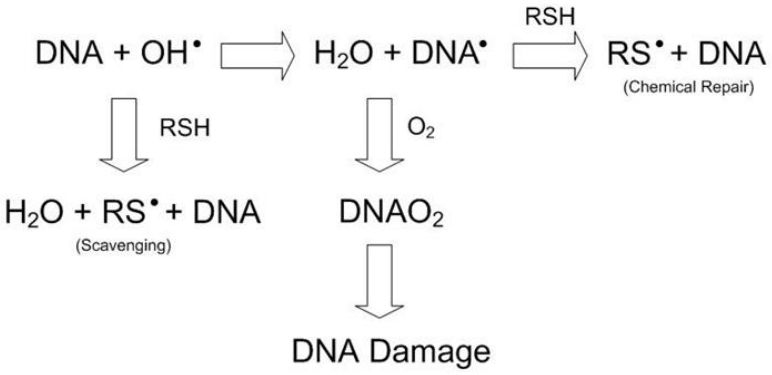

Fig. 16.4: Plasma sterilization mechanism

16.8 Oscillating Magnetic Fields (OMF)

OMF applied in the form of pulses reverses the charge for each pulse, and the intensity of each pulse decreases with time to about 10% of the initial intensity. Preservation of foods with OMF involves sealing food in a plastic bag and subjecting it to 1 to 100 pulses in an OMF with a frequency between 5 to 500 kHz at temperatures in the range of 0 to 50°C for a total exposure time ranging from 25 to 100 ms. Frequencies higher

than 500 kHz are less effective for microbial inactivation and tend to heat the food material. Magnetic field treatments are carried out at atmospheric pressure and at moderate temperatures. The temperature of the food increases 2-5°C. Exposure to magnetic fields causes inhibition in the growth and reproduction of microorganisms. OMF of intensity of 5 to 50T and frequency of 5 to 500 kHz was applied and reduced the number of microorganisms by at least 2-log cycles. Within the magnetic field of 5-50 T, the amount of energy per oscillation coupled to 1 dipole in the DNA is 10^{-2} to 10^{-3} EV. Inactivation of microorganisms may be based on the theory that the OMF may couple energy into the magnetically active parts of large critical molecules such as DNA. Within 5-50 T range, the amount of energy per oscillation coupled to 1 dipole in the DNA is 10^{-2} to 10^{-3} EV. Several oscillations and collective assembly of enough local activation may result in the breakdown of covalent bonds in the DNA molecule and inhibition of the growth of microorganisms. Examples of food preserved with OMF milk, yoghurt and orange juice.

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Lesson 17

Combination Methods of Preservation

17.1 INTRODUCTION

Hurdle technology is a method of ensuring that pathogens in food products can be eliminated or controlled. This means the food products will be safe for consumption, and their shelf life will be extended Leistner in germany referred the combination preservation as hurdle technology. Hurdle technology usually works by combining more than one approach. L. Lestner in germany referred the combination preservation as hurdle technology in 1980's. These approaches can be thought of as "hurdles" the pathogen has to overcome if it is to remain active in the food. The right combination of hurdles can ensure all pathogens are eliminated or rendered harmless in the final product. Hurdle technology has been defined as an intelligent combination of hurdles which secures the microbial safety and stability as well as the organoleptic and nutritional quality and the economic viability of food products .

17.2 COMMON HURDLE TECHNOLOGY

The most important hurdles generally used in food preservation are temperature (high or low), water activity (a_w), acidity (pH), redox potential (Eh), preservatives (e.g., nitrite , sorbate, sulfite), and competitive microorganisms (e.g., lactic acid bacteria). However, more than 60 potential hurdles for foods, which improve the stability and/or quality of the products, have been already described, and the list of possible hurdles for food preservation is by no means complete. Some hurdles (e.g., Millard reaction products) will influence the safety and the quality of foods, because they have antimicrobial properties and at the same time improve the flavour of the products. The same hurdles could have a positive or a negative effect on foods, depending on its intensity. For instance, chilling to an unsuitable low temperature is detrimental to some foods of plant origin ('chilling injury'), whereas moderate chilling will be beneficial for their shelf life. Another example is the pH of fermented sausage which should be low enough to inhibit pathogenic bacteria , but not so low as to impair taste. If the intensity of a particular hurdles in a food is too small it should be strengthened, if it is detrimental to the food quality it should be lowered. By this adjustment, hurdles in foods can be kept in the optimal range, considering safety as well as quality, and thus the total quality of a food. For each stable and safe food a certain set of hurdles is inherent, which differs in quality and intensity depending on the particular product, but in any case the hurdles must keep the 'normal' population of microorganisms in this food under control. The microorganisms present ('at the start') in a food should not be able to overcome ('leap over') the hurdles present during the storage of a product; otherwise the food will spoil or even cause food poisoning .

Severe heat treatments can impair the organoleptic properties and nutritional value of foods. Excessive Low temperature treatment may reduce food quality by destroying food surface (chilling injury) or due to release of enzymes by dead microbes. Too low pH of a food may impair taste of a food. MAP in excess may change color, flavor, and texture of food. Irradiation at high doses can be harmful to food like production of free radicals. Hurdle effect was first introduced in 1978. Leistner and co-workers acknowledged that the complex interactions of temperature, water activity, acidity, redox potential, preservatives etc; are significant for the microbial stability and safety of most foods. In modern food preservation techniques using bacteriolytic enzymes, irradiation, high pressure or pulsed technologies, secondary hurdles are employed to achieve the desired preservation. Pulsed electric fields can be combined with other hurdles such as pH, aw, temperature or preservatives. Effect of high pressure can be improved if combined with heat, antimicrobials or ionizing radiations.

Various factors like the microbial load determines the type and amount of hurdles required.

· If only few microorganisms are present at the start, than a few or low hurdles are sufficient for the stability of the product

· If microbes present are sub lethally injured, they lack vitality & are easier to inhibit by few hurdles

· Food rich in nutrients and vitamins will enhance the growth of microorganisms (booster or trampoline effect), thus the number & intensity of hurdles should be increased

· Water content is an essential component of food, if an increased water activity is compensated by other hurdles (pH, Eh etc.), the food becomes more economical

· If energy preservation is the goal, than energy consuming hurdles such as refrigeration are replaced by other hurdles that do not demand energy but still ensures a stable and safe food

17.2.1. Basic aspects of hurdle technology

17.2.1.1 Homeostasis

Strong tendency of organisms to maintain their internal environment stable and balanced so that homeostasis is in balanced condition. If homeostasis is disturbed by preservative factors (hurdles) in foods, they will remain in lag phase or even die before their homeostasis is re-established. Repair of disturbed homeostasis demands much energy, thus restriction of energy supply inhibits repair mechanism and leads to synergistic effect of preservative factors. Energy restrictions are caused by anaerobic conditions, low a_w, low pH and low redox potential.

17.2.1.2 Metabolic exhaustion

It leads to auto sterilization of foods. Counts of variety of bacteria, yeasts and molds that survive the mild heat treatment decrease quite fast in the products during unrefrigerated storage because the hurdles applied do not allow growth. Microorganisms in hurdle technology foods try every possible repair mechanism for their homeostasis. By doing this, they completely use up their energy and die, that leads to auto sterilization of foods.

17.2.1.3 Stress reactions

Bacteria become more resistant or even more virulent under stress – heat shock proteins. Protective stress shock proteins are induced by heat, pH, Aw, ethanol, starvation etc. Activation of shock protein genes would be more difficult if different stresses are received at the same time.

17.2.1.4 Multi target preservation

A Synergistic effect could be achieved if hurdles in a food hit, at the same time, different targets (e.g., cell membrane, DNA, enzyme system, RNA) within the microbial cell and thus disturb the homeostasis of the microorganisms. Repair of homeostasis as well as the activation of stress shock proteins would become more difficult. Nisin, damages the cell membrane, in combination with Lysozyme and citrate, which then easily penetrate the cell and disturb the homeostasis with different targets.

Example for application of hurdle technology:

The hurdle technology approach is used for non fermented foods like Italian pasta. In this reduced aw, mild heating are principle hurdles with modified atmosphere in packaging and chilling during storage are the hurdles.

Other food items preserved by the hurdle \ technology are foods, dairy products, fish, meat and cereals for shelf stable food preparations.

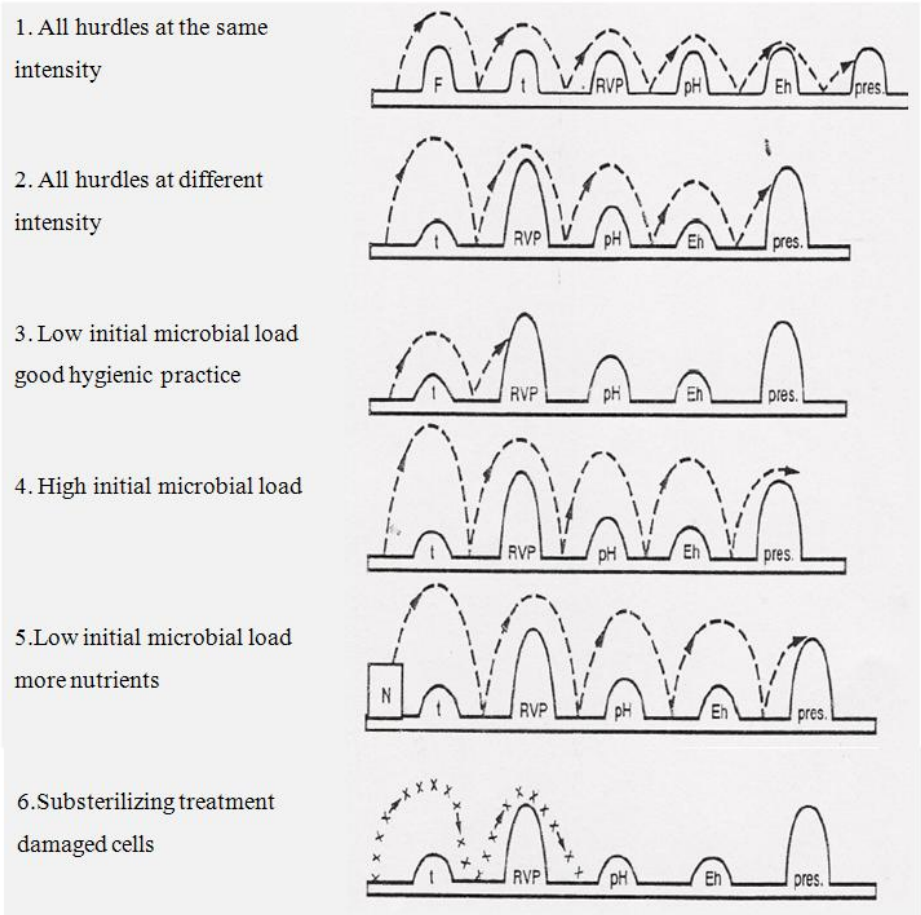

Fig. 17.1: Example for application of hurdle technology

17.3 THE HURDLE CONCEPT

In the hurdle concept, multiple factors or techniques are employed to affect the control of microorganisms in foods. Barrier technology, combination preservation, and combined methods are among some of the other descriptions of this concept. Referred to as "hurdle technology" since the mid-1980s by L. Leistner in Germany, the practice has been applied to some foods for over a century. A simple example of the hurdle concept or barrier technology is demonstrated by preventing the germination of spores of proteolytic or group I strains of *Clostridium botulinum*. Among the intrinsic and extrinsic parameters that are known to prevent their germination and growth are: pH <14.6; *a*w <0.914; NaCl of 10% or more; NaNO2 ca. 120 ppm; incubation temperature<10◦C; and a large aerobic bacterial biota. Foods that employ the hurdle concept in their formulation would embody a series of the above, thus making for a multitargeted approach to preventing germination and growth of these spores. In order for *C. botulinum* to grow, it must "hurdle" a series of the barriers noted. Note that the hurdles listed above include "aerobic bacterial biota", which is microbial interference. The important parameters of pH and a_w may be controlled by the growing food biota, especially the lactic acid bacteria. The concept of growth/no growth (G/NG) has been advanced to better quantify the hurdle concept by employing the synergy that exists between two or more parameters. Implicit in this concept is the interaction between two or more parameters to a point where growth ceases,the G/NG interface. Precise definitions and determinations of those factors/parameters that permit and prevent growth of a given organism should make it possible to devise models for the hurdle concept.

Lesson 18

Biotechnology in Food Preservation

18.1. INTRODUCTION

In response to consumer demand for more natural food preservation methods, biotechnology has been used to find ways of replacing synthetic preservatives in food. Some of the examples of food preservation methods that employ biotechnology include: recombinant antifreeze proteins that extend the shelf life of frozen dairy products and fruits, fermented whey that is high in acids to preserve cheese and rosmarinic acid produced from rosemary plant cell cultures as an oxidant. Moreover the microbial cultures and their metabolites have been used for the preservation of foods.

Since the role of micro-organisms in spontaneous food fermentation processes became clear, man has tried to apply 'controlled' fermentations in order to preserve food products. An increasing number of consumers prefer minimally processed food products, prepared with less or without chemical preservatives. The consumer wants food products to be 'fresh', 'natural', 'healthy' and 'convenient'. Many of the new ready-to-eat and novel food types bring along new health hazards and new spoilage associations. Against this background and relying on improved understanding and knowledge of microbial interactions, milder preservation approaches such as bio preservation have been

18.2. BIOPRESERVATION

Biopreservation or biological preservation can be defined as a preservation method to improve safety and stability of food products in a natural way by using 'desired' microorganisms (cultures) and/or their metabolites without changing the sensory quality

Cultures can be defined as protective or antagonistic micro-organisms that are added to a food product only to inhibit pathogens and/or to extend the shelf-life, while changing the sensory properties of the product as little as possible. These cultures differ from starter cultures in their functional objectives. Starter cultures are, by definition, used in food fermentations in order to modify the raw material to give it new sensory properties and this relying on the metabolic activity (acid production) of the culture, while the preservation effect (antimicrobial action) is of secondary importance. For a protective culture, the functional objectives are the inverse.

Biopreservation can be applied in food products by two basic methods:

Ø Adding crude, semi-purified or purified microbial metabolites;

Ø Adding pure and viable micro-organisms .

The use of micro-organisms or their metabolites as food preservatives is not meant as a primary means of preservation but as a way to contribute to the hurdle approach in food preservation.

18.3 ANTIMICROBIAL METABOLITES OF LACTIC ACID BACTERIA

18.3.1 Organic acids

The most important and best characterized antimicrobials produced by LAB are lactic and acetic acid. The amount and type of acids produced during fermentation influence the subsequent microbial activity in the fermented material. Acetic acid, for example, is more antagonistic against yeasts compared to lactic acid. Some oxidative yeasts are able to utilize organic acids as a carbon and energy source and consequently cause spoilage through deacidification in fermented, especially plant material where they are naturall y present . The inhibitory effect of organic acids is mainly caused by undissociated form of the molecule, which diffuses across the cell membrane towards the more alkaline cytosol and interferes with essential metabolic functions. The toxic effects of lactic and acetic acid include the reduction of intracellular pH and dissipation of the me mbrane potential .

18.3.2 Hydrogen peroxide

Antimicrobial activity of hydrogen peroxide is attributed to its strong oxidizing effect on the bacterial cell and to the destruction of basic molecular structures of cell proteins . In raw milk, hydrogen peroxide produced by lactic acid bacteria can, after being catalyzed by lactoperoxidase, oxidise endogenous thiocyanate. The oxidized intermediary products are toxic to different bacteria . Hydrogen peroxide production has been considered as the main metabolite of LAB that could protect against urogenital infections, especially in the case of bacterial vaginosis .

18.3.3 Carbon dioxide

The influence of carbon dioxide on product preservation is twofold. Namely, except for its own antimicrobial activity, it creates an anaerobic environment by replacing the existent molecular oxygen. The antifungal activity of CO_2 is due to the inhibition of enzymatic decarboxylations and to its accumulation in the membrane lipid bilayer resulting in dysfunction in perm eability .

18.3.4 Reuterin

Reuterin is a pH neutral, water soluble, low molecular weight substance, which is non-bacteriocin and resistant to nuclease, protease and lipolytic enzymes. It is active over a wide range of pH values and capable of inhibiting growth of a wide spectrum of microorganisms, but it is labile to heat (100^0C for 10 minutes). The unique and most attractive feature of reuterin is its strong antimicrobial activity. It has been found that concentrations of reuterin in the range of 15-30 µg/ml effectively inhibit growth of Gram-positive and Gram-negative bacteria, and lower eukaryotic organisms including yeast, fungi and protozoa.

18.3.5 Bacteriocins

The bacteriocins most studied for their biopreservative effect in food products, and more specific in meat and meat products, include nisin, pediocins and sakacins Nisin, produced by *Lc. lactis* subsp. *lactis*, is the only bacteriocin that has found practical application in food products. It is mainly applied in the prevention of late-blowing of cheese by inhibiting the outgrowth of *Clostridium* spores and in selected pasteurised cheese spreads to inhibit *Clostridium* and *Listeria*. Typical levels that are used in food products range from 2.5 to 100 ppm. Pediocins, in particular pediocin PA-1 (also AcH) from *P. acidilactici*, have been used successfully to control growth of *L. monocytogenes* in cottage cheese, half-and-half cream and cheese sauce, raw or fresh meat, cooked meat products and fermented meat products. Pediocin PA-1 was also found to be active towards *L. curvatus* in a

meat product model. In general, the pediocins seem to be more effective in meat products than nisin but they are not approved for use.

18.4 FERMENTATES OF SELECTED ORGANISMS

The Microgard™ products marketed by Danisco are fermentates of *Propionibacterium freundenreichii* subsp. *shermanii* that are commonly used commercially as biopreservatives in cottage cheese. Their inhibitory activity is attributed to propionic acid, acetic acid and a heatstable peptide (Guinane et al., 2005). Alta™ and Perlac™ are fermented whey-based products used as shelf-life extenders.

18.5 BIOPRESERVATION BY MEANS OF PROTECTIVE LACTIC CULTURES

Since lactic acid bacteria are commonly used as starter cultures in food fermentations, investigators have explored the use of bacteriocin producers as protective cultures. Bioprotective culture may act as starter cultures in the food fermentation process or they may protect foods without any detrimental organoleptic changes. Natural bacteriocin producers, such as *Lactobacillus plantarum, Pediococcus acidilactici, Enterococcus faecalis* and *Enterococcus faecium* have been used as protective cultures in various products e.g. the outgrowth of clostridia spores in cheese milk was completely prevented when a nisin A producing strain was mixed at 10% rate with the starter culture. Similarly the application of bacteriocin- producing LAB in the meat industry also offers a promising way of natural food preservation.

Table 18.1 Bacteriocin based bioprotective cultures

BS-10®	Nisin producing *L. lactis* spp. *Lactis, Chris Hansen*
BIOPROFIT™	*L. rhamnosus* LC705, BioGaia
BOVAMINE Meat Cultures™	Texas Tech University
HOLDBAC™ DANISCO	*L. plantarum*, *L. rhamnosus*, *L. sakei*, *L. paracasei* a *Propionibacterium freundenreichii* spp. *Shermanii,*

Table 18.2 Desired properties of a protective LAB-culture

1. No health risks
   - No toxin production
   - No production of biogenic amines or other metabolites detrimental to health
   - Non pathogenic
2. Bring about beneficial effects in product
   - Adaptation to product/storage conditions (e.g. psychrotrophic, salt tolerant)
   - Reliable/consistent protective activity
   - Predictability of metabolic activity under given conditions (e.g. lactic acid/gas production)
   - Competitiveness against endogeneous microbial flora
3. No negative sensory effects on product
   - No slime/gas/acid formation
   - No discolouration
   - No proteolytic or lipolytic activity
4. Function as indicator under abuse conditions

18.7 APPLICATIONS OF PROTECTIVE LAB IN DIFFERENT FOOD PRODUCTS

The effectiveness of Protective culture has been studied in different food products (Table 18.3.). The majority of these inoculation experiments were performed with the intention of demonstrating the effectiveness of bacteriocinogenic strains in controlling *L. monocytogenes.*

Table 18.3 Studies on the effect of non-bacteriocinogenic protective cultures in different types of food products

Protective culture	Target organism	Food product
Lactobacillus sakei TH1	*L. monocytogenes*	Cooked ham and servelat sausage
Lactobacillus casei D6 *Lactobacillus paracasei* I5	*L. monocytogenes*	Frankfurters
Lactobacillus alimentarius (FloraCarn L-2)	Spoilage flora	Cooked ham
Lactobacillus alimentarius (FloraCarn L-2)	*L. monocytogenes*	Ground beef
Lactobacillus alimentarius (FloraCarn L-2)	Ropy slime producing *Lactobacillus sakei*	Frankfurters
Lactobacillus alimentarius (FloraCarn L-2)	*L. monocytogenes*	Bacon cubes
Lactobacillus and *Lactococcus* strains	*B.cereus, L. monocytogenes*	Milk
Lactobacillus reuteri BPL-36	*Salmonella, S. aureus, L. monocytogenes,* Spoilage flora (*Pseudomonas* spp), *E. coli* O157:H7	Milk, Cheese

18.7.1 Milk and Dairy Products

Cheese suffers from spoilage through *Clostridium* spp. (late blowing) and is, furthermore, susceptible to contamination with *L. monocytogenes.* This latter problem arises mainly in cheeses in which the pH increases during ripening, such as the Italian cheeses Taleggio, Gorgonzola and Mozarella. The addition of this paired nisin producing starter system to make cheddar cheese provided enough nisin to increase the shelf life of pasteurised processed cheese, made from this cheddar, from 14 to 87 days at 22°C and to control *L. monocytogenes, Cl. sporogenes* and *S. aureus.* Antilisterial effects were also observed for a bacteriocinogenic *Enterococcus faecium* strain during Taleggio production.

18.7.2 Vegetable products

Bacteriocinogenic LAB are reported to have potential for the biopreservation of foods of plant origin, especially minimally processed vegetables and fermented vegetables. In minimally processed vegetables such as pre-packaged mixed salads and different types of sprouts, bacteriocinogenic LAB have been found to act on coliforms and enterococci and on *L. monocytogenes*). Moreover, bacteriocinogenic starter cultures may be useful for the fermentation of sauerkraut or olives to prevent spoilage. Biocompetitive control or the use of biocompetitive micro-organisms to inhibit mycotoxin forming moulds can be obtained by (1) the use of biocompetitive non-aflatoxinogenic moulds or (2) the use of antagonistic yeasts or bacteria.

18.7.3 Fish, fish products and seafood

Spoilage of fresh fish is generally caused by Gram-negative bacteria. However, when vacuum packaged the spoilage of fresh fish, smoked fish and seafood is dominated by mainly Gram-positive bacteria, in particular LAB, and also *L. monocytogenes* can cause problems. The potential of *Carnobacterium* spp. to control *L. monocytogenes* in cold-smoked salmon, the divercin V41 producing*Carnobacterium divergens* V41 the bacteriocinogenic *Carnobacterium maltaromaticum* (previous *piscicola*) A9b and the non-bacteriocinogenic *C. maltaromaticum* A10a have been used.

18.7.4 Other food products

Bacteriocin-producing and acid producing LAB have applications of in refrigerated ready-to-eat food products, e.g. soups, meals and salads, to prevent them from growth of food born pathogens, in particular *Cl. botulinum* and/or *L. monocytogenes*. A mixture of a nisin-producing *Lc. lactis* and a pediocin A-producing *P. pentosaceus* are effective to prevent growth of *Cl. botulinum* and botulinal toxin formation after 10 days at 10°C.

18.8 FOOD PRESERVATİON THROUGH FERMENTATİON

Fermentation is the process of bioconversion of organic substances by microorganisms and/or enzymes (complex proteins) of microbial, plant or animal origin. It is one of the oldest forms of food preservation which is applied globally. Indigenous fermented foods such as bread, cheese and wine, have been prepared and consumed for thousands of years and are strongly linked to culture and tradition, especially in rural households and village communities. It is estimated that fermented foods contribute to about one-third of the diet worldwide. During fermentation processes, microbial growth and metabolism i.e. the biochemical processes whereby complex substances and food are broken down into simple substances, result in the production of a diversity of metabolites. D uring fermentation breakdown of carbohydrates under limited supply of oxygen or under anaerobic conditions take place e.g. yeasts converts sugar to alcohol and CO_2 . Some aerobic, specific conversions may a lso be referred as fermentation such as *Acetobacter*convert ethylalcohol to acetic acid in the presence of oxygen . In nature, natural fermentations occur continuously. In technically advanced societies, fermented foods are produced to add special tastes to human diet, in less developed areas fermentation is still one of the major preservation methods. In contrast to most preservation methods, fermentation encourages growth and multiplication of selected microorganisms in foods. The application of microorganisms to food preservation practices must be such that a positive protection is available to control contamination. Lactic acid fermentation are of great importance in food preservation. The sugar in foodstuff may be converted to lactic acid and other end products and in such amounts that the environment is controlling over other organisms. Lactic acid fermentation is efficient and the fermenting organisms grow rapid ly . Natural inoculations are such that in a suitable environment the lactic acid bacteria will dominate, as in souring of milk. There is another fermentation which involves much gas production. It is useful in food preservation. In gassy fermentations sugar molecules are altered to form acids, alcohols and carbon dioxide. It is usually necessary to include some other controlling influence, such as adding sodium chloride to a substrate, with this form of fermentation.

Sodium chloride is useful in a fermentation process of foods by limiting the growth of putrefactive organisms and by inhibiting the growth of large numbers of other organisms. Sodium chloride is one of the most important food adjuncts in food preservation. In fermentations salt can exert a role in sorting the organisms permitted to grow. Fermentation is globally applied in the preservation of a range of raw agricultural materials (cereals, roots, tubers, fruit and vegetables, milk, meat, fish etc.). Commercially produced fermented foods which are marketed globally include dairy products (cheese, yogurt, fermented milks), sausages and soy sauce. Foods fermented by Lactic acid bacteria are cucumbers, olives, cabbage, coffee cherries, vanilla beans, meat, dairy, by Yeasts are malt, grapes, wines, wines, bread doughs and by mould are soybeans.

18.9 GENETICALLY MODIFIED ORGANISMS IN PRESERVATION

Microorganisms are an integral part of the processing system during the production of fermented foods. Microbial cultures can be genetically improved using both traditional and molecular approaches and improvement of bacteria, yeasts and moulds is done. One of the traits which have been considered for commercial food applications in both developed and developing countries include the ability to produce antimicrobial compounds (e.g. bacteriocins, hydrogen peroxide) for the inhibition of undesirable microorganisms. In many developing countries, the focus is also on the degradation or inactivation of natural toxins (e.g. cyanogenic glucosides in cassava), mycotoxins (in cereal fermentations) and anti-nutritional factors (e.g. phytates).

18.10 RECOMBINANT ANTIFREEZE PROTEIN

Antifreeze proteins are potent cryogenic protection agents for the cryopreservation of food and pharmaceutical materials. A food-grade expression and fermentation system (BSE- and antibiotic-free) for the production and secretion of high levels of rAFP was developed. A novel recombinant type I antifreeze protein analogue (rAFP) was produced and secreted by *Lactococcus lactis*, a food-grade microorganism of major commercial importance. Lyophilized, crude rAFP produced by L. lactis was tested in a frozen meat and frozen dough processing model. The frozen meat treated with the antifreeze protein showed less drip loss, less protein loss, and a high score on juiciness by sensory evaluation. Frozen dough treated with the rAFP showed better fermentation capacity than untreated frozen dough. Breads baked from frozen dough treated with rAFP acquired the same consumer acceptance as fresh bread.

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Lesson-19
Overview of Bioprocessing

19.1. INTRODUCTION

Bioprocessing is the use of biological materials (organisms, cells, organelles, enzymes) to carry out a process for commercial, medical or scientific reasons.

Bioprocess operations should ideally manufacture new products and destroy harmful wastes. Use of microorganisms to transfer biological material for production of fermented foods has been an essential part of many foods, chemical and pharmaceutical industries. Since then, bioprocesses have been developed for an enormous range of commercial products, from relatively cheap materials such as industrial alcohol and organic solvents to expensive speciality chemicals such as antibiotics, therapeutic proteins and vaccines.

Advantages of bioprocessing:

· They are specific in their action

· They are extremely efficient

· They are biodegradable

· Safer,

· They work in mild conditions and thus energy saving.

Tools of modern biotechnology such as recombinant DNA, gene probes, cell fusion and tissue culture offer ways to produce new products or improve bioprocessing methods. Modern Biotechnology has allowed us to envision sophisticated medicines, cultured human tissues and organs, biochips for new-age computers, environmentally-compatible pesticides and powerful pollution-degrading microbes.

19.2. A BRIEF ON H ISTORICAL DEVELOPMENTS IN BIOPROCESSING

Modern bio-process technology is an extension of ancient techniques for developing useful products by taking advantage of natural biological activities.

Earliest example of bioprocess is alcoholic beverages that are a combination of yeast cells and nutrients (cereal grains) to form a fermentation system in which the organisms consumed the nutrients for their own growth and produced by-products (alcohol and carbon dioxide gas) that helped to make the beverage.

Modern biotechnology was started in the 19th century when knowledge about biological system, their components, and interaction between them grew. In the first half of the 20th century the first large scale fermentation processes, namely citric acid and penicillin, were realized. The process of recombinant gene technology then led to a substantial increase in the number of bioprocesses and their production volume starting with insulin, the first product manufactured with recombinant technology, in the early 1980s.

The development of genetic engineering and monoclonal antibody technology, which started in the 1970s, has led to the introduction of a large number of new products with application in many different areas. The most highly visible applications have been in the area of human health care, with product such as human insulin, interferon's, tissue plasminogen activator, erythropoietin, colony-stimulating factors, and monoclonal antibody-based products.

New products for agriculture, food industry, fine chemicals and the environmental protection are also under intense development.

Today, the bio-industries have reached a critical size and are additionally based on a broad understanding of genomics, proteomics, bioinformatics, genetic transformation and molecular breeding. However, this knowledge waits to be transfer to technology and market products. The knowledge of molecular breeding, stem cell technology and pharmacogenomics might lead to strongly personalized therapies and therapeutics.

19.3. INDUSTRIAL FERMENTATION PRODUCTS & PRODUCER ORGANISMS

The word fermentation comes from the Latin verb *fevere*, which means to boil. It originated from the fact that early at the start of wine fermentation gas bubbles are released continuously to the surface giving the impression of boiling.

It has three different connotations when used in industrial microbiology

a) The first meaning relates to microbial physiology. In strict physiological terms, fermentation is defined in microbiology as the type of metabolism of a carbon source in which energy is generated by substrate level phosphorylation and in which organic molecules function as the final electron acceptor (or as acceptors of the reducing equivalents) generated during the break-down of carbon-containing compounds or catabolism.

b) The second usage of the word is in industrial microbiology, where the term 'fermentation' is any process in which micro-organisms are grown on a large scale, even if the final electron acceptor is not an organic. Thus, the production of penicillin, and the growth of yeast cells which are both highly aerobic, and the production of ethanol or alcoholic beverages which are fermentations in the physiological sense, are all referred to as fermentations.

c) The third usage concerns food. A fermented food is one, the processing of which microorganisms play a major part. Microorganisms determine the nature of the food through producing the flavor components as well deciding the general character of the food, but microorganisms form only a small portion of the finished product by weight.

19.4. TYPES OF END PRODUCTS OF FERMENTATION

Types of end products of fermentation include:

• Microbial cells (e.g. bacteria, yeast, fungal spores)

• Microbial enzymes (e.g. milk clotting enzymes or rennets, recombinant fungal and bacterial rennets for cheese manufacture)

• Microbial metabolites (e.g. alcohols– ethanol, butanol, 2, 3-butanediol, isopropanol; chemicals– lactate, propionate, proteins, vitamins, antibiotics; and fuels– methane)

• Recombinant products (e.g. hormones)

Fermentation products can be broadly divided into two categories: high volume, low value products or low volume, high value products. Examples of the first category include most food and beverage fermentation products, whereas many fine chemicals and pharmaceuticals are in the latter category.

Products of industrial microorganisms may also be divided into two broad groups, those which result from primary metabolism and others which derive from secondary metabolism.

19.4.1. Products of Primary Metabolism

Primary metabolism is the inter-related group of reactions within a microorganism which are associated with growth and the maintenance of life. Primary metabolism is essentially the same in all living things and is concerned with the release of energy, and the synthesis of important macromolecules such as proteins, nucleic acids and other cell constituents. When primary metabolism is stopped the organism dies. Products of primary metabolism are associated with growth and their maximum production occurs in the logarithmic phase of growth in a batch culture. Primary catabolic products include ethanol, lactic acid, and butanol while anabolic products include amino-acids, enzymes and nucleic acids. Single-cell proteins and yeasts would also be regarded as primary products (Table 19.1)

Table-19.1: Examples of industrial products resulting from primary metabolism

Anabolic Products	*Catabolic Products*
1. Enzymes	1. Ethanol and ethanol-containing products, e.g. wines
2. Amino acids	2. Butanol
3. Vitamins	3. Acetone
4. Polysaccharides	4. Lactic acid
5. Yeast cells	5. Acetic acid (vinegar)
6. Single cell protein	
7. Nucleic acids	
8. Citric acid	

Source: Okafor, 2007

19.4.2. Products of Secondary Metabolism

In contrast to primary metabolism which is associated with the growth of the cell and the continued existence of the organism, secondary metabolism (Table 19.2), which was first observed in higher plants, has the following characteristics

- Secondary metabolism has no *apparent* function in the organism.
- Secondary metabolites are produced in response to a restriction in nutrients.
- Secondary metabolism appears to be restricted to some species of microorganisms.
- Secondary metabolites usually have ‘unusual chemical structures and several closely related metabolites may be produced by the same organism in wild-type strains.
- The ability to produce a particular secondary metabolite is easily lost.
- Owing to the ease of the loss of the ability to synthesize secondary metabolites, secondary metabolite production is believed to be controlled by plasmids rather than by the organism’s chromosomes.

The factors which trigger secondary metabolism, the inducers, also trigger morphogenesis.

Table-19.2. Examples of Industrial products of microbial secondary metabolism

Product	*Organism*	*Use/Importance*
Antibiotics		
Penicillin	*Penicillium chrysogenum*	Clinical use
Streptomycin	*Streptomyces griseus*	Clinical use
Anti-tumor Agents		
Actinomyin	*Streptomyces antibioticus*	Clinical use
Bleomycin	*Streptomyces verticulus*	Clinical use
Toxins		
Aflatoxin	*Aspergiulus flavous*	Food toxin
Amanitine	*Amanita* sp	Food toxin
Alkaloids		
Ergot alkaloids	*Claviceps purpurea*	Pharmaceutical
Miscellaneous		
Gibberellic acid	*Gibberalla fujikuroi*	Plant growth hormone
Kojic acid	*Aspergillus flavus*	Food flavor
Muscarine	*Clitocybe rivalosa*	Pharmaceutical
Patulin	*Penicillium urticae*	Anti-microbial agent

Source: Okafor, 2007

19.5. CRITERIA FOR SELECTION OF INDUSTRIALLY IMPORTANT MICROORGANISMS

Since prehistoric times, by means of trial and error, people developed strains of microbes that were used in the production of 'beverages, food, textiles, and antibiotics without knowing that microbes were the responsible agents. With the discovery that microorganisms existed, and the subsequent development of culture methods, came the birth of modem biological technology or biotechnology. Expanded development of microbial screening and cultural techniques has brought us to a point where microbially produced products are a major part of our life. While production of food, drink, and textiles remains a large part of the biotechnology industry, the discovery of penicillin in the first half of this century revolutionized microbial screening for other useful products such as antibiotics, enzymes, and speciality chemicals.

During the past 40-50 years, screening of industrially important microorganisms has evolved steadily, although somewhat haphazardly, while still relying on the original underlying techniques of enrichment, pure culture, mutagenesis, and sheer labor. Classical methods are still used extensively with modifications resulting from the use of chemical analogs, coupled

Colorimetric reactions, membrane technology, immunological techniques, and advances in instrumentation.

Although the well-known ubiquity of microorganism implies that almost any natural ecological entity–water, air, leaves, tree trunks – may provide microorganisms, the soil is the preferred source for isolating organisms, because it is a vast reservoir of diverse organisms. In recent times, other ‘new’ habitats, especially the marine environment, have been included in habitats to be studied in searches for bioactive microbial metabolites or ‘bio-mining’.

Identification and isolation of required micro-organisms is very critical for any microbiological process, since some micro-organisms may be toxic to the useful microbes and may use up the nutrients all by themselves, producing metabolites that are different from the desired ones. Isolation of micro-organisms also helps to screen them to determine, if they can be used for any industrial process. Such microorganisms should satisfy some specified criteria.

19.5.1. Important criteria in the choice of organism:

- The nutritional characteristics of the organism:
- The optimum temperature of the organism
- The reaction of the organism with the equipment to be employed and the suitability of the organism to the type of process to be used
- T he stability of the organism and its amenability to genetic manipulation
- The productivity of the organism, measured in its ability to convert substrate into product and to give a high yield of product per unit time
- The ease of product recovery from the culture
- Irrespective of the origins of an industrial microorganism,

Other features that may be exploited are thermophilic or halophilic properties, which may be useful in a fermentation environment. Also, particularly for cells grown in suspension, they should grow well in conventional bioreactors to avoid the necessity to develop alternative systems. Consequently, they should not be shear sensitive, or generate excessive foam, nor be prone to attachment to surfaces.

19.5.2. Some general screening methods are described below.

1. Isolation *de novo* of Organisms Producing Metabolites of Economic Importance

1.1. Enrichment with the substrate utilized by the organism being sought

1.1. Enrichment with toxic analogues of the substrate utilized by the organism being sought

1.2. Testing microbial metabolites for bioactive activity

1.2.1. Testing for anti-microbial activity

1.2.2. Testing for enzyme inhibition

1.2.3. Testing for morphological changes in fungal test organisms

1.2.4. Conducting animal tests on the microbial metabolites

19.5.3. Strain Improvement

Several options are open to an industrial microbiology organization seeking to maximize its profits in the face of its competitors' race for the same market. The operations in the fermentor may also be improved by its use of a more productive medium, better environmental conditions, better engineering control of the fermentor processes, or it may genetically improve the productivity of the microbial strain it is using. Of all the above options, strain improvement appears to be the one single factor with the greatest potential for contributing to greater profitability.

To appreciate the basis of strain improvement it is important to remember that the ability of any organism to make any particular product is predicated on its capability for the secretion of a particular set of enzymes. The production of the enzymes, themselves depends ultimately on the genetic make-up of the organisms. Improvement of strains can therefore be put down in simple term as follows

1. Regulating the activity of the enzymes secreted by the organisms

2. Increasing the permeability of the organism so that the microbial products can find their way more easily outside the cell.

3. Selecting suitable producing strains from a natural population

4. Manipulation of the existing genetic apparatus in a producing organism

5. Introducing new genetic properties into the organism by recombinant DNA

6. Technology or genetic engineering

19.5.3.1. Examples of targets for strain improvement

- Rapid growth
- Genetic stability
- Non-toxicity to humans
- Large cell size, for easy removal from the culture fluid
- Ability to use cheaper substrates
- Modification of submerged morphology
- Elimination of the production of compounds that may interfere with downstream processing
- Catabolite derepression
- Phosphate deregulation
- Permeability alterations to improve product export rates
- Metabolite resistance
- Production of additional enzymes and compounds to inhibit contaminant microorganisms

19.5.3.2. Manipulation of the genome of industrial organisms in strain improvement can be done by mainly two ways:

1. *Methods not involving foreign DNA*

1.1 Conventional mutation

2. *Methods involving DNA foreign to the organism (i.e. recombination)*

1.2.5. Transduction

1.2.6. Conjugation

1.2.7. Transformation

1.2.8. Heterokaryosis

1.2.9. Protoplast fusion

1.2.10. Genetic engineering

1.2.11. Metabolic engineering

1.2.12. Site-directed mutation

19.6. MEDIA FOR INDUSTRIAL INOCULUMS DEVELOPMENT

The use of a good, adequate, and industrially usable medium is as important as the deployment of a suitable microorganism in industrial microbiology. Unless the medium is adequate, no matter how innately productive the organism is, it will not be possible to harness the organism's full industrial potentials. Indeed not only may the production of the desired product be reduced but toxic materials may be produced.

Fermentation media must satisfy all the nutritional requirements of the microorganism and fulfill the technical objectives of the process. The nutrients should be formulated to promote the synthesis of the target product, either cell biomass or a specific metabolite.

19.6.1 The main factors that affect the final choice of individual raw materials are as follows .

(a) Cost of the material

The cheaper the raw materials the more competitive the selling price of the final product will be. Due to these economic considerations the raw materials used in many industrial media are usually waste products from other processes. Corn steep liquor and molasses are, for example, waste products from the starch and sugar industries, respectively.

(b) Ready availability of the raw material

The raw material must be readily available in order not to halt production. If it is seasonal or imported, then it must be possible to store it for a reasonable period.

(c) Transportation costs

Proximity of the user-industry to the site of production of the raw materials is a factor of great importance, because the cost of the finished material and its competitiveness can all be affected by the transportation costs.

(d) Ease of disposal of wastes resulting from the raw materials

The disposal of industrial waste is rigidly controlled in many countries. When choosing a raw material therefore the cost, if any, of treating its waste must be considered.

(e) Uniformity in the quality of the raw material and ease of standardization

The quality of the raw material in terms of its composition must be reasonably constant in order to ensure uniformity of quality in the final product and the satisfaction of the customer and his/her expectations.

(f) Adequate chemical composition of medium

The medium must have adequate amounts of carbon, nitrogen, minerals and vitamins in the appropriate quantities and proportions necessary for the optimum production of the commodity in question.

g) Presence of relevant precursors

The raw material must contain the precursors necessary for the synthesis of the finished product. Precursors often stimulate production of secondary metabolites either by increasing the amount of a limiting metabolite, by inducing a biosynthetic enzyme or both.

❤️ **Satisfaction of growth and production requirements of the microorganisms**

Many industrial organisms have two phases of growth in batch cultivation: the phase of growth, or the trophophase , and the phase of production, or the idiophase . Often these two phases require different nutrients or different proportions of the same nutrients.

19.6.2 Components of media for industrial inoculums development:

The media should support the metabolic process of the microorganisms and allow bio-synthesis of the desired products.

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

Media are designed based on the above equation using minimum components required to produce maximum product yield. Important components of the medium are carbon sources, nitrogen sources, minerals, growth factors, chelating agents, buffers, antifoaming agents, air, steam, and fermentations vessels.

1. Carbon sources

A carbon source is required for all biosynthesis leading to reproduction, product formation and cell maintenance. In most fermentations it also serves as the energy source. Carbohydrates are traditional carbon and energy sources for microbial fermentations, although other sources may be used, such as alcohols, alkanes and organic acids. Animal fats and plant oils may also be incorporated into some media, often as supplements to the main carbon source.

a. ***Molasses***

Pure glucose and sucrose are rarely used for industrial scale fermentations, primarily due to cost. Molasses, a byproduct of cane and beet sugar production, is a cheaper and more usual source of sucrose. This material is the residue remaining after most of the sucrose has been crystallized from the plant extract. 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.

b. ***Malt Extract***

Aqueous extracts of malted barley can be concentrated to form syrups that are particularly useful carbon sources for the cultivation of filamentous fungi, yeasts and actinomycetes. The composition of malt extracts varies to some extent, but they usually contain approximately 90% carbohydrate, on a dry weight basis. This comprises 20% hexoses (glucose and small amounts of fructose), 55% disaccharides (mainly maltose and traces of sucrose), along with 10% maltotriose, a trisaccharide. Malt extracts also contain some vitamins and approximately 5% nitrogenous substances, proteins, peptides and amino acids.

c. ***Starch and Dextrins***

These polysaccharides are not as readily utilized as monosaccharides and disaccharides, but can be directly metabolized by amylase-producing microorganisms, particularly filamentous fungi.

d. *Sulphite Waste Liquor*

Sugar containing wastes derived from the paper pulping industry are primarily used for the cultivation of yeasts. Waste liquors from coniferous trees contain 2–3% (w/v) sugar, which is a mixture of hexoses (80%) and pentoses (20%). Hexoses include glucose, mannose and galactose, whereas the pentose sugars are mostly xylose and arabinose. Usually the liquor requires processing before use as it contains sulphur dioxide.

e. *Cellulose*

Cellulose is predominantly found as lignocellulose in plant cell walls, which is composed of three polymers: cellulose, hemicellulose and lignin. Lignocellulose is available from agricultural, forestry, industrial and domestic wastes. Relatively few microorganisms can utilize it directly, as it is difficult to hydrolyse. It is potentially a very valuable renewable source of fermentable sugars once hydrolysed, particularly in the bioconversion to ethanol for fuel use.

f. *Whey*

Whey is an aqueous byproduct of the dairy industry. This material is expensive to store and transport. Therefore, lactose concentrates are often prepared for later fermentation by evaporation of the whey, following removal of milk proteins for use as food supplements. Lactose is generally less useful as a fermentation feedstock than sucrose, as it is metabolized by fewer organisms. *S. cerevisiae*, for example, does not ferment lactose.

g. *Alkanes and Alcohols*

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

2. Fats and Oils

Hard animal fats that are mostly composed of glycerides of palmitic and stearic acids are rarely used in fermentations. However, plant oils (primarily from cotton seed, linseed, maize, olive, palm, rape seed and soya) and occasionally fish oil, may be used as the primary or supplementary carbon source, especially in antibiotic production.

3. Nitrogen sources

Most industrial microbes can utilize both inorganic and organic nitrogen sources. Inorganic nitrogen may be supplied as ammonium salts, often ammonium sulphate and di ammonium hydrogen phosphate, or ammonia. Ammonia can also be used to adjust the pH of the fermentation. Organic nitrogen sources include amino acids, proteins and urea. Nitrogen is often supplied in crude forms that are essentially byproducts of other industries, such as corn steep liquor, yeast extracts, peptones and soya meal.

a. Corn *Steep Liquor*

Corn steep liquor is a byproduct of starch extraction from maize. The exact composition of the liquor varies depending on the quality of the maize and the processing conditions. Concentrated extracts generally contain about 4% (w/v) nitrogen, including a wide range of amino acids, along with vitamins and minerals.

b. *Yeast Extracts*

Yeast extracts may be produced from waste baker's and brewer's yeast, or other strains of *S. cerevisiae*. Alternate sources are*Kluyveromyces marxianus* grown on whey and *Candida utilis* cultivated using ethanol, or wastes from wood and paper processing.

c. Peptones

Peptones are usually too expensive for large-scale industrial fermentations. They are prepared by acid or enzyme hydrolysis of high protein materials: meat, casein, gelatin, keratin, peanuts, soy meal, cotton seeds, etc..

4. Water

All fermentation processes, except solid-substrate fermentations, require vast quantities of water. In many cases it also provides trace mineral elements and is important for ancillary equipment and cleaning. Before use, removal of suspended solids, colloids and microorganisms is usually required. When the water supply is 'hard', it is treated to remove salts such as calcium carbonate.

5. Minerals

Normally, sufficient quantities of cobalt, copper, iron, manganese, molybdenum, and zinc are present in the water supplies, and as impurities in other media ingredients. Occasionally, levels of calcium, magnesium, phosphorus, potassium, sulphur and chloride ions are too low to fulfil requirements and these may be added as specific salts.

6. Vitamins and growth factors

Many bacteria can synthesize all necessary vitamins from basic elements. For other bacteria, filamentous fungi and yeasts, they must be added as supplements to the fermentation medium. Most natural carbon and nitrogen sources also contain at least some of the required vitamins as minor contaminants. Other necessary growth factors, amino acids, nucleotides, fatty acids and sterols, are added either in pure form or, for economic reasons, as less expensive plant and animal extracts. Pharmamedia, cornsteep powder, distillers solubles and malt sprouts are some examples of media ingredients.

7. Precursors

Some fermentations must be supplemented with specific precursors, notably for secondary metabolite production. Examples include phenylacetic acid or phenylacetamide added as side-chain precursors in penicillin production. Threonine is used as a precursor in isoleucine production by *Serratia marsescens*, and anthranillic acid additions are made to fermentations of the yeast *Hansenula anomala* during tryptophan production.

8. Inducers and elicitors

If product formation is dependent upon the presence of a specific inducer compound or a structural analogue, it must be incorporated into the culture medium or added at a specific point during the fermentation. Inducers are often necessary in fermentations of genetically modified microorganisms (GMMs).

9. Inhibitors

Inhibitors are used to redirect metabolism towards the target product and reduce formation of other metabolic intermediates; others halt a pathway at a certain point to prevent further metabolism of the target product. An example of an inhibitor specifically employed to redirect metabolism is sodium bisulphite, which is used in the production of glycerol by *S. cerevisiae.*

10. Cell permeability modifiers

These compounds increase cell permeability by modifying cell walls and/or membranes, promoting the release of intracellular products into the fermentation medium. Compounds used for this purpose include penicillins and surfactants.

11. Oxygen

Depending on the amount of oxygen required by the organism, it may be supplied in the form of air containing about 21% (v/v) oxygen, or occasionally as pure oxygen when requirements are particularly high. For most fermentations the air or oxygen supply is filter sterilized prior to being injected into the fermenter.

12. Antifoams

Antifoams are necessary to reduce foam formation during fermentation. Foaming is largely due to media proteins that become attached to the air–broth interface where they denature to form astable foam. If uncontrolled the foam may block air filters, resulting in the loss of aseptic conditions; the fermenter becomes contaminated and microorganisms are released into the environment. Natural antifoams include plant oils (e.g. from soya, sunflower and rapeseed), deodorized fish oil, mineral oils and tallow.

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Lesson-20

Growth Curve, Growth Measurement, Growth Cultivation

20.0. INTRODUCTION

Growth is an orderly increase in the quantity of cellular constituents. It depends upon the ability of the cell to form new protoplasm from nutrients available in the environment. In most bacteria, growth involves the following steps.

1. Increase in cell mass and number of ribosomes

2. Duplication of the bacterial chromosome

3. Synthesis of new cell wall and plasma membrane

4. Partitioning of the two chromosomes

5. Septum formation

6. Cell division.

This asexual process of reproduction is called binary fission . For unicellular organisms such as the bacteria, growth can be measured in terms of two different parameters: changes in cell mass and changes in cell numbers .

20.1. GROWTH CURVE

20.1.1. Batch Experiment

It has been observed that if one of the essential requirements for growth is present in only limited amounts, the limiting factor affects on the rate of growth.

A batch culture system is one containing a limited amount of nutrient, which is inoculated with the microorganism. Cells grow until some component is exhausted or until the environment changes so as to inhibit growth. During batch fermentations the population of microorganisms goes through several distinct growth phases:

1. Lag phase
2. Accelerating growth phase
3. Log (exponential growth) phase
4. Declining growth phase
5. Stationary phase
6. Death or declining death phase

Log death phase

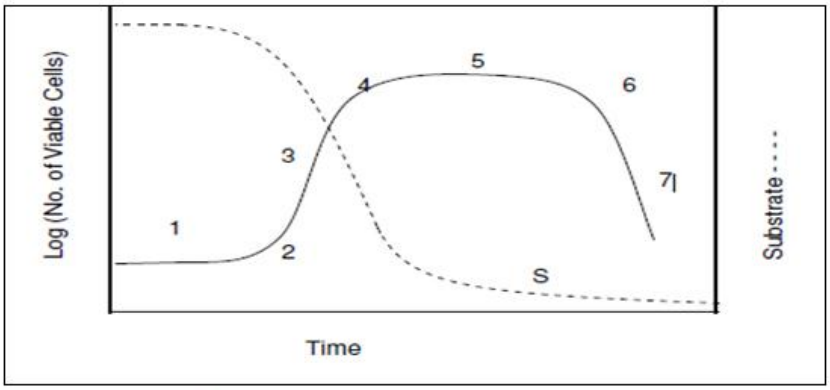


Fig. 20.1: Growth Phases of a Simple Batch Culture (Liu et al., 2003)

The salient points of the growth phases are as under

1. Lag Phase

a. **Adaptation (acclimation) period**

The lag phase is the initial phase which represents the period (time) required for bacteria to adapt to their new environment.

b. **Constant number of cells**

During this phase, the individual bacterial cells increase in size, but the number of cells remains unchanged.

c. **Physiologically active**

They are very active physiologically and are synthesizing new enzymes and activating factors.

2. Accelerating Growth Phase

Transition period from the lag phase to the log phase. .Cell is beginning to grow (increase in numbers) noticeably as enzyme systems are gearing up.

3. Log (Exponential Growth) Phase

a. Exponential growth during this phase, the bacterial cells divide regularly at a constant rate.

b. Straight line on semilog scale - The logarithms of the number of cells plotted against time results in a straight line.

c. Maximum Rate of Substrate utilization. A maximum growth rate occurs under optimal conditions, and substrate is removed from the medium at the maximum rate. The growth rate is limited only by the bacteria's ability to process the substrate. Food is in excess (not limiting) so that the rate of growth is only limited by the ability to process the food. Sometimes called "0-order growth" and growth rate is constant and maximum.

4. Declining Growth Phase

a. Transition period from the log phase to the stationary phase.

b. Decreasing growth rate

c. Exhaustion of essential nutrients

d. Accumulation of toxic metabolic products - the growth rate can be limited either by the exhaustion of essential nutrients or by the accumulation of toxic metabolic products. - food becomes limiting factor and therefore growth rate and mass of bacteria are dependent on the amount of food present.

5. Stationary Phase

a. The number of cells remains constant perhaps as a results of complete cessation of division or the balancing of reproduction rate by an equivalent death rate. Growth of new cells is balanced by the death of old cells. No increase in cell mass - population is "stable". net growth rate = 0

6. Death or declining Phase

a. The number of viable cells decreases slowly while the total mass may remain constant due to the fact that the death rate exceeds the production rate of new cells.

b. Depletion of essential nutrients

c. Accumulation of inhibitory products. - Death occurs primarily as a result of depletion of essential nutrients and/or the accumulation of inhibitory products.

7. Log Death Phase

a. Exponential death - "wholesale die-off" - system is dead - even if you add food, you will get no growth.

20.2 Microbial Growth Kinetics

The exponential growth phase is the most important phase of the growth cycle when the product you are trying to produce is, either the biomass itself or a growth associated product. Quantification of exponential growth rate (i.e. how fast cells grow) is the first fundamental step in the quantification of culture kinetics.

The establishment of exponential growth is dependent on a number of factors.

a. A viable inoculum

b. A suitable energy source

c. The presence of excess nutrients and growth factors

d. The absence of inhibitors

e. A suitable environment (i.e. temperature, dissolved oxygen)

Modelling and simulation of microbial cell growth is important both theoretically and practically. Although the Monod model has been the most widely used for the prediction of cell growth, it only fits the exponential growth phase of the growth, without any inhibition. The lag growth phase, decreased growth phase and the stationary growth phase of a typical microbial batch culture growth curve cannot be predicted using the Monod model.

When t = 0 (when exponential growth begins)

where $X = X_0$ (the biomass concentration at the start of the fermentation).

When t = t, X = X.

If μ is constant w.r.t. (which in most cases it is), then rearranging Equation (1) and integrating:

$$\int_{X_0}^{X} \frac{dX}{X} = \int_0^t \mu dt$$

$$Ln \frac{X}{X_0} = \mu t \quad \text{or} \quad X = X_0 e^{\mu t} \qquad (2)$$

Equation (2) applies only to the duration of the exponential growth phase, beyond which either substrate limitation or toxin accumulation become rate determining.

Doubling Time i s the time required to double the quantity of biomass, that is growing exponentially.

X: $X_0 \rightarrow 2X_0$ (The amount of biomass at the start must double)

t: $0 \rightarrow td$ (Within a finite time, td, or doubling time)

$$\frac{X}{X_0} = e^{\mu t}$$

Subbing in $2X_0$ for X and td for t

$$\frac{2X_0}{X_0} = e^{\mu t_d}$$

Logging both sides and cancelling out the two X_0's

$$\ln 2 = \mu td$$

therefore

$$td = \frac{\ln 2}{\mu} = \frac{0.693}{\mu}$$

Specific growth rate (μ) can be defined as any point during the growth cycle. During the exponential growth period μ is constant and at a maximum for that process under the specified conditions.

It is often stated that μ , the specific growth rate is a function of growth limiting substrate concentration

μ = *f* (S)

S = concentration of growth rate limiting substrate

Relationship between growth rate and substrate concentration : This is generally defined in terms of the Monod Equation. Applies only where a single substrate (S) is limiting and the accumulation of growth associated toxins can be ignored (which is generally the case in dilute microbial suspensions). It is very similar to the shape of the Michaelis Menten equation for enzyme kinetics.

$$\mu = \frac{\mu_{max} S}{K_S + S} \Rightarrow \frac{dX}{dt} = \frac{\mu_{max} SX}{K_S + S}$$

μ_{max} = maximum specific growth rate (@S >> K_S) (hr^{-1})

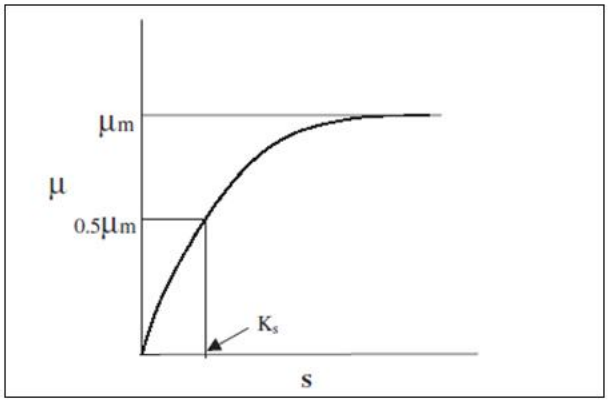
K_S = saturation constant (g/L of substrate)

= Rate-limiting substrate conc. (g/L) when $\mu = \frac{\mu_{max}}{2}$

μ_{max} and K_S are constants for a given organism for a specified substrate.

μ_{max} is an indication of how fast the organism grows in conditions where all limiting substrates are in excess.

K_S is an indication of how quickly μ migrate from μ_{max} to 0 as the concentration of limiting substrate (maybe glucose) moves towards 0.



(Liu et al., 2003)

Where,

μ = Specific growth rate

μ_m = maximum specific growth rate

S = concentration of growth rate limiting substrate

K_S = saturation constant

20.3 MONITORING MICROBIAL GROWTH IN CULTURE

During fermentation, methods are required for the routine determination of the microbial population, cell number and / or biomass, in order to monitor its progress. Numerous direct and indirect methods are available for this purpose. Direct procedures involve dry weight determination, cell counting by microscopy and plate counting methods. Indirect methods include turbidimetry, spectrophotometer, estimation of cell components (protein, DNA, RNA, or ATP), and online monitoring of carbon dioxide production or oxygen utilization.

20.3.1 Classical cultural methods

Conventional methods for the enumeration of bacteria in food are colony count methods. If low numbers of bacteria are suspected to be present in the food samples, numbers may be estimated by means of the most probable number method (MPN).

20.3.2 Automation as alternative method

Many improvements in this field have been made that permit laboratories to increase the efficiency and the number of samples processed such as agar preparation machines, automated dilutors, automated counting devices and spiral plate. As an example, the spiral plate is a semi-automated plating technique that greatly reduces manpower and material costs normally associated with the classical cultural method, in particular the colony count method.

20.3.3 Chromogenic and fluorogenic isolation media

The recognition of colonies of presumptive target organisms has been facilitated by the introduction of chromogenic and fluorogenic media. These are microbiological growth media that contain enzyme substrates linked to a chromogen (colour reaction), fluorogen

(fluorescent reaction) or a combination of both. The incorporation of such fluorogenic or chromogenic enzyme substrates into a selective medium can eliminate the need for subculture and further biochemical tests to establish the identity of certain micro-organisms.

20.3.4 Modified cultural methods

A variety of rapid methods have been elaborated, which predominantly aim to reduce the workload and facilitate the work flow by reducing the manipulations and/or the necessity for a full lab infrastructure and not necessarily shorten the time for detection. Some of these modified cultural methods are based upon the colony count method e.g. 3M Petrifilm and Compact Dry, whereas others make use of the principle of the MPN method e.g. TEMPO and SimPlate.

20.3.4.1. 3M Petrifilm

Rather than a Petri dish, 3M Petrifilm makes use of thin plastic film as carrier of the culture medium. Generally the 3M Petrifilm plate comprises a cold-water-soluble gelling agent, nutrients and indicators for activity and enumeration. An important advantage of the 3M Petrifilm plate is the fact that it is very thin (a film), saving space in the incubator. After incubation, typical colonies can be counted either manually (facilitated by the grid on the background of the film and characteristic colored colonies) or automatically.

20.3.4.2 Compact Dry (Nissui Pharmaceutical Co., LTD.)

The Compact Dry plates also have a dedicated user-friendly small plastic dish format that contains dehydrated nutrients and differentiating components. Similar to the 3M Petrifilm Compact Dry plates are thin, light and convenient to handle.

20.3.4.3 SimPlate (BioControl systems)

Detection and enumeration of micro-organisms by the SimPlate methods rely on a binary detection technology. It uses IDEXX's Multiple Enzyme Technology (MET) to detect bacteria in food and in water. Visible colour changes occur as a result of bacterial enzyme interaction with substrates present in the liquid culture medium. The counting range is from <1 to 738 per plate (more than double of a standard pour plate).

20.3.4.4. TEMPO (Bio-Mérieux)

The TEMPO test is an automated MPN enumeration method and consists of a vial of culture medium and a card, which are specific to the test. Dedicated equipment and software support the inoculation and reading of the cards. Target micro-organism multiply in the culture medium resulting in a signal detected by the TEMPO Reader (based up on fluorescent pH indicator, β-glucuronidase activity, etc.).

20.3.4.5 Colilert_ (IDEXX Laboratories)

Colilert is used for the simultaneously detection and enumeration of total coliforms and *E. coli* in water and waste water based on the MPN principle. Colilert uses the patented Defined Substrate Technology (DST) and two chromogenic nutrient-indicators, ortho-nitrophenyl-β-D-galactopyranoside (ONPG) and 4-methylumbelliferyl-β-D-glucuronide (MUG).

20.3.4.6. Soleris (Neogen)

The Soleris technology monitors changes in the chemical characteristics of microbial liquid growth medium and detects micro-organisms with pH and other sensitive reagents. The reagents change their spectral patterns as the

metabolic process takes place which can be detected photometrically by an optical instrument and monitored at predetermined time intervals. Sensitivity ranges from a single organism per vial to 10^8 cfu/ml (upper limit), but the time at which growth is first detected is inversely proportional to the log number of bacteria in the sample.

20.3.5 Bio-chemically based enumeration methods

20.3.5.1. Impedance

This method is based on the principle that bacteria actively growing in a culture medium produce positively or negatively charged end-products (early stages of breakdown of nutrients) that cause an impedance variation of the medium. This variation, which is proportional to the change in the number of bacteria in the culture, makes it possible to measure bacterial growth. The time at which growth is first detected, referred to as detection time (DT), is inversely proportional to the log number of bacteria in the sample, which means that bacterial counts can be predicted from DT.

20.3.5.2. ATP bioluminescence

This technique measures light emission produced due to the presence of ATP, which is involved in an enzyme substrate reaction between luciferin and luciferase (bioluminescence). The quantity of light produced (measured as Relative Light Units RLUs) is proportional to the concentration of ATP and, thus, to the number of micro-organisms in the original sample. ATP bioluminescence can be used for enumeration of total count but it is only applicable if high numbers of bacteria are present (>10,000 cfu/g). As such this technique is mostly used to estimate the total surface cleanliness, including the presence of organic debris and microbial contamination, providing results within less than 5 min. The Milliflex Rapid system is an ATP based system provided by MilliPore.

20.3.6. Microscopic based enumeration methods

20.3.6.1. Flow cytometry

Flow cytometry quantitatively measures the optical characteristics of cells as they are presented separately in front of a focused light beam (from a high-pressure mercury vapour lamp or an assortment of lasers). As particles pass through the light beam three parameters are measured using photomultiplier tubes, the forward scatter, the side scatter and fluorescence. For routine analyses of milk quality, an automated instrument (Bactoscan 8000 method) was developed. This flow cytometry method uses ethidium bromide (intercalating with DNA) to stain bacteria in milk. The disturbing milk components are reduced and dispersed by treatment with detergent and enzyme at 50 ^{o}C, and provides a result after 8 min.

20.3.6.2. Direct Epifluorescent Filter Technique (DEFT)

The DEFT is a microscopic cell counting method. A pre-treated sample is filtered over a polycarbonate membrane. The microbial cells are concentrated and stained with fluorescent dyes. Incident light illumination (epifluorescence) is used to examine the filter surface. The actual staining and counting takes less than 0.5-1 h, but sample pre-treatment steps lengthen the total detection time. The detection limit is 10^4 to 10^5 cells/ml. COBRA instrument provided by Cobra Biomanufacturing Plc is an example of a DEFT based method.

20.3.7. Future trends for enumeration methods

20.3.7.1. Quantitative real-time polymerase chain reaction (Q-PCR)

PCR is a technique which actually quantifies genomic copies, the relation between genomic copies and the number of cells present in the sample needs to be determined as well.

20.3.8 Immunoassays

immunoassays are based on the highly specific binding reaction between antibodies and antigens. The selection of an appropriate antibody (monoclonal or polyclonal) is the determinant factor for the method's performance. Usually, any positive result for pathogens obtained with immunoassays is considered as presumptive and requires further confirmation. Detection limit is approximately 10^4 to 10^5 cfu/ml, depending upon the type of antibody and its affinity for the corresponding epitope. Several types of immunoassays are available in food diagnostics of which lateral flow devices (LFD), Enzyme Linked Immunosorbent Assays (ELISAs) & Enzyme linked fluorescent assays (ELFAs) are widely used. Immunomagnetic separation (IMS) assays, although a sample preparation tool instead of a detection method, have been developed as an aid in reducing the time for the enrichment step prior to detection. and are therefore discussed in this paragraph.

20.3.8.1. Enzyme linked immunosorbent assay (ELISA) & enzyme linked fluorescent assays (ELFA)

ELISA is a biochemical technique that couples an immunoassay with an enzyme assay. In most of the alternative methods a sandwich ELISA is used. The sandwich ELISA comprised different steps. Specific antibodies are affixed to the surface of the wells of a 96 well microtiter plate. The sample, with an unknown amount of target antigen, is added and allowed to bind to the affixed antibodies. In the final step a substrate is added that the enzyme can convert to a detectable signal. Validated ELISA methods are available from BIO A.R.T., R-Biopharm AG, BioControl Systems, Rayal and 3M. The VIDA system developed by Bio-Mérieux

20.3.8.2. Immunomagnetic separation and concentration

Superparamagnetic particles can be coated with antibodies, allowing specific capture and isolation of intact cells directly from a complex sample suspension without the need for column immobilization or centrifugation. Monosized superparamagnetic polymer particles known as "Dynabeads" are available commercially from Invitrogen-Dynal. Pathatrix, an automated system, is a patented re-circulating immunomagnetic separation technology.

20.3.9. Bacteriophage-based detection methods

Phages are extremely host-specific. Bacteriophages or proteins of bacteriophages have been included in various ways in detection methods for pathogens. The specific bacteriophage tail-associated proteins can be attached to paramagnetic beads to capture bacteria in suspension. The bacteria-bead complex can be integrated in fast detection protocols.

20.3.10 Molecular based detection methods

20.3.10.1. Fluorescent in situ hybridization (FISH)

Fluorescence in situ hybridization (FISH) with ribosomal RNA (rRNA) targeted oligonucleotide probes is the most commonly applied technique among the 'non-PCR-based' molecular techniques. FISH is commercially exploited by e.g. Vermicon, which has detection kit for different pathogenic and non-pathogenic micro-organisms.

20.3.10.2. Conventional, real-time and multiplex polymerase chain reaction (PCR)

It is a three-step cyclic in vitro procedure based on the ability of the DNA polymerase to copy a strand of DNA. The presence of even 1 copy of the template within the reaction mixture can be detected within a couple of hours as about a million-fold of copies are created. In the early 1990s, the "second" generation of PCR technologies was introduced by the use of fluorescent double-stranded DNA dyes. Validated PCR methods are available from Bio-Rad, Roche, Qualicon/Oxoid, Genesystems, AES Chemunex, Applied BioSystems, Idaho Technology Inc., Lantmännen, IEH Laboratries and Consulting Group, ADNucleis and BioControl systems.

20.3.11. Future trends

20.3.11.1. Microarrays

Microarrays or gene chips provide a miniaturized system for the simultaneous analysis of hybridization of fluorescent-labelled single strand nucleotide chains to an array of oligonucleotide probes immobilized on a support such as glass or a synthetic membrane. PCR amplification is often used prior to hybridization to increase sensitivity of detection. DNA microarrays may be very useful for detecting multiple bacteria simultaneously on a single glass slide .

20.3.11.2 Biosensors

Biosensors are defined as analytical devices that combine biospecific recognition systems with physical or electrochemical signaling. Biosensors for the detection of pathogens in the food industry consist of immobilized biologically active material, like enzymes, antibodies, antigens or nucleic acids, in close proximity to a receiving transducer unit. Target recognition results in the generation of an electrical, optical or thermal signal that is proportional to the concentration of target molecules. Biosensors have the potential to shorten the time between sampling and results, but their future lies in reaching selectivity and sensitivities comparable to established methods at a fraction of the cost.

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Lesson-21

Growth Cultivation- Batch and Continuous Type

21.1 INTRODUCTION

The fermentation process involves actual growth of the microorganism and formation of

the product under agitation and aeration, to provide uniform environment and adequate

oxygen to the cell for growth, survival, and product formation.

A fermentation system is usually operated in one of the following modes: batch, fed batch, or continuous fermentation. The choice of the fermentation mode is dependent on the relation of consumption of substrate to biomass and products.

The fermentation unit in industrial microbiology is analogous to a chemical plant in the chemical industry. A fermentation process is a biological process and, therefore, has requirements of sterility and use of cellular enzymatic reactions instead of chemical reactions aided by inanimate catalysts, sometimes operating at elevated temperature and pressure. Industrial fermentation processes may be divided into two main types, with various combinations and modifications. These are batch fermentations and continuous fermentations.

21.2. BATCH FERMENTATIONS

A tank of fermenter (Figure 21.3) is filled with the prepared mash of raw materials to be fermented. The temperature and pH for microbial fermentation is properly adjusted, and occassionally nutritive supplements are added to the prepared mash. The mash is steam-sterilized in a pure culture process. The inoculum of a pure culture is added to the fermenter, from a separate pure culture vessel. Fermentation proceeds, and after the proper time the contents of the fermenter, are taken out for further processing. The fermenter is cleaned and the process is repeated. Thus each fermentation is a discontinuous process divided into batches.

21.3. CONTINUOUS FERMENTATION

Growth of microorganisms during batch fermentation confirms to the characteristic growth curve, with a lag phase followed by a logarithmic phase. This, in turn, is terminated by progressive decrements in the rate of growth until the stationary phase is reached. This is because of limitation of one or more of the essential nutrients. In continuous fermentation, the substrate is added to the fermenter continuously at a fixed rate. This maintains the organisms in the logarithmic growth phase. The fermentation products are taken out continuously. The design and arrangements for continuous fermentation are somewhat complex.

21.3 BATCH GROWTH

It involves a closed system where all nutrients are present at the start of the fermentation within a fixed volume.

Batch cultivations are generally employed to grow cells for desired metabolite production as it is simple and involves least requirement of labor and equipments as opposed to other modes of cultivation (Fed-batch/ Continuous cultivations). However these cultivations work as closed system and thereby feature highly dynamic

growth conditions and have less yield and productivity of desired product. This is particularly because of high non production time (Cleaning/Sterilization/Cooling etc) & due to significantly long Lag/Stationery phases in this mode of cultivation. Generally the higher activity featuring exponential growth (Balanced growth wherein all components of the cell grow by same proportion) of the culture is observed for only 25-40 % of total cultivation time of batch cultivation wherein the culture produces the metabolites of interest in growth associated fermentation processes. Growth rate in batch fermentation is normally uncontrolled and is highest at the start. Productivity of batch fermentation: The productivity of batch fermentation is calculated by the final concentration of biomass or product being produced divided by the complete time of batch, which includes fermentation time and turnaround time (time for emptying, cleaning, sterilizing, and refilling) (Figure 21.2).

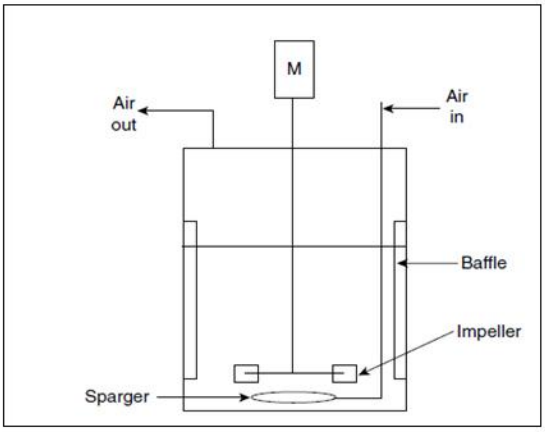

Fig. 21.1: Batch fermentation system (Crueger and A.Cruegar, 2000)

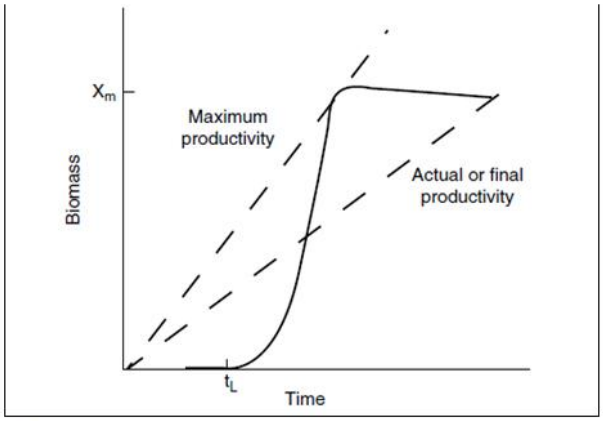

Fig. 21.2: Productivity in batch fermentation (Crueger and A.Cruegar, 2000)

If however, during the exponential phase of growth, a constant volume is maintained by ensuring an arrangement for a rate of broth outflow which equals the rate of inflow of fresh medium, then the microbial density (i.e., cells per unit volume) remains constant. This is the principle of continuous culture.

21.5. CONTINUOUS CULTURE

The concept of the continuous cultures dates from the 19th Century when a continuous process for the conversion of waste beers and wines to vinegar was developed. In this reactor, natural acetic acid bacterial populations were immobilized on wood shavings. Beer or wine was added through the top of the reactor and allowed to trickle through the shavings. Vinegar was collected at an outlet located at the base of the reactor.

It is an approach that provides a spectrum of exciting possibilities for studying bacteria under conditions that more closely resemble the way they grow naturally is continuous culture. In continuous culture, microorganisms are placed in an environment where the feed rate to the system and from the system is fixed. Thus, microorganisms experience a constant, and steady supply of limiting substrate and nutrients. Consequently, they can (over time) adjust their enzyme levels, pH and osmotic gradients, macromolecular composition etc. to achieve an “optimal growth”. This situation is generally referred to as “steady state” and may take up to 10-21 generations to achieve.

Continuous culture systems can be operated as chemostats or as turbidostats. In a chemostat the flow rate is set at a particular value with the help of a flow rate regulator and the rate of growth of the culture adjusts to this flow rate. That is, the sterile medium is fed-into the vessel at the same rate as the media containing microorganisms is removed.

21.5.1 . Chemostat

Continuous fermentation is an open system to maintain cells in a state of balanced growth by continuously adding fresh medium and removing the culture medium at the same rate. Essentially, the two modes of operation for continuous fermentation are chemostats and auxostats. The commonly used auxostats include turbidostats, the pHauxostat, and the nutristat. One type of system that is widely used for continuous cultivation is the chemostat. This system depends on the fact that the concentration of an essential nutrient within the culture vessel controls the growth rate of the cells. In general, one nutrient is limited to an amount that restricts growth, and the culture is removed at the same rate as nutrients are added (Figure 21.3).

The chemostat invented in the early 1940's marked the advent of serious continuous fermentation. Here, planktonic cultures in a fermentor are fed with a nutrient solution to maintain a bacterial population in the exponential or log phase of growth.The continuous culture reaches "balanced growth" in which the levels of bacteria, bacterial products, media components, and waste products are constant. This condition is referred to as "steady-state" growth. The culture volume and the cell concentration are both kept constant by allowing fresh, sterile medium to enter the culture vessel at the same rate that "spent" medium, containing cells, is removed from the growing culture. Under these conditions, the rate at which new cells are produced in the culture vessel is exactly balanced by the rate at which cells are being lost through the overflow from the culture vessel.

Presently, the chemostat is the most widely used apparatus for studying microorganisms under constant environmental conditions. It is a continuous fermentation

process performed in a Continuous Stirred Tank Reactor (CSTR). A CSTR operates by maintaining a growth rate through continuously feeding a growth limiting nutrient and withdrawing part of medium at the same rate, thereby achieving steady state growth. The growth limiting nutrient may be carbon, nitrogen, phosphorus, or any other essential nutrient, which influences the specific growth rate. A significant advantage of chemostat mode over batch mode is that by changing the feed rate of growth limiting nutrient, the growth rate can be varied .

21.5.1.1. Auxostat: An auxostat is a continuous culture technique wherein the dilution rate is regulated based on an indication of the metabolic activity of the culture. A chemostat is essentially used for operation at moderate to low dilution rates, but an auxostat is used at high dilution rates.

21.5.1.2. pHauxostat: In pH auxostat the feed rate is regulated by measurement and control of the pH of the fermentation medium. This can be applied only if there is a change in pH consequent to the growth of microorganism. The pHauxostat has been used for continuous mass cultivation of bacteria for isolation of intracellular products.

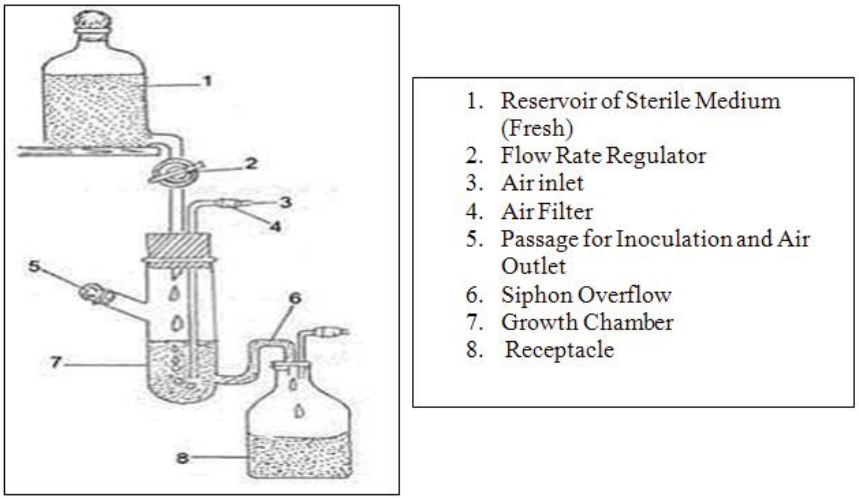

Fig. 21.3: Schematic representation of a chemostat (Najafpour, 2007)

21.5.2. Turbidostat

A turbidostat is a continuous culturing method where the turbidity of the culture is held constant by manipulating the rate at which medium is fed. If the turbidity tends to increase, the feed rate is increased to dilute the turbidity back to its set point. When the turbidity tends to fall, the feed rate is lowered so that growth can restore the turbidity to its setpoint. The problem of growth or other materials fouling the optical surfaces of whatever method is used to measure turbidity has not been solved. While a turbidostat may operate well for a brief time, the control signal for turbidity soon becomes unreliable. In a turbidostat the system includes an optical sensing device (photoelectric device) which continuously monitors the culture density in the growth vessel and controls the dilution rate to maintain the culture density at a constant rate (Figure 21.4). If the culture density becomes too high the dilution rate is increased, and if it becomes too low the dilution rate is decreased. The turbidostat differs from the chemostat in many ways (Table 21.1). The dilution rate in a turbidostat varies rather then remaining constant, and its culture medium lacks a limiting nutrient. The turbidostat operates best at high dilution rates; the chemostat is most stable and effective at low dilution rates.

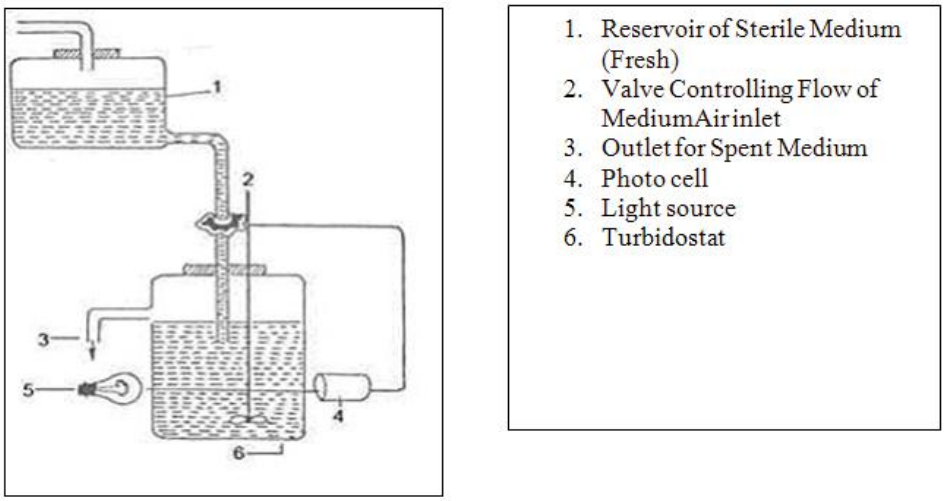

Fig. 21.4: Schematic representation of a turbidostat (Najafpour, 2007)

Table-21.1: Comparison between turbidostat and chemostat continuous flow cultures

Parameter	Turbidostat	Chemostat
Method of growth rate control	Internal	External
Growth rate of culture	At or close to μ_{max}	From just above zero to just below μ_{max}
Effect of increasing concentration of all nutrients in the medium reservoir	Biomass concentration will only be increased if the control settings on the photometer are changed	Increase in biomass concentration
Culture volume	Constant	Constant
Environmental conditions	Constant	Constant
Duration of culture	Indefinite	Indefinite
Types of mutants selected by prolonged culture	μ_{max} mutants and a range of neutral mutants	μ_{max} mutants at high dilution rates, K_s mutants at low dilution rates, and a range of neutral mutants

As discussed above, the stationary phase sets in partly because of the exhaustion of various *nutrients* and partly because of the introduction of an unfavorable environment produced by *metabolites* such as acid. Either of these two groups of factors can be used to maintain the culture at a constant density. In fed-batch systems fresh medium or medium components are fed continuously, intermittently or are added as a single supplement and the volume of the batch increases with time.

21.6. FED BATCH FERMENTATION

Fed batch fermentation, which is a technique in between batch and continuous fermentation, is a more recent development in industrial fermentation systems. Neither batch nor continuous fermentation is suitable for non growth associated products. In order to produce such products, it is first necessary to build up a high concentration of cells in the growth or batch phase, and then switch the metabolism of the

cell to arrest cell growth by feeding product precursors, carbon, and oxygen at a rate sufficient to meet the maintenance and product synthesis requirements. Essentially, fed batch fermentation involves two phases: growth phase and production phase. After the initial growth phase, one or more of the nutrients are supplied to the fermentor while cells and product remain in the fermentor.

Fed batch fermentation is well suited for production of compounds during very slow growth where there is no possibility of cell washout. Fed batch fermentation is well suited for producing product or cells when: (1) Substrate is inhibitory and there is a need to maintain low substrate concentration to avoid the cells being inhibited (e.g., citric acid, amylase), and (2) Product or biomass yields at low substrate concentrations are high (e.g.,baker's yeast, antibiotic production).

There are two methodologies in the fed batch approach, namely fixed volume fed batch and variable volume fed batch. In fixed volume fed batch fermentation, a very concentrated feed nutrient is fed to the fermentor so that there is no appreciable increase in volume. The specific growth rate decreases with time and the biomass increases directly with time. In variable volume fed batch fermentation, there is an increase in volume due to nutrient inflow and no outflow. The specific growth rate is solely dependent on the concentration of the limiting nutrient.

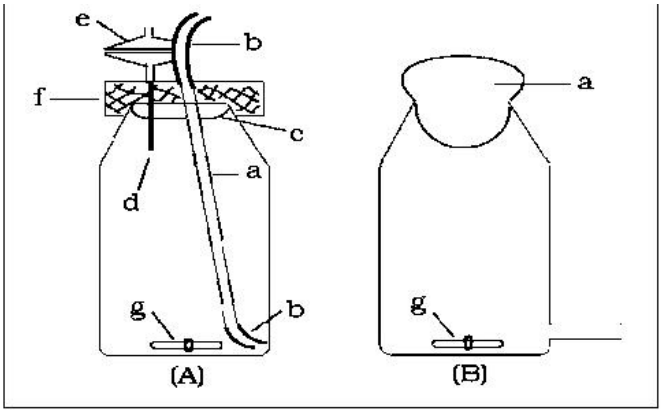

Fig.21.5: Holding vessels for batch type culture cultivation (Crueger and Cruegar, 2000)

Screw-neck borosilicate glass vessel with medium/inoculum addition assembly. (a) Stainless steel rod; (b) Silicon tubing; (c) Silicon disc; (d) Hypodermic needle; (e) Air vent; (f) Screw cap; (g) Magnetic bar. B. Aspirator-type vessel for introducing an inoculum of filamentous fungi into the fermentor. (a) Cotton-wool plug; (b) Magnetic stirrer bar.

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Module 6. Fermentation process strategies

Lesson-22

Types of Fermentation- Submerged, Solid and Surface Type

22.1 INTRODUCTION

Fermentation is one of the oldest technologies used for food preservation. Over the centuries, it has evolved, been refined and diversified. Today a variety of fermented foods is produced both in industrialised and developing countries using this technology at the s. A wide range of raw materials is used as substrates and panoply of products is concocted. Foods derived from fermentation are major constituents of the human diet all over the world. Although advances in food science and technology have given rise to a wide range of new food technologies, fermentation has remained an important technology throughout the history of mankind. Many benefits are attributed to fermentation. It preserves and enriches food, improves digestibility, and enhances the taste and flavour of foods. It is also an affordable technology and is thus accessible to all populations. Furthermore, fermentation has the potential of enhancing food safety by controlling the growth and multiplication of a number of pathogens in foods. Thus, it makes an important contribution to human nutrition, particularly in developing countries, where economic problems pose a major barrier to ensuring food safety.

Fermentation systems may be **liquid**, also known as **submerged** or **solid state**, also known as **surface**. Most fermentors used in industry are of the submerged type, because the submerged fermentor saves space and is more amenable to engineering control and design.

Fig. 22.1: Submerged fermentation unit (www.biocon.com)

22.2. SUBMERGED LIQUID FERMENTATIONS

Submerged liquid fermentations are traditionally used for the production of microbially derived enzymes. Submerged fermentation involves submersion of the microorganism in an aqueous solution containing all the nutrients needed for growth.

Fermentation takes place in large vessels (fermenter) with volumes of up to 1,000 cubic metres. The fermentation media sterilises nutrients based on renewable raw materials like maize, sugars and soya. Most industrial enzymes are secreted by microorganisms into the fermentation medium in order to break down the

carbon and nitrogen sources. Batch-fed and continuous fermentation processes are common. In the batch-fed process, sterilised nutrients are added to the fermenter during the growth of the biomass. In the continuous process, sterilised liquid nutrients are fed into the fermenter at the same flow rate as the fermentation broth leaving the system. Parameters like temperature, pH, oxygen consumption and carbon dioxide formation are measured and controlled to optimize the fermentation process.

Next in harvesting enzymes from the fermentation medium one must remove insoluble products, e.g. microbial cells. This is normally done by centrifugation. As most industrial enzymes are extracellular (secreted by cells into the external environment), they remain in the fermented broth after the biomass has been removed. The enzymes in the remaining broth are then concentrated by evaporation, membrane filtration or crystallization depending on their intended application. If pure enzyme preparations are required, they are usually isolated by gel or ion exchange chromatography.

Several types of submerged type of fermentors are known and they may be grouped in several ways: shape or configuration, whether aerated or anaerobic and whether they are batch or continuous. The most commonly used type of fermentor is the Aerated Stirred Tank Batch Fermentor.

22.2.1. Aerated Stirred Tank Batch Fermentor

A *typical* fermentor of this type (as shown in Fig 22.1 and Fig. 22.2) is an upright closed cylindrical tank fitted with one or more baffles attached to the side of the wall, a water jacket or coil for heating and/ or cooling, a device for forcible aeration (known as sparger), a mechanical agitator usually carrying a pair or more impellers, means of introducing organisms and nutrients and of taking samples, and outlets for exhaust gases. Modern fermentors are highly

automated and usually have means of continuously monitoring, controlling or recording pH, oxidation-reduction potential, dissolved oxygen, effluent O2 and CO2, and chemical components. Further diagrams of stirred tank fermentors are shown below

22.2.1.1. Aeration System (Sparger)

Sparger is a device for introducing air into fermenter. Aeration provides sufficient oxygen for organism in the fermenter. Fine bubble aerators must be used. Large bubbles will have less surface area than smaller bubbles which will facilitate oxygen transfer to a greater extent. Agitation is not required when aeration provides enough agitation which is the case Air lift fermenter. But this is possible with only for medium with low viscosity and low total solids.

For aeration to provide agitation the vessel height/diameter ratio (aspect ratin) should be 5:1.

Air supply to sparger should be supplied through filter.

There are three types of sparger viz. porous sparger, orifice sparger and nozzle sparger.

1. Porous sparger: made of sintered glass, ceramics or metal. It is used only in lab scale-non agitated vessel. The size of the bubble formed is 10-100 times larger than pore size. There is a pressure drop across the sparger and the holes tend to be blocked by growth which is the limitation of porous sparger.

2. Orifice sparger: used in small stirred fermenter. It is a perforated pipe kept below the impeller in the form of crosses or rings. The size should be ~ ¾ of impeller diameter. Air holes drilled on the under surfaces of the tubes and the holes should be at least 6mm diameter. This type of sparger is used mostly with agitation. It is also used with out agitation in some cases like yeast manufacture, effluent treatment and production of SCP.

3. Nozzle sparger: Mostly used in large scale. It is single open/partially closed pipe positioned centrally below the impeller. When air is passed through this pipe there is lower pressure loss and does not get blocked.

4. Combined sparger agitator: This is air supply via hallow agitator shaft. The air is emitted through holes in the disc or blades of agitator.

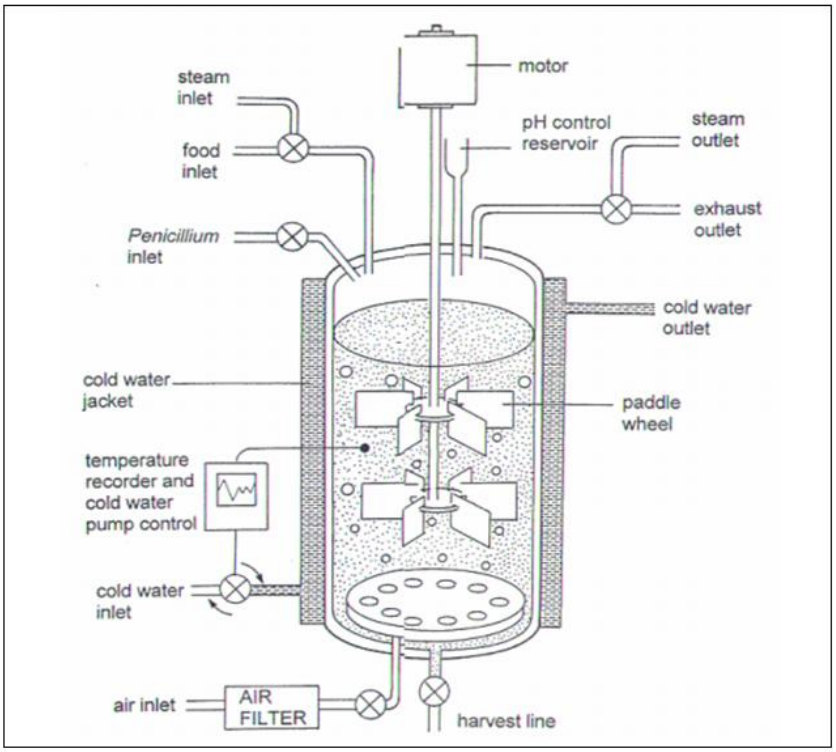

Fig. 22.2: An aerated stirred tank fermentor (Najafpour, 2007)

1.lid, **2** air-outlet, **3** fermenter, **4** module base permeable for air, **5** cultivation substrate, **6** cooling device, **7** air-inlet, **8** water supply, **9** water discharge, **10** heat resistant seal, **11** support device for the module base, **12** edge of the module base, **13** quick coupling, **14** pipe for the inflow and outflow of the cooling liquid, **15** heat resistant seal, **16** exterior ring, **17** coupling

22.3 Surface fermentation

In the surface techniques, the microorganisms are cultivated on the surface of a liquid or solid substrate. These techniques are very complicated and rarely used in industry. *A. niger* forms a mycelium layer on the liquid surface of the aluminum or stainless steel trays. These trays are stacked in fermentation rooms supplied with filtered air which serves both to supply oxygen and to control the temperature of fermentation. Surface fermentation is easy to control and to implement. It needs no aeration or agitation of the fermentation broth, so it needs no instrumentation for aeration and agitation. The separation of citric acid from the mycelium is easy because the microorganism is not dispersing into the medium. Only the temperature and humidity of the fermentation chamber need controlling. It can be used easily in small plants as well as in third world countries. With surface fermentation, the fermentation broth is concentrated due to a high evaporation rate during fermentation. Thus, expenses and losses during recovery and purification are low. However, surface fermentation has the following disadvantages: Building investment costs are high. Personnel expenses are high in developed industrial countries with extremely high wages. Fermentation time is long and therefore productivity is low.

Submerged fermentation is favored over surface fermentation for the following reasons

- Lower total investment costs;
- Improved process control;
- Reduced fermentation time;
- Reduced floor space requirements;
- Lower labour costs;
- Simpler operations; and
- Easier maintenance of aseptic conditions on an industrial scale.

However, submerged fermentation has some disadvantages compared to surface fermentation: expenses for equipment are higher; consumption of electrical energy is higher; and the process is very sensitive to short interruptions or breakdowns in aeration and vulnerable to infections, which result not only in losses of yield, but also in a total breakdown of respective batches

22.4 SOLID-STATE FERMENTATION (SSF)

The origin of Solid-state fermentation can be traced back to bread-making in ancient Egypt. Solid substrate fermentations also include a number of well known microbial processes such as soil growth, composting, silage production, wood rotting and mushroom cultivation. In addition, many familiar western foods such as mold-ripened cheese, bread, sausage and many foods of Asian origin including miso, tempeh and soy sauce are produced using SSF. Beverages derived from SSF processes include ontjom in Indonesia, shao-hsing wine and kaoliang (sorghum) liquor in China and sake in Japan.

SSF is used for the production of bioproducts from microorganisms under conditions of low moisture content for growth. The medium used for SSF is usually a solid substrate (e.g., rice bran, wheat bran, or grain), which requires no processing. In order to optimize water activity requirements, which are of major importance for growth, it is necessary to take into account the water sorption properties of the solid substrate during the fermentation. In view of the low water content, fewer problems due to contamination are observed. The power requirements are lower than submerged fermentation. Inadequate mixing, limitations of nutrient diffusion, metabolic heat accumulation, and ineffective process control renders SSF generally applicable for low value products with less monitoring and control. There exists a potential for conducting SSF on inert substrate supports impregnated with defined media for the production of high value products.

It involves the growth of microorganisms on moist solid particles, in situations in which the spaces between the particles contain a continuous gas phase and a minimum of visible water. Although droplets of water may be present between the particles, and there may be thin films of water at the particle surface, the inter-particle water phase is discontinuous and most of the inter- particle space is filled by the gas phase. The majority of the water in the system is absorbed within the moist solid-particles the more general term “solid-substrate fermentation” is used to denote any type of fermentation process that involves solids, including suspensions of solid particles in a continuous liquid phase and even trickling filters.

Advantages of Solid State Fermentation over Submerged Fermentation

1. Higher volumetric productivity
2. Usually simpler with lower energy requirements
3. Might be easier to meet aeration requirements
4. Resembles the natural habitat of some fungi and bacteria
5. Easier downstream processing
6. The fungal hyphae are bathed in a liquid medium and do not run the risk of desiccation;

7. Temperature control is typically not overly difficult, such that the organism is exposed to a constant temperature throughout its growth cycle;
8. The availability of O_2 to the biomass can be controlled reasonably well at a particular level of saturation of the medium
9. The availability of the nutrients to the organism can be controlled within relatively narrow limits if desired, through the feeding of nutrient solutions.
10. Although shear forces do occur within mechanically stirred bioreactors, the nature and magnitude of these forces are well understood and it is possible to use bioreactors that provide a low-shear environment, if the organism is highly susceptible to shear damage, such as bubble columns or air lift bioreactors;
11. pH control is relatively easy to provide.

In contrast, the environment in SSF can be quite stressful to the organism. For example:

1. Fungal hyphae are exposed to an air phase that can desiccate them;
2. Temperatures can rise to values that are well above the optimum for growth due to the inadequate removal of waste metabolic heat. In other words, the temperature to which the organism is exposed can vary during the growth cycle;
3. O_2 is typically freely available at the surface of the particle, however, there may be severe restrictions in the supply of O_2 to a significant proportion of the biomass that is within a biofilm at the surface or penetrating into the particle;
4. The availability of nutrients to the organism may be poor, even when the average nutrient concentration within the substrate particle, determined after homogenizing a sample of fermenting solid particles, is high. In other words, there tend to be large concentration gradients of nutrients within the particles; movement of the particles of the solid substrate can cause impact and shear damage. In the case of fungal processes the hyphae can suffer severe damage
5. It may be difficult to provide pH control under some circumstances.

However, there are certain instances in which, despite being more problematic, SSF may be appropriate:

1. When the product needs to be in a solid form (e.g., fermented foods);
2. When a particular product is only produced under the conditions of SSF or, if produced in both SLF and SSF, is produced in much higher levels in SSF. For example, certain enzymes are only induced in SSF and some fungi only sporulate when grown in SSF, in which the hyphae are exposed directly to an air phase. For example, *Monascus* pigment and many fungal spores are produced in much higher yields in SSF
3. If it is desired to use genetically unmodified organisms in a process for the production of such a product, then SSF may be the only option;
4. When socio-economic conditions mean that the fermentation process must be carried out by relatively unskilled workers. Some SSF processes can be relatively resistant to being overtaken by contaminants;
5. When the product is produced in both SSF and SLF, but the product produced in SSF has desirable properties which the product produced in SLF lacks. For example, spore-based fungal biopesticides produced in SSF processes are usually more resistant to adverse conditions than those produced in SLF, and are therefore more effective when spread in the field;
6. When it is imperative to use a solid waste in order to avoid the environmental impacts that would be caused by its direct disposal. This is likely to become an increasingly important consideration as the ever-increasing population puts an increasing strain on the environment.

Some examples of traditional SSF processes are:

1. Tempe, which involves the cultivation of the fungus *Rhizopus oligosporus* on cooked soybeans.

2. The *koji* step of soy sauce manufacture, which involves the cultivation of the fungus *Aspergillus oryzae* on cooked soybeans.
3. 'ang-kak', or "red rice", which involves the cultivation of the fungus *Monascus purpureus* on cooked rice.

Beyond this, over the last three decades, there has been an upsurge in interest in SSF technology, with research being undertaken into the production of a myriad of different products, including:

15. Enzymes such as amylases, proteases, lipases, pectinases, tannases, cellulases, and rennet;
16. Pigments;
17. Aromas and flavor compounds;
18. "Small organics" such as ethanol, oxalic acid, citric acid, and lactic acid;
19. Gibbrellic acid (a plant growth hormone);
20. Protein-enriched agricultural residues for use as animal feeds;
21. Animal feeds with reduced levels of toxins or with improved digestibility;
22. Antibiotics, such as penicillin and oxytetracycline;
23. Biological control agents, including bioinsecticides and bioherbicides;
24. Spore inocula (such as spore inoculum of *Penicillium roqueforti* for blue cheese production).
25. Decolorization of dyes;
26. Biobleaching;
27. Biopulping;
28. Bioremediation.

Bacteria, yeast and fungi can all grow on solid substrates and have applications in SSF processes. However, filamentous fungi are the best adapted species for SSF and dominate in the research and practical applications around the world. Bacterial SSF fermentations are rarely used for large scale enzyme production, but are very important in nature and in the fermented food industry. Filamentous fungi are the most important group of microorganisms for enzyme production in SSF. The hyphal mode of growth gives a major advantage to filamentous fungi over unicellular microorganisms in the colonization of solid substrates and the utilization of available nutrients. The filamentous fungi have the power to penetrate solid substrates. Hydrolytic enzymes are excreted at the hyphal tip, without large dilution. This makes the action of hydrolytic enzymes very efficient and allows penetration into most solid substrates. This is critical for the growth of the fungi. Fungi cannot transport macromolecular substrates across the cell wall, so the macromolecule must be hydrolyzed externally into soluble units that can be transported into the cell.

Fundamentally, there are 6 types of solid-state fermenters:

1. Tray bioreactor

2. Packed bed bioreactor

3. Rotary drum bioreactor

4. Swing solid state bioreactor

5. Stirred vessel bioreactor

6. Air solid fluidized bed bioreactor

The simplest SSF reactor is the tray. In a tray bioreactor a relatively thin layer of substrate is spread over a large horizontal area. There is no forced aeration, although the base of the tray may be perforated and air forced

around the tray. Mixing, if any, is by simple automatic devices or manual. Internal temperature may vary with ambient temperature; or the tray may be placed in a temperature-controlled room. Tray bioreactors have been used successfully at laboratory, pilot, semi-commercial and commercial scale.

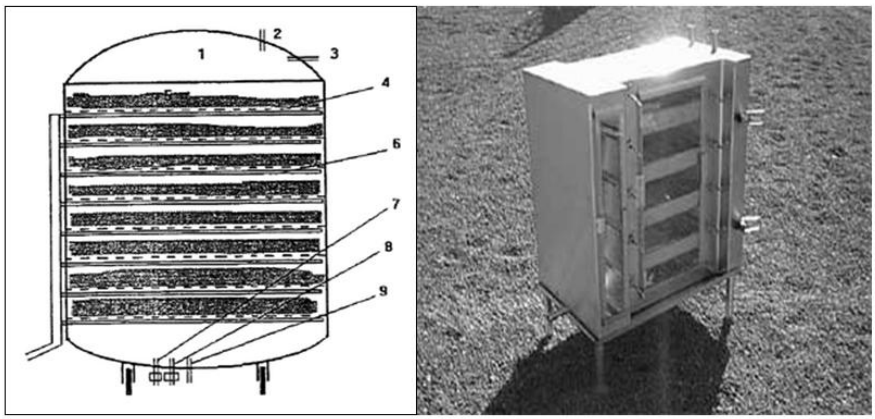

Fig 22.3 Structure of a tray type solid state fermentation system (Okafor, 2007)

22.4.1. Factors affecting enzyme production in solid state fermentation systems

The major factors that affect microbial synthesis of enzymes in a SSF system include: selection of a suitable substrate and microorganism; pre-treatment of the substrate; particle size (inter-particle space and surface area) of the substrate; water content and a_w of the substrate; relative humidity; type and size of the inoculum; control of temperature of fermenting matter/removal of metabolic heat; period of cultivation; maintenance of uniformity in the environment of SSF system, and the gaseous atmosphere, i.e. oxygen consumption rate and carbon dioxide evolution rate.

Current trends on SSF have focused on application of SSF for the development of bioprocess such as bioremediation and biodegradation of hazardous compounds, biological detoxification of agro-industrial residues, biotransformation of crops and crop-residues for nutritional enrichment, biopulping, and production of value-added products such as biologically active secondary metabolites, including antibiotics, alkaloids, plant growth factors, enzymes, organic acids, biopesticides, including mycopesticides and bioherbicides, biosurfactants, biofuel, aroma compounds, etc. SSF systems, which during the previous two decades were termed as a 'low-technology' system appear to be a promising ones for the production of value-added 'low volume–high cost' products such as biopharmaceuticals. SSF processes offer potential advantages in bioremediation and biological detoxification of hazardous and toxic compounds.

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Module 7. Bioreactor configuration

Lesson-23

Fermentors: Types, Functions, Design and Control

23.1. INTRODUCTION

The reliable operation of a fermentation system to achieve process objectives depends on two factors: the fermentor design and the fermentation process. Therefore, rational design of a fermentor requires careful consideration of how it is integrated into the process design. The design should consider from the outset such factors as plant scheduling, space constraints, relationships between fermentor productivity and throughput rates of downstream equipment, containment and validation requirements, utilities requirements, potential interruptions in normal plant operation, overall labor requirements and operating versus capital costs. Consistency, safety, cost, and compliance with statutory requirements usually are of prime concern for production equipment.

23.2. FERMENTOR AND BIOREACTOR

In general terms, a fermentor is something that, as its name would suggest, ferments. The process of fermentation has been known of for thousands of years, but has been mainly used over this time to the glucose found in various fruits, seeds and tubers into alcohol, later used for human consumption. In recent times, however, a fermentor is simply an optimal environment for bacteria and / or fungi to grow in, and the cultivation of said organisms will yield a desirable substance.

A bioreactor is a vessel in which is carried out a chemical process which involves organisms or biochemically active substances derived from such organisms. Bioreactors are commonly cylindrical, ranging in size from some liter to cube meters, and are often made of stainless steel. In brief bioreactor can be considered as a large scale operation whose volume/capacity ranges to several litres. Bioreactor is a system used for the growth and maintenance of a population of mammalian or insect cells whereas Fermentor is a system used for the growth and maintenance of a population of bacterial or fungal cells. There is also geometrical difference between Fermentor and Bioreactor taller vessels are used for bacterial processes to improve oxygen mass transfer whereas Shorter vessels for mammalian cel culture to improve mixing.

23.2.1. Bioreactor Configuration

23.2.1.1. Design Criteria

The objective of fermentor design is to produce fermentation systems necessary to build economical production facilities that satisfy well considered performance criteria. Reactor design and scale up considerations are driven by the need to provide the organism optimal conditions for producing the desired product uniformly in the reactor.

a) . Mechanical Aspects

Mechanical design aspects are important for the successful operation of any fermentation

plant. Some practical aspects of vessel design are:

1. Space requirements: The vessel dimensions must be chosen to meet plant space limitations. Poor choice of equipment sizes can cause inordinately high building costs.

2. Transportation: Shop built vessels are usually less expensive and of higher quality than field built vessels.

3. Special heads: A hemispherical bottom gives better mixing and fewer shears than does a standard dished head.

b) . Process aspects

The fermentation process guidelines commonly employed without proper consideration of the process aspects do very little to promote good design. These include:

1. Aeration rate: The airflow rate to the fermentor must be generally one volume of air per liquid volume per minute (1 vvm). However, in practice, the aeration rates depend on oxygen solubility in media, and 1 vvm may be too high for a particular fermentation process when operated at a larger scale.
2. Impeller tip speed: The tip speed of a fermentor impeller must not exceed 7.6 m/s.
3. Arrest of fermentative metabolism: At the end of fermentation, the fermentor broth must be cooled immediately and stored at 4°C, to arrest the fermentative metabolism.
4. The maximum production rate cell mass theory: Process optimization is achieved by obtaining very high cell mass concentrations at very high growth rates.
5. Oxygen transfer rate: The consequences of increasing OTR by increasing air flow rate and agitation could lead to foaming, increased gas holdup, higher gas velocities, higher vessel pressure, and oxygen enrichment.
6. Heat transfer rate: Heat transfer usually is the limiting constraint for highly aerobic large scale fermentors.

c). Jackets and coils

Jackets are used for the circulation of steam and cooling water during the heating and cooling cycles of sterilization of the fermentor. Since microbial reactions are exothermic, the heat produced during fermentations leads to a rise in the temperature of the broth, necessitating the need to maintain the temperature at the optimal value. Normally, steam is used as the heating fluid; and water, chilled water, or chilled brine is used as cooling fluids. The contact surface area of the jacket with the fermentor should be maximal and the pressure drop of the circulating fluids in the jacket should be minimal for better process performance. The layout of the external jackets could be a double jacket, a full pipe, or a half pipe (limpet coil), depending on the required area for heat transfer, the heating or cooling medium, and the circulation velocity. If internal cooling coils are used, they can not only remove the excess heat from fermentation, but also act as baffles for better mixing.

d). Safety codes

The safety of the vessel should be the foremost consideration in vessel design. The vessel must be fabricated in accordance with the Standard Code for unfired pressure vessels and tested at design conditions to insure that the vessel can withstand all forces generated under the specified operating conditions. If operation is required at high operating pressures, one should consider ways to minimize the metal thickness to allow the use of cold rolled sheet rather than plate. This results in better heat transfer, a better interior finish, and a lower price.

e). Material of construction

Stainless steel is the more commonly employed material for the fabrication of biotechnology equipment. The selection of the right steel quality in biotechnology is based on a compromise between material costs, availability, and the physical and chemical requirements of the process. The low carbon steels, SS 304L and SS 316L, are known worldwide as standard steels. Generally, vessels used in biological processes are fabricated with 316 or 316L steel. The vessels widely used in food technology or harvest storage tanks are fabricated with a cheaper and less corrosion resistant steel of grade 304 or 304L.

The selection of a vessel material for fabrication should take into consideration:

1. Sensitivity of the organism, particularly eukaryotic cells

2. Extent of vessel corrosion on exposure to fermentation media and utilities

3. Aseptic operation requires use of SS316, SS316L, SS304, or SS304L.

f). Baffles

Baffles are usually welded to the interior of the vessel. Baffles usually take the form of metal strips, roughly one tenth of the vessel diameter in width, extending vertically down the height of the vessel and attached radially to the wall (Figure 23.1). Removable baffles mean unsealed joints. Baffles should be set off from the wall, to minimize solids build up and to simplify cleaning. Slots between the baffle and the vessel wall prevent the formation of dead spots. The provision of baffles increases the turbulence in the liquid and results in a more efficient utilization of power.

Fig. 23.1: Baffles (www.googleimages.com)

g). Sparger

A device for introducing air below the liquid level in the fermentor vessel is called a sparger (Figure 23.2). The use of spargers with very small orifices is more efficient than a single orifice delivering the same volume of air. However, aerobic fermentations, e.g., penicillin, produce large quantities of mycelium and mechanical agitation

must be used in order to ensure adequate dispersion of air and other nutrients in large scale fermentations. As the degree of agitation is increased, the relative efficiencies of the various types of spargers tend to converge, and at high agitation levels, all spargers give approximately the same performance. The types of spargers in general use may be classified as nozzle spargers, orifice spargers, and porous spargers.

Fig. 23.2: Sparger (www.googleimages.com)

h). Nozzles and Manways

Nozzle design must take into consideration the following:

1. To ensure aseptic connections to external piping,
2. In order to facilitate free draining
3. For proper and safe cleaning of the nozzles, protrusions inside the vessel should be as minimal as possible.
4. Addition of feed through nozzles to the fermentor to take care that the added liquids do not dribble down the interior surfaces.
5. The use of manways obviates the need for full opening heads on larger vessels.

i). Piping and Valves

Piping materials: The most commonly used piping materials for biotechnology plants, in order of usage, are stainless steel, thermoplastics (polypropylene, polyethylene, polyvinylidine fluoride), carbon steel, copper, iron, glass, and lined pipe (glass and plastic liners). In sterile fermentation processes, a great deal of attention is necessary for the layout of lines and the construction of joints. Lines for sterile air and lines for transferring sterile mash are the subject of special care during installation to prevent the formation of pockets where liquid can collect. Adequate slopes, continuous in one direction, have to be given in what would be normally horizontal lines. Loops in lines are best excluded, but if unavoidable have to be provided with drain points so that residual mash and steam condensate can be removed. For purposes of sterilization, steam is introduced wherever possible at the highest point or points in the system, with the steam condensate removed from the lowest points. Valves used on sterile lines have given cause for thought for a considerable time. For robust industrial processes such as alcohol fermentation, or even for yeast cultivation, the use of the standard type of gate valve is normally acceptable. However, for fermentation systems more prone to contamination, the use of a standard valve has obvious shortcomings. The introduction of a diaphragm capable of standing up to steam for longer periods into the valve has encouraged a gradual changeover to the use of the diaphragm type of valve. Presently, with a diaphragm life of 3–4 months, the use of this type of valve for aseptic applications is justified.

j). Steam Locks

A good steam lock assembly should have the following features:

1. Elimination of the chances of dead spots with inefficient sterilization
2. Thorough steaming of sterile lines to be done during non usage to avoid contamination
3. Lines from feed addition tanks should be able to be steamed through their entire lengths at any time after they are connected to the assembly
4. Steam locks to be checked for leakage of steam during steaming
5. Self draining of condensate through steam traps
6. Easy cleaning and maintenance of the steam lock assembly

k). Welds and joints

Vessel welds of high quality are required to adhere to code purposes, ensure maximum smoothness and cleanability, and to minimize corrosion problems. Welding for aseptic fermentors should be carried out under an inert gas shield to minimize oxidation and flux residue, and create smoother, pit free welds. A flanged joint for sterile fermentation processes should be constructed with smooth bore continuity throughout the joint. Smooth bore continuity is important, and can be compromised by such factors as tolerances in bore and circularity of commercial tubing, coupled with the tolerances in the bore of such things as slip on welding flanges, and the standard clearance holes for flange bolts. If in addition, the joint is badly cut or badly placed, focal points for lack of sterility with subsequent contamination can easily be produced.

l). Surface treatment and finish

The surface treatment of a vessel is required for any surface that comes in contact with the product. It is imperative that all stainless steel surfaces are treated and cleaned in a way that prevents corrosion under the operating conditions. Stainless steels are corrosion resistant due to the formation of a microscopically thin, invisible chromium oxide layer, which occurs on clean metal and polished surfaces only. The three main surface treatment methods used are mechanical, chemical, and electrochemical.

Mechanical Surface Treatment: It is possible to improve the surface quality of steel parts in contact with the product by means of mechanical treatment. The surface finish qualities of the vessel exterior are governed more by aesthetic rather than functional attributes.

Chemical surface treatment: The two main types of chemical surface treatment are cauterization and passivation. The impurities that require cauterization may be interior surfaces with oxidation tints, welding slag residues, and fine scales or overlaps generated

during the working process. Passivation is used either after cauterization or as a final treatment of ground, brushed, or polished steels with special surface structures. A natural passive layer is invariably formed when stainless steel is exposed to air.

Electrochemical surface treatment: Electropolishing, or mirror polishing, is an electrochemical treatment which smoothes and polishes rough, dull surfaces. The vessel is immersed in an electrolyte and serves as an anode.

The quality of the surface and welds after surface treatments are checked with following methods:

1. Visual inspection
2. Detection of ferric contaminations with the ferroxly test
3. Palladium testing to check the surface passivation
4. Detection detergent residues
5. Detection of chloride or sulfur contamination
6. Detection of pickling damage

m). Agitation system design

Typical agitation equipment consists of the prime mover (usually an electric motor) coupled to the shaft through a reduction gear. Impellers and baffles are fitted to the shaft and vessel, respectively, to give the desired liquid motion. The shaft may enter from the top, side or bottom, and is usually fitted with a mechanical seal at the vessel wall. The number and location of impeller units depends on the vessel. In a smooth walled tank, the liquid swirls round in the same general direction as the agitator. As the impeller speed is increased, a vortex is formed and the liquid level at the wall is raised above the average liquid level. This is normally undesirable for the following reasons:

1. Power is wasted in holding up the liquid at the wall

2. The relative speed of the impeller to the liquid is reduced

3. Slight radial movements of the vortex cause the liquid to swirl unevenly, and undesirable side thrusts are set up

23.3. Biofermentor controls

Bioreactor or fermentation processes are the core manufacturing process in the biotech industry. Implementation delays and process upsets can result in the loss of millions of dollars in revenue through lost product and downtime. Because the bioreactor is such a critical component, getting it into production as quickly as possible and keeping it running, are essential to the profitability of a biotech operation. During implementation, many end users strive to reduce the I/O footprint of their control devices, since bioreactors use a wide-range of varied signals. Analog I/O points measure pressure, temperature and bring in flow rates for buffer and media. Discrete I/O controls peristalsic/ metering pumps and valves. Additionally, analytical probes control pH, dissolved oxygen and conductivity. Throughout the process, bioreactors need to maintain

precise control speed in the agitator to minimize shear. If the agitator creates too much turbulence, the microorganisms being grown may be torn apart. High rates of oxygen flowing through the sparge tube and improper agitator design can add to the problem of shear. The various types of biofermentor are shown in Figure 23.3. A comprehensive depiction of fermentor controls is shown in Figure 23.4.

23.3.1. Temperature Control

The temperature control system is designed using PID control algorithms. The liquid temperature is sensed and compared it with the desired temperature to form the error signal. The error signal is processed using control algorithms to produce the desired output to the heater driver. The feed back control system operates on the heating system to maintain the temperature at the required set value by reducing the error towards zero. Continuously variable controllers are designed to produce power to the heater proportional to the error signal. As the measured value approaches its desired value, the power fed to the heater progressively reduced. PID controllers are most commonly used controllers in temperature control.

23.3.2. pH control

In real life, bioreactors actually use on-off control for pH. The two position control system is designed; so that the element controlling reagent addition is always set in one of two positions, either fully open or fully closed. The important consideration in pH control is hold time, which is required to provide time for neutralization reaction to go to completion.

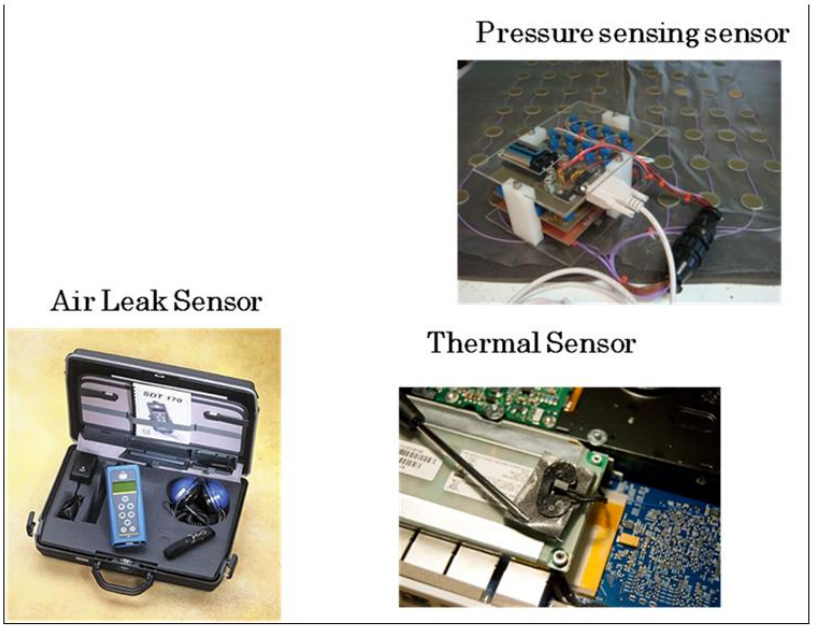

Fig.23.3: Biofermentor controls (www.googleimages.com)

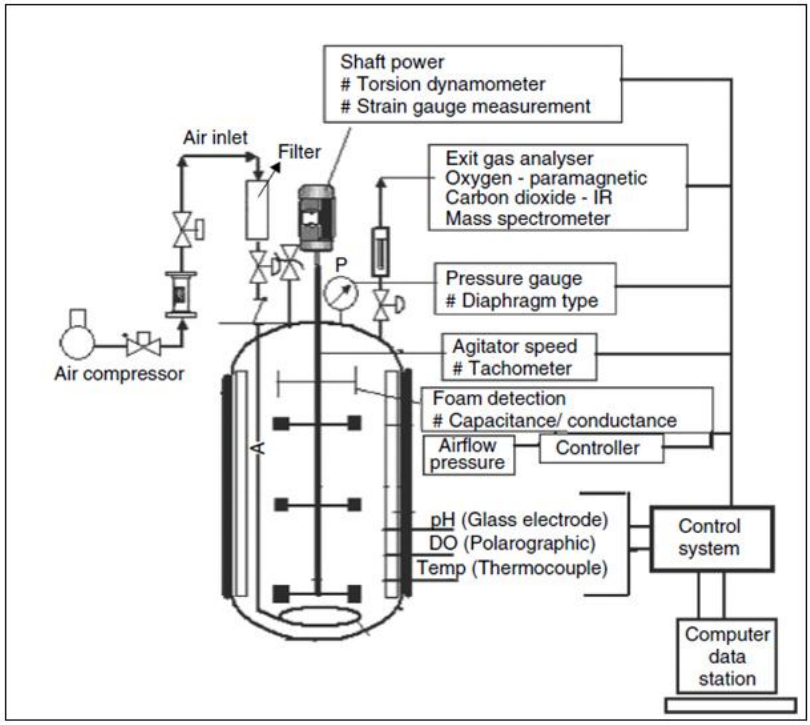

Fig. 23.4: A comprehensive depiction of fermentor controls (Okofer et al., 2007)

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Lesson-24

Submerged Fermentor System and Their Types

24.1. INTRODUCTION

The fermentor is the heart of any biochemical process in which microbial, mammalian, or plant cell systems are employed for the economic production of fermentation products. A properly designed fermentor should be used to provide an aseptic, controlled environment to facilitate optimal growth and product formation of a particular cell system.

24.2. STIRRED TANK BIOREACTOR (STR)

The most commonly used bioreactor for industrial applications is the conventional stirred tank reactor (STR) (Figure 24.1). The STR offers the advantages of high oxygen transfer rates required for high biomass productivity coupled with low investment and operating costs, which form the basis for any successful aerobic fermentation process. STRs typically have height to diameter ratios of 1:3 to 1:6. The agitator may be top driven or bottom driven depending on the scale of operation and other operational aspects. The choice of impeller depends on the physical and biological characteristics of the fermentation broth. Usually, a ring-type sparger with perforations is used to supply air to the fermentor. Baffles are provided to avoid vortex formation and improve mixing. Most fermentation processes use complex medium ingredients like corn steep liquor, molasses, and soybean flour as inexpensive nutritional sources (for carbon and nitrogen), supplemented with vital growth factors (amino acids, proteins, and vitamins) . The high turbulence imparted by the impellers in an STR can result in foaming due to the presence of proteinaceous substrates. Although chemical antifoaming agents (silicone or polypropylene glycol) can be added to control the foam, these can have detrimental effects on microbial growth and product recovery. In order to overcome this, mechanical methods of foam suppression such as rakes on the stirrer shaft mounted above the critical level have also been adopted. The emphasis on asepsis of the bioreactor, right from the end of the sterilization cycle to the end of the fermentation, has led to the maintenance of a minimum positive pressure in the fermentor to ensure sterility. A most important aspect of sterility is the point of contact between agitator shaft and vessel, which can be effectively sealed with a lubricated double mechanical seal. The sampling devices and injection ports must be contained in steam sterilizable closures.

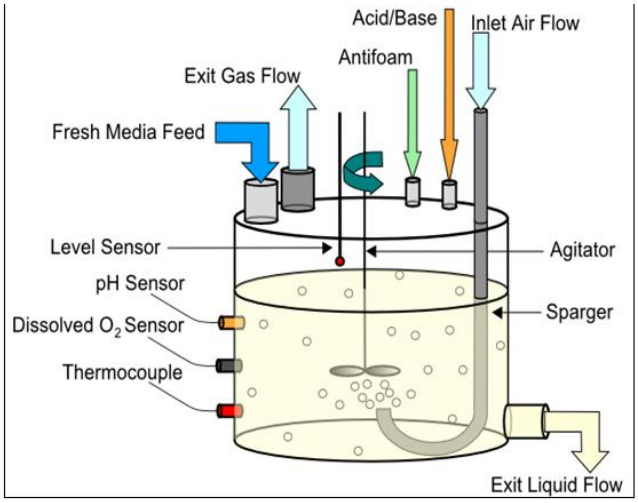

Fig. 24.1: Conventional stirred tank reactor (www.googleimages.com)

A Few important types are briefly described below

24.2.1. Stirred tank reactors

In these reactors, mechanical stirrers (using impellers) are used to mix the reactor to distribute heat and materials (such as oxygen and substrates)

24.2.2. Bubble column reactors

These are tall reactors which use air alone to mix the contents

24.2.3. Air lift reactors

These reactors are similar to bubble column reactors, but differ by the fact that they contain a draft tube. The draft tube is typically an inner tube which improves circulation and oxygen transfer and equalizes shear forces in the reactor.

24.2.4. Fluidized bed reactors

In fluidized bed reactors, cells are "immobilized" on small particles which move with the fluid. The small particles create a large surface area for cells to stick to and enable a high rate of transfer of oxygen and nutrients to the cells

24.2.5. Packed bed reactors

In packed bed reactors, cells are immobilized on large particles. These particles do not move with the liquid. Packed bed reactors are simple to construct and operate but can suffer from blockages and from poor oxygen transfer.

24.2.6. Flocculated cell reactors

Flocculated cell reactors retain cells by allow them to flocculate.

24.2.7. Air Lift Bioreactor

For fermentations that have low shear and energy requirements, an air lift reactor can be useful (Figure 24.2). The amount of air required for the fermentation process is usually sufficient to act as the sole source of liquid mixing. In this process, air pumped from the bottom of the reactor creates buoyant, bubbles, which exert a drag on the surrounding fluid. A riser and a “down comer” inside the bioreactor impose a circulating fluid pattern of movement, which provides for oxygenation and mixing of the fermentation broth. The bottlenecks associated with large scale air lift bioreactors are inadequate sterilization, higher capital investment, and aeration requirements. Since mixing in an air lift is solely caused by aeration, the power required for fluid circulation and dispersion can be higher than that needed by an agitator in a stirred tank bioreactor.

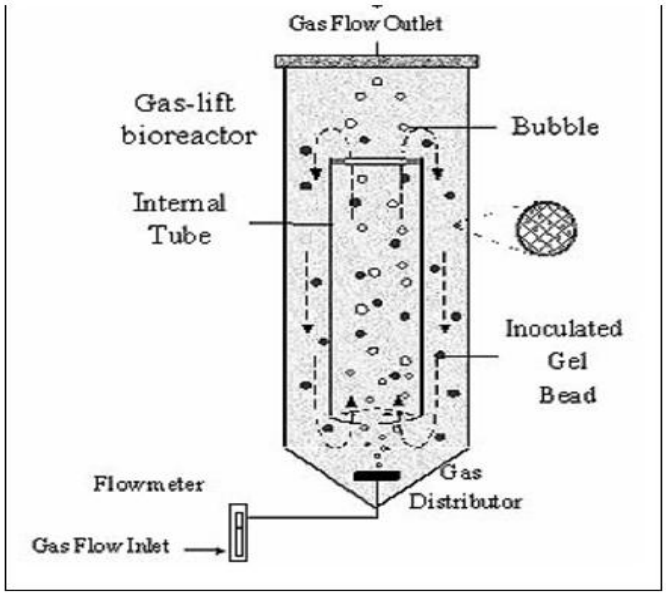

Fig. 24.2: Air lift bioreactor (Okofer et al., 2007)

Fluidized Bed Bioreactor

In the last few decades, there has been a significant increase reported in the application of fluidized bed reactor systems. These have been mainly used for cells that have been immobilized onto particulate matter. This has the advantage that a high density of particles can be used, and that the flow velocity required for the fluidization can be achieved independently of the reactor throughput. The main advantages of a fluidized bioreactor system as observed in ethanol production from *S.cerevisiae*) are superior mass and heat transfer characteristics, very good mixing between the three phases, relatively low energy requirements, and low shear rates (which makes a fluidized bed reactor suitable also for shear sensitive cells such as mammalian and plant cells). Fluidized bed reactors have been used with cells adsorbed inside the carrier, made either of glass or of ceramics. The upward feed flow rate in a fluidized bed bioreactor is high enough to provide fluidization of carriers, resulting in improved mixing properties and medium distribution; but this can also induce carrier abrasion and damage. In addition, fluidization of glass and ceramic carriers may require high medium flow rates that could result in higher pumping costs and eventually cell leakage. Gas liquid solid fluidized bed bioreactors have been employed for production of ligninolytic enzymes, treatment of wastewater from refineries, and raw wastewater.

In this type of bioreactor, no mechanical agitation is provided, but the medium can be manually agitated *in situ* or it can be transferred into a kneading machine and reloaded into the basket. The majority of mammalian cells need a solid surface such as a microcarrier or a packed

bed upon which to grow. The growth of cells on microcarrier beads depends directly on the surface available for growth up to the point where the microcarrier particles reach sufficient concentration to inhibit the cells and thus reduce cell yield. The toxicity of the support can cause long lag phases, death of the cells in the early stages of development, and limited cell yields. Microcarrier bioreactor systems (Figure 24.3) have been used for cultivation of human fibroblast cells to produce cell mass and in the production of interferon. A great advantage of microcarriers is the high surface area for cell growth provided under low shear conditions, while still allowing conventional fermentor equipment to be used. However, bead to bead and bead to impeller collisions, and hydrodynamic shear forces, may cause reduced viability.

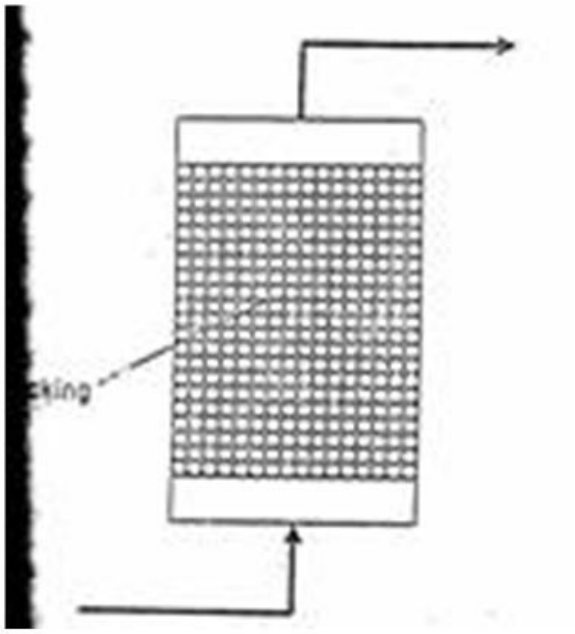

Fig. 24.3: Microcarrier bioreactor

24.2.9. Membrane Bioreactor

Membrane bioreactors comprising hollow fiber systems have been developed and tested for the growth of mammalian and plant cells, and for the immobilization of bacteria, yeast and enzymes. Hollow fiber reactors have been used in the enzymatic hydrolysis of cellulose, penicillin, starch, haemoglobin, protein synthesis, and the culture of plant cells and mammalian cells. The advantages of using a hollow fiber reactor for microbial systems include high density of cell growth, using a perfusion system for simultaneous separation of product and biomass, and biocatalyst regeneration. However, a major disadvantage is the difficulty in monitoring and controlling the growth and metabolism of the culture. Other process constraints associated with microbial hollow fiber reactors are low oxygen transfer rates at high cell density and blockage, and rupture of the membranes due to excessive growth. The accumulation of toxic products in the hollow fiber might also inhibit the metabolic activity of the cell system.

24.2.10. Photobioreactor

Microalgae have been used successfully, with high productivity compared to higher plants. The high productivity in these systems is due to the high biomass produced in the bioreactor. Microalgae have been used for preparation of vitamins, pigments, antioxidants, and fatty acids, and as feed for aquaculture. The cultivation techniques employed are open systems and closed or semiclosed outdoor photobioreactors. The common photobioreactors used are tubular-type and plate-type reactors The cyanobacterium *Spirulina platensis* has been studied in batch and continuous photobioreactors under varying conditions of incident light energy and nutrient limitations.

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Lesson-25

Solid Fermentor System and Their Types

25.1. INTRODUCTION

The main difference between submerged and solid-state fermentations is the amount of free liquid in the substrate. Solid-state fermentations (SSF) exhibit a poor conductive gas phase between the particles as compared to submerged fermentation. The presence of a wide variety of SSF matrices in terms of composition, size of solid substrate, mechanical resistance to air flow, porosity, and water holding capacity renders bioreactor design and control more difficult for the regulation of two important parameters, namely temperature and water content of the solid medium. Other factors that influence the bioreactor design are fungal morphological characteristics, resistance to mechanical agitation, and degree of asepsis required for the fermentation process.

25.2. CATEGORIES OF BIOREACTOR

Two categories of bioreactor exist for the SSF processes:

(i) At laboratory-scale, using quantities of dry solid medium from a few grams up to few kilograms, (ii) at pilot and industrial-scale, where several kilograms up to several tons are used. The first category comprises many designs, more or less sophisticated, while the second category, which is used mainly at industrial level, is markedly less varied .

However, based on similarities in design and operation, SSF bioreactors can be divided into groups on the basis of how they are mixed and aerated

Group I:

Bioreactors in which the bed is static, or mixed only very infrequently (i.e., once or twice per day) and air is circulated around the bed, but not blown forcefully through it. These are often referred to as “tray bioreactors”.

Group II:

Bioreactors in which the bed is static or mixed only very infrequently (i.e., once per day) and air is blown forcefully though the bed. These are typically referred to as “packed-bed bioreactors”.

Group III

Bioreactors in which the bed is continuously mixed or mixed intermittently with a frequency of minutes to hours, and air is circulated around the bed, but not blown forcefully through it. Two bioreactors that have this mode of operation, using different mechanisms to achieve the agitation, are “stirred drum bioreactors” and “rotating drum bioreactors”.

Group IV

Bioreactors in which the bed is agitated and air is blown forcefully through the bed. This type of bioreactor can typically be operated in either of two modes, so it is useful to identify two subgroups.

Group IVa bioreactors are mixed continuously while **Group IVb** bioreactors are mixed intermittently with intervals of minutes to hours between mixing events. Various designs fulfill these criteria, such as "gas-solid fluidized beds", the "rocking drum", and various "stirred-aerated bioreactors".

25.3. LABORATORY SCALE SSF BIOREACTOR

Small scale SSF equipment can be classified as those without forced aeration and agitation to include Petri dishes, jars, widemouth Erlenmeyer flasks, Roux bottles and roller bottles, and those incorporating continuous agitation of the solid medium such as a rotating drum bioreactor, a perforated drum bioreactor and a horizontal paddle mixer. The former are easy to operate in large numbers and commonly used for the screening of substrates or microorganisms for research purposes, while the latter offer the advantage of temperature control due to continuous agitation.

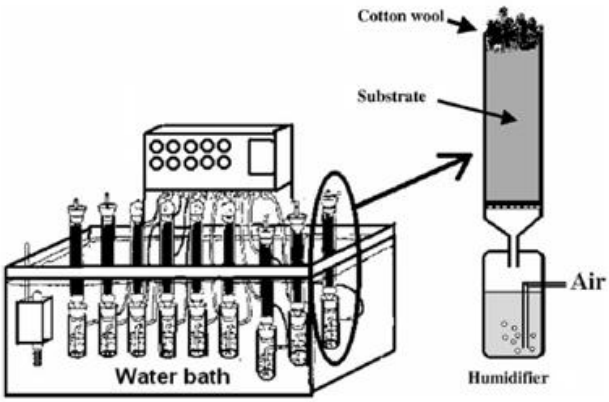


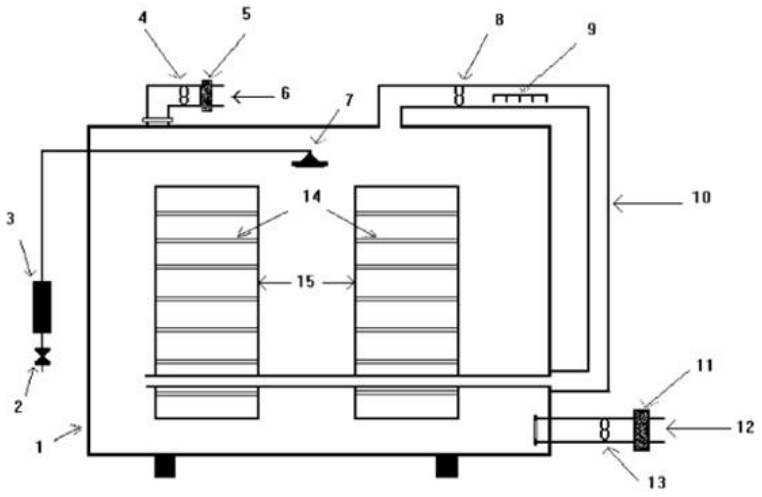
Fig. 25.1: Small scale SSF equipment (Okafor, 2007)

25.4. INDUSTRIAL SCALE SSF BIOREACTOR

Industrial scale SSF bioreactors can be built with or without aeration. Those without forced aeration can exhibit limitation of heat and mass transfer as the fermentation progresses, changing the properties of the microorganism involved, particularly in light of associated complexities like heat build up and inadequate oxygen transfer. However, with aeration strategies like circulation of air around the substrate layer or passing air through the substrate layer, these limitations are reduced to a certain extent.

25.4.1. SSF Bioreactor without forced aeration

On an industrial scale, this bioreactor is generally a tray fermentor. The trays containing the solid medium are stacked in tiers and placed in humidity and temperature controlled chamber. This technology has the limitations of not conforming to asepsis conditions, and of high labour requirements. However, it is easily scaled up by the incorporation of additional trays.



(Okafor, 2007)

Fig 25.2: Tray type bioreactors for making Koji: (1) Koji room, (2) water valve, (3) UV tube, (4, 8, 13) air blowers, (5, 11) air filters, (6) air outlet, (7) humidifier, (9) heater, (10) air recirculation, (12) air-inlet, (14) trays, (15) tray holders.

25.4.2. SSF Bioreactor with forced aeration and no mixing

In this type of bioreactor, no mechanical agitation is provided, but the medium can be manually agitated *in situ* or it can be transferred into a kneading machine and reloaded into the basket. However, this type of device without agitation is limited by the metabolic heat produced. Considerable temperature gradients can exist within the substrate bed. As the majority of the heat is removed and water is evaporated by forced aeration, the bed dries out, reducing fermentation efficiency. Periodic water addition in the form of spray is required to maintain the moisture content at desiredlevels.

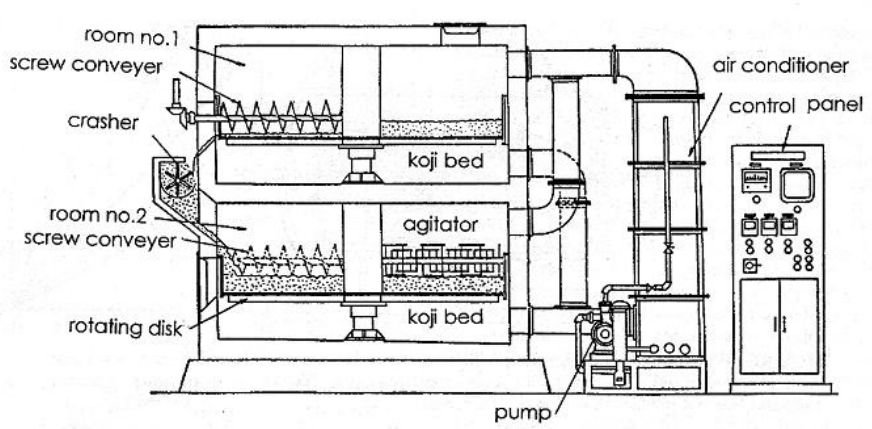


Fig- 25.3: Packed bed fermentor (Shetty et al., 2005)

Fig.25.4: Discontinuosly rotating drum (Shetty et al., 2005)

25.4.3. SSF Bioreactor with Continuous Mixing and Forced Aeration

A rotating drum bioreactor with continuous mixing maximizes the exposure of each substrate

particle to a thermostatic air circulating unit in the headspace. A large reactor, capable of handling 10 kg of steamed wheat bran as substrate, has been reported. Large scale use of unagitated SSF is limited by the difficulty in maintaining temperature during the fermentation. However, in a rotating drum bioreactor, efficient heat transfer is possible by convective and evaporative cooling. As the scale of fermentation increases, evaporative cooling becomes significant, because the ratio of the heat produced to the surface area available for convection decreases. The inherent difficulties encountered in the operation of solid-state fermentation systems on a large scale has led to new developments aimed at improving the efficiency of the fermentation process.

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Lesson-26

Upstream Processing and Downstream Processing

26.1. INTRODUCTION

Industrial fermentation involves upstream and downstream processes (Figure 26.1).

Upstream processes include selection of a microbial strain characterized by the ability to synthesize a specific product having the desired commercial value. This strain then is subjected to improvement protocols to maximize the ability of the strain to synthesize economical amounts of the product. Included in the upstream phase is the fermentation process itself which usually is carried out in large tanks known as fermenters or bioreactors. In addition to mechanical parts which provide proper conditions inside the tank such as aeration, cooling, agitation, etc., the tank is usually also equipped with complex sets of monitors and control devices in order to run the microbial growth and product synthesis under optimized conditions. The processing of the fermentation reactions inside the fermenter can be done using many modifications of engineering technologies. One of the most commonly used fermenter types is the stirred-tank fermenter which utilizes mechanical agitation principles, mainly using radial-flow impellers, during the fermentation process.

Downstream processing , the various stages that follow the fermentation process, involves suitable techniques and methods for recovery, purification, and characterization of the desired fermentation product. A vast array of methods for downstream processing, such as centrifugation, filtration, and chromatography, may be applied. These methods vary according to the chemical and physical nature, as well as the desired grade, of the final product.

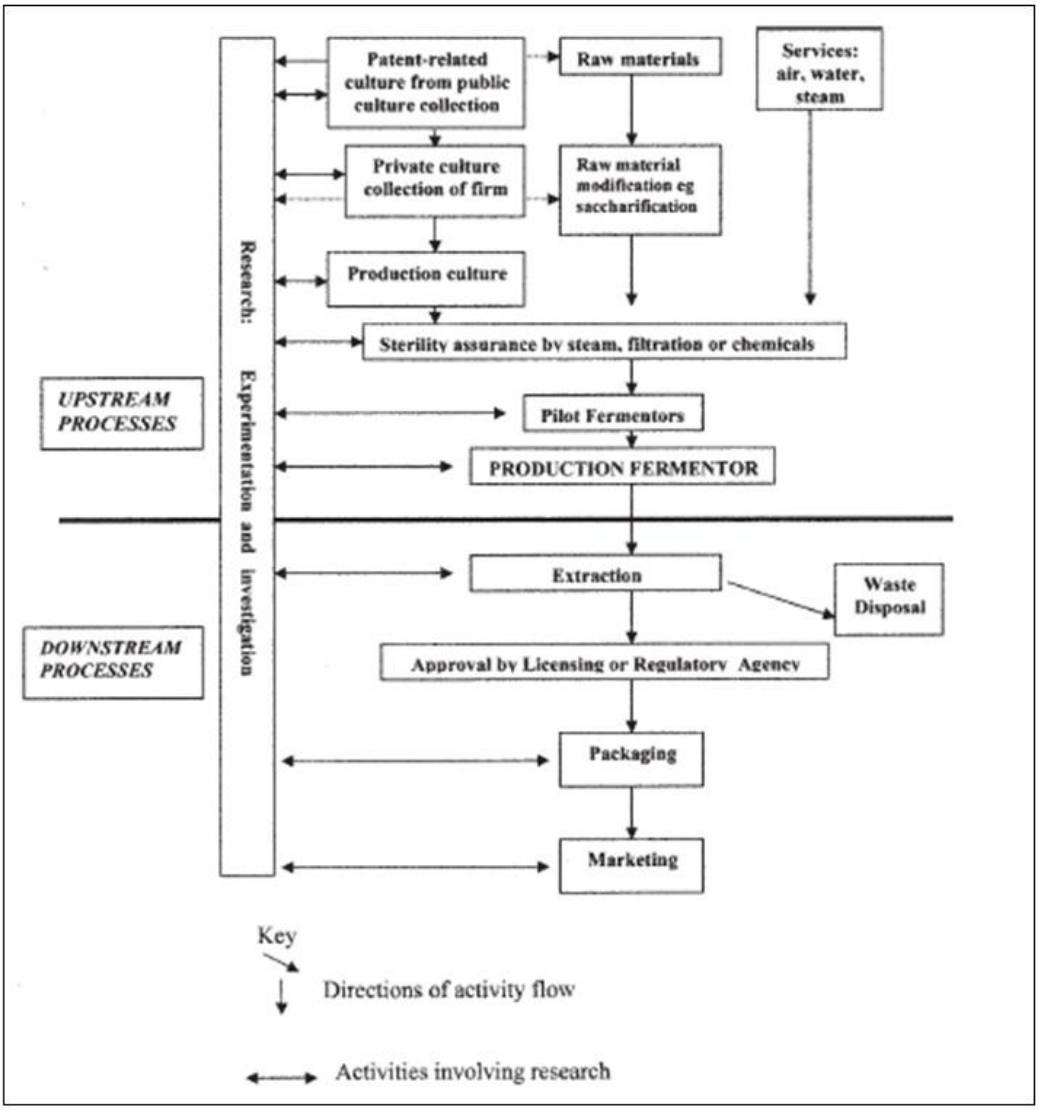

Fig. 26.1: Stages in a Fermentation Process (Waites et al., 2001)

26.2. OVERVIEW OF UPSTREAM PROCESSING

Upstream processing normally deals with three important points.

The first relates to fermentation media, especially the selection of suitable cost effective carbon and energy sources, along with other essential nutrients. The media optimization is a vital aspect of process development to ensure maximization of yield and profit.

The second aspect involves aspects associated with the producer microorganism. They include the strategy for initially obtaining a suitable microorganism, industrial stain improvement to enhance productivity and yield, maintenance of strain purity, preparation of a suitable inoculums and continuing development of selected strains to increase the economic efficiency of the process.

The third component relates to the **fermentation** which is usually performed under rigorously controlled conditions developed to optimize the growth of the organism or the production of a target microbial product.

26.2.1. Fermentation medium

The medium used for fermentation may be classified as defined, complex or technical medium. Defined medium consists only of precisely chemically defined substrates. Complex medium is composed of substrates with undefined composition, such as extracts or hydrolysates from waste products, which are cheap substrates commonly used in industrial production. Relatively expensive substrates, such as yeast extract, brain heart infusion, peptone, and tryptone are often used for complex medium. Technical media are used on an industrial scale and are cheaper. The substrate sources can also be derived from industrial waste, and are often highly impure mixtures, requiring pretreatment before they could be used for a fermentation process. Examples are soy meal, whey, fishmeal, malt extract, and sulfite waste liquor. Wastewater from monosodium glutamate production, which contains high levels of chemical oxygen demand (COD), sulphate, and ammoniacal nitrogen at a low pH, has been used as the nitrogen and water source, with sugar beet pulp as the carbon source, for the production of pectinase.

Media sterilization is necessary to ensure that only the desired microorganism is present to carry out the fermentation, that products are made of predicted quality, that the environment is protected from undesirable contamination, and that deterioration (microbial spoilage) of products is prevented. Sterilization by high temperature achieved by direct or indirect steam or electric heating, membrane filtration, microwave irradiation, high voltage pulses and photosemiconductor powders which involve the rupture of the cell membrane by increasing the transmembrane electric field strength beyond a certain threshold.

Inoculation is the transfer of seed material or inoculum into the fermentor. Inoculation of a laboratory fermentor is generally done using presterilized tubing and a peristaltic pump. However, on a larger scale, inoculum transfer is done by applying a positive pressure on the inoculum fermentor and connecting it aseptically to the production fermentor. The connecting lines are sterilized before being used for transfer of inoculum. Heat susceptible substances such as amino acids and some vitamins must be dissolved in small volumes of water, sterilized by filtration and added separately to the final medium aseptically.

26.2.2. Fermentation systems

A fermentation system is usually operated in one of the following modes: batch, fed batch, or continuous fermentation. The choice of the fermentation mode is dependent on the relation of consumption of substrate to biomass and products. The systems are batch, continuous and fed batch systems that were described earlier in lesson 21.

Today the most common type of upstream processing of proteins utilizes two tools: bioreactors a nd suspension (or attached) cells transformed with expression vectors genetically engineered to contain one (or more) human genes that produce copious amounts of their protein(s).

26.2.3. Innoculum

Upstream processing of proteins using bioreactors and cells usually begins with the preparation of the inoculum which proceeds in scale-up steps until enough inoculum is made to aseptically inoculate the final, sterile, media-filled bioreactor.

During the culture period samples are removed, aseptically, and various parameters are measured by fermentation technicians or operators including optical density (OD) and live cell count. Samples are also brought to quality control where other parameters may be measured such as the levels of glucose, lactate and ammonia, as well as the identity and concentration of the human protein that the cells are producing. Also part of upstream processing are the initial purification steps which could include centrifugation and/or filtration in order to separate cells from media. The cells or the media would be discarded to the kill tank, depending on

where the protein was located. In this course we are using glass bioreactors and representative of three types of cells used in upstream processing of human protein pharmaceuticals: bacterial, animal, and fungal cells. In bacteria, such as biotechnology's workhorse, *Escherichia coli* , *Pichia pastoris* , proteins are secreted into the media so the media is saved for later isolation and purification of the protein of interest in downstream processing. proteins remain inside the cell so the cells are separated from the media and the media is discarded to the kill tank. In animal cells, such as Chinese Hamster Ovary (CHO) cells, and in fungal cells, such as the yeast

26.3. OVERVIEW OF DOWNSTREAM PROCESSING

Downstream processing encompasses all processes following the fermentation. It has the primary aim of efficiently, reproducibly and safely recovering the target product to the required specifications (biological activity, purity) while maximizing recovery yield and minimising costs. The target product may be recovered by processing the cells or the spent medium depending upon whether it is in intracellular or extracellular product. The level of purity that must be achieved is usually determined by the specific use of the product.

Each stage in the overall recovery procedure is strongly dependent on the protocol of the preceding fermentation. Fermentation factors affecting downstream processing include the properties of microorganisms, particularly morphology, flocculation characteristics, size and cell wall rigidity. These factors have major influences on the filterability, sedimentation and homogenization efficiency. The presence of fermentation by-products, media impurities and fermentations additives such as antifoams may interfere with downstream processing steps and accompanying product analysis.

The products of fermentation are usually found in complex mixtures of dilute solutions and must be concentrated and purified. The separation of the product of interest from the

fermentation broth depends on the accumulation of the product, which may be intracellular or extracellular.

The typical downstream operations and the unit operations involved in the processing of fermentation broth are:

1. Cell disruption (high pressure homogenization, wet milling, and lysis)
2. Clarification of extract (centrifugation, extraction, dead end filtration, and cross flow filtration)
3. Enrichment (precipitation, batch adsorption, ultrafiltration, and partition)
4. High resolution techniques (ion exchange, affinity, hydrophobic, gelfiltration, adsorption chromatography, and electrophoresis)

Concentration (sterile filtration, diafiltration, ultrafiltration, freeze drying, spray drying, and precipitation).

Fig. 26.2: Continuous centrifugation wherein the media and cells get in & clarified media go out

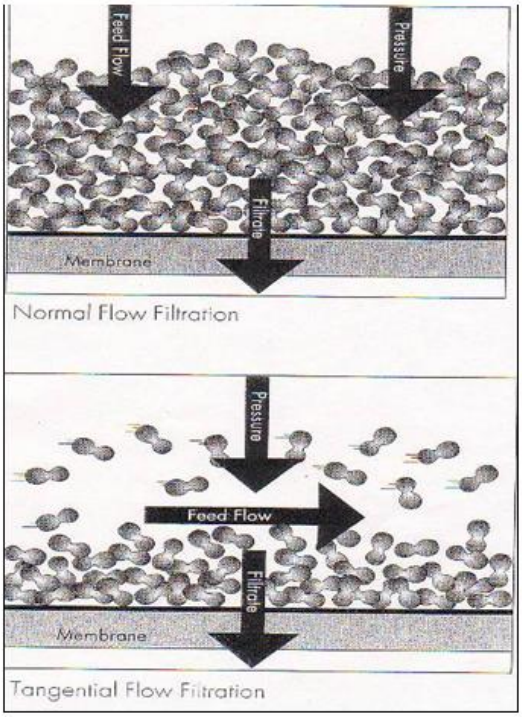

Fig. 26.3: Filtration methods wherein separation of particles from liquid occur by applying a pressure to the solution to force the solution through a filter.

Fig. 26.4: Large scale tangential flow filtration methods (wwww.biomanufacturing.com)

26.3.1. High resolution techniques like Chromatography techniques

The molecules of interest are adsorbed or stuck to beads packed in the column. The higher the affinity of the molecule (protein) for the bead the more will be bound to the column at any given time. Proteins with a high affinity travel slowly through the column because they are stuck a significant portion of the time. Molecules

with a lower affinity will not stick as often and will elute more quickly. We can change the relative affinity of the protein for the column (retention time) and mobile phase by changing the mobile phase (the buffer).

The most common type of adsorption chromatography is ion exchange chromatography. The others used in commercial biopharmaceutical production are affinity, hydrophobic interaction and gel filtration.

Column chromatography Separates molecules by their chemical and physical differences

Most common types: Size exclusion (Gel filtration): separates by molecular weight, Ion exchange: separates by charge, Affinity chromatography: specific binding Hydrophobic Interaction: separates by hydrophobic/hydrophilic characteristics.

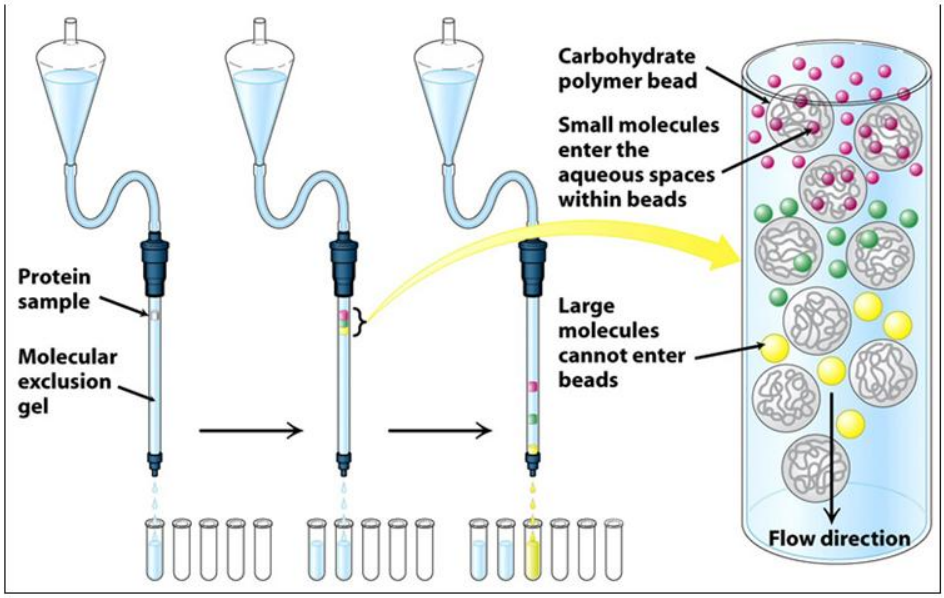

Fig. 26.5: Gel filtration chromatography (wwww.biomanufacturing.com)

26.3.2. Ion Exchange Chromatography

Ion Exchange Chromatography relies on charge-charge interactions between the protein of interest and charges on a resin (bead). Ion exchange chromatography can be subdivided into cation exchange chromatography, in which a positively charged protein of interest binds to a negatively charged resin; and anion exchange chromatography, in which a negatively charged protein of interest binds to a positively charged resin.

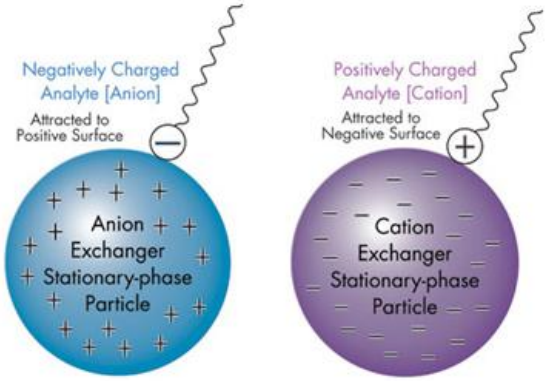

Fig. 26.6: Ion Exchange Chromatography (wwww.biomanufacturing.com)

26.3.3. Isoelectric Focusing

Once the pI of your protein is known (or the pH at which your protein is neutral), you can place it in a buffer at a lower or higher pH to alter its charge. If the pH of the buffer is less than the pI, the protein of interest will become positively charged. If the pH of the buffer is greater than the pI, the protein of interest will become negatively charged.

26.3.4. Affinity chromatography

Affinity chromatography separates the protein of interest on the basis of a reversible interaction between it and its antibody coupled to a chromatography bead (here labeled antigen). With high selectivity, high resolution, and high capacity for the protein of interest, purification levels in the order of several thousand-fold are achievable.

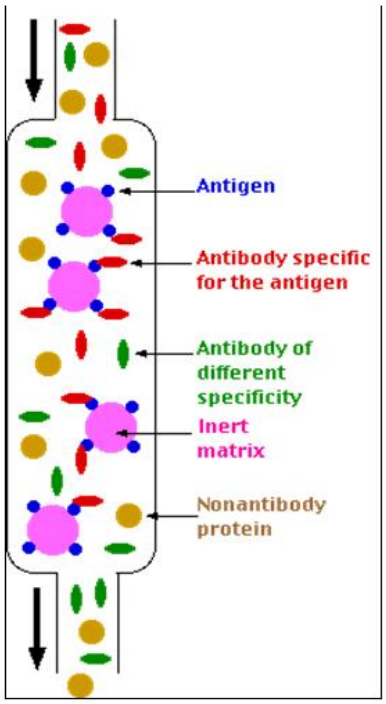

Fig. 26.7: Affinity Chromatography (wwww.biomanufacturing.com)

26.3.5. Hydrophobic Interaction Chromatography (HIC)

HIC is finding dramatically increased use in production chromatography. Antibodies are quite hydrophobic and therapeutic antibodies are the most important proteins in the biopharmaceutical pipeline. Usually HIC media have high capacity and are economical and stable. Adsorption takes place in high salt and elution in low salt concentrations.

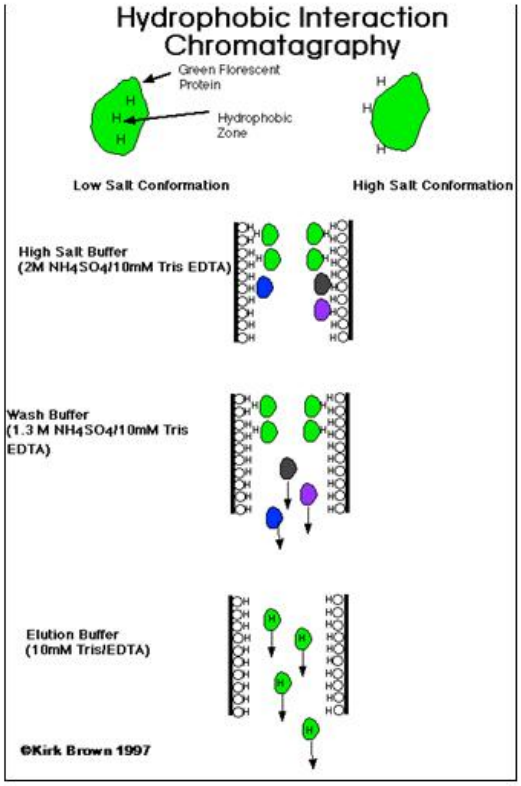

Fig. 26.8: Hydrophobic Interaction Chromatography (HIC)

Table-26.1: Common process compounds and methods of purification or removal

Component	Culture Harvest Level	Final Product Level	Conventional Method
Therapeutic Antibody	0.1-1.5 g/l	1-10 g/l	Ultra Filtration (UF)/ Cromatography
Isoforms	Various	Monomer	Chromatography
Serum and host proteins	0.1-3.0 g/l	< 0.1-10 mg/l	Chromatography
Cell debris and colloids	10^6/ml	None	Membrane Filtration (MF)
Bacterial pathogens	Various	$<10^{-6}$/dose	MF
Virus pathogens	Various	$<10^{-6}$/dose (12 LRV)	virus filtration
DNA	1 mg/l	10 ng/dose	Chromatography
Endotoxins	Various	<0.25 EU/ml	Chromatography
Buffer	Growth media	Stability media	UF
Extractables/leachables	Various	<0.1-10 mg/l	UF/ Chromatography
Purification reagents	Various	<0.1-10mg/l	UF

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Module 9. Microbial Process Strategies

Lesson-27

Single Cell Protein and Industrial Alcohol

27.1. INTRODUCTION

Microbial primary metabolites used in the food and feed industries include: alcohols (ethanol), amino acids (monosodium glutamate, lysine, threonine, phenylalanine, and tryptophan), flavor nucleotides, organic acids (acetic, propionic, succinic, fumaric, and lactic), polyols (glycerol, mannitol, erythritol, and xylitol), polysaccharides (xanthan and gelan), sugars (fructose, ribose, and sorbose), and vitamins (riboflavin, cyanocobalamin, and biotin). Over the last three decades, traditional industrial microbiology, using the tools of molecular biology, has led to the development of recombinant organisms aimed at production of high value biopharmaceutical products such as erythropoietin, human growth hormones and interferons. The major microbial hosts for the production of recombinant proteins are *E. coli*, *B. subtilis*, *S. cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha* and *Aspergillus niger*.

27.2. SINGLE CELL PROTEIN

In the 1950s and 1960s concern grew about the 'food gap' between the industrialized and

the less industrialized parts of the world. As a result of this concern, alternate and unconventional sources of food were sought. It was recognized that protein malnutrition is usually far more severe than that of other foods. The hope was that microorganisms would help meet this world protein deficiency.

However, the limitations of conventional sources of proteins were recognized. These include: (a) possible crop failure due to unfavorable climatic conditions in the case of plants; (b) the need to allow a time lapse for the replenishment of stock in the case of fish; (c) the limited land available for farming in the case of plant production.

On the other hand the production of Single cell protein (SCP) has a number of attractive features: (a) it was not subject to the vicissitudes of the weather and can be produced every minute of the year. (b) Microorganisms have a much more rapid growth than plants or animals.

SCP is itself not entirely lacking in disadvantages. One of the most obvious is that many developing countries, where protein malnutrition actually exists, lack the expertise and/or the financial resources to develop the highly capital intensive fermentation industries involved. But this short-coming can be bridged by the use of improvised fermentors and recovery methods which do not require sophisticated equipments. Other criticisms of SCP are that microorganisms contain high levels of RNA and that its consumption could lead to uric acid accumulation, kidney stone formation and gout

Organisms to be used in SCP production should have the following properties:

(a) Absence of pathogenicity and toxicity: It is obvious that the large-scale cultivation of

organisms which are pathogenic to animals or plants could pose a great threat to health and therefore, should be avoided. The organisms should also not contain or produce toxic or carcinogenic materials.

(b) Protein quality and content: The amount of protein in the organisms should not only be high but should contain as much as possible of the amino acids required by man.

(c) Digestibility and organoleptic qualities: The organism should not only be digestible,

but it should possess acceptable taste and aroma.

(d) Growth rate: It must grow rapidly in a cheap, easily available medium.

(e) Adaptability to unusual environmental conditions

In order to eliminate contaminants and hence reduce the cost of production, environmental conditions which are antagonistic to possible contaminants are often advantageous. Thus, strains which grow at low pH conditions or at high temperature are often chosen. The heterotrophic microorganisms currently used are bacteria (and actinomycetes and fungi (moulds and yeasts); protozoa have not been used in SCP production. Of the substrates currently in use, the gaseous hydrocarbons (methane, propane, butane) are almost exclusively attacked by bacteria. Liquid hydrocarbons (n-paraffins, gas oil, diesel oil) and alcohols are utilized by both bacteria and yeasts. Fungi have the advantage that they are lower in RNA content and are easily harvested.

Table-27.1: Organisms and substrates which have been used for single cell protein production

Gaseous hydrocarbons	*Bacteria*	*Fungi*
i) Methane	*Methanomones* sp. *Methylococius capsulatus* *Pseudononas* sp. *Hyphomisobium* sp. Mixed *Acinetobacter* sp.	
ii) Propane	*Flavobacterium* sp. *Arthrobacter simplex*	
iii) Butane	*Nocardia paraffinica* *Nocardia paraffinica*	
Liquid Hydrocarbons:		
i) n-Alkanes (C_{10}-C_{30}) carbon length	*Mycobacterium phlei* *Nocardia* sp.	*Candida guillermondi* *Candida lipolytica*
ii) n-Alkanes (unspecified)		*Candida Kofuensis* *Candida tropicals*
iii) Liquified petroleum gas		*Candida lipolylica* *Candida rigida*
iv) Gas oil	*Acinetobacter* *Pseudomonas*	*Candida tropicalis* *Candida lipolytica*
v) Diesel oil	*Acromobacter delcavate*	
Alcohols	*Methylomonas Methanolica*	*Torulopsis methansoba*
Methanol	*Methyliphilus (Pseudomonas) methylotrophus*	*Toralopsis methanolove* *Candida boidini*
Ethanol	*Methylomonas clara*	*Hansemula polymorpha* *Candid ethanomorphium* *Candida tropicalis*

Plant/Wood Wastes		
Sulphite liquor	*Thermomonespore fusca*	*Paecylomyces variotii*
Cellulose pulping fines	*Brevibacterium* sp.	*Candida utilis*
Mesquite wood		
Wheat bran	*Rhodopseudomonas glatinosa*	
Wastes from carb,		*Fusarium sp*
Palms, papaya, etc.		*Aspergillus sp*
Starch Wastes		
Potato hydrolysate		*Rhodotromla rubra*
Tapioca (Cassava) starch		*Candida tropicalis*
Diary Wastes		*Endomycopsis*
		Libuligera
Whey		*Kluyveromyces fragilis*
		Trichosporon cutaneum
Sugar Wastes		
Molasses		*Candida utilis*
Chemical Industries Wastes:		
Oxanone Waste Water		*Trichosporon cutaneum*
		Candida pseudotropicalis
Waste polyethylene		

27.2.1. Nucleic Acids and their Removal from SCP

Apart from the fears of carcinogenicity and toxicity from petroleum derivatives mentioned above, both of which fears have been allayed in extensive studies, another area of concern in SCP feeding is the consumption of high levels of nucleic acid. Man has lost the enzyme uricase which oxidizes uric acid to the soluble and excretable allantoin. When nucleic acid is eaten by man, it is broken up by nucleases present in the pancreatic juice, and converted into nucleosides by intestinal juices before absorption. Guanine and adenine are converted to uric acid, which as had been pointed out earlier cannot be converted to the soluble and excretable allantoin. As a result when foods rich in nucleic acid are consumed in large amounts, an unusually high level of uric acid occurs in the blood plasma. Owing to the low solubility of uric acid, uricates may be deposited in various tissues in the body including the kidneys and the joints when the diseases known as kidney stones and gout may respectively result.

27.2.2. Various ways have been devised for the removal of nucleic acids from SCP.

(a) **Growth and cell physiology method** : The RNA content of cell is dependent on growth rate: the higher the dilution rate (in continuous cultures) the higher the RNA/ protein ratio. In other words the higher the growth rate the higher the RNA content. The growth rate is therefore reduced as a means of reducing nucleic acid. It must however be borne in mind that high growth is one of the requirements of reducing costs in SCP, hence the method may have only limited usefulness.

(b) **Extraction with chemicals** : Dilute bases such as NaOH or KOH will hydrolyze RNA

easily. Hot 10% sodium chloride may also be used to extract RNA. The cells usually have to be disrupted before using these methods. In some cases the protein may then be extracted, purified and concentrated. Use of pancreatic juice: RNAase from bovine pancreatic juice, which is heat-stable, has been used to hydrolyze yeast RNA at 80°C at which temperature the cells are more permeable.

(d) *Activation of endogenous RNA*: The RNAase of the organism itself may be activated

by heat-shock or by chemicals. The RNA content of yeasts have been reduced in this way.

27.2.3. The fermentation process

The fermentation process requires a pure culture of the chosen organism that is in the correct physiological state, sterilization of the growth medium which is used for the organism, a production fermenter which is the equipment used for drawing the culture medium in the steady state, cell separation, collection of cell free supernatant, product purification and effluent treatment.

Fermenters can vary in size from laboratory experimental models of one or two litres capacity, to industrial models of several hundred litres capacity. The most commonly used principle has been the chemostat: a perfectly mixed suspension of biomass into which medium is fed at a constant rate and the culture is harvested at the same rate so that the culture volume remains constant. Among the various designs which have been put to effect, air-lift has enjoyed the greatest success as the configuration of choice for continuous SCP production. The control of key process variables is a critical element of SCP production, from oxygen transfer, substrate and product concentration, to the appearance of minimal amounts of toxic compounds through undesired metabolic processes, which may compromise the quality of the final product. The biomass from yeast fermentation processes is harvested normally by continuous centrifugation. Filamentous fungi are harvested by filtration. The biomass is then treated for RNA reduction and dried in steam drums of spray driers. Drying is expensive, but results in stabilized product with shelf lives of years. Generally, under combined conditions of low water activity and presence of intractable solid substrate, fungi show luxuriant growth. Hence, proper growth of fungi in Solid state fermentation gives much higher concentration of the biomass and higher yield when compared to **submerged fermentation** .

The advantage in SSF process is the unique possibility of efficient utilization of waste as the substrate to produce commercially viable products. The process does not need elaborate prearrangements for media preparation. The process of SSF initially concentrated on enzyme production. But presently, there is worldwide interest for (SCP) production due to the dwindling conventional food resources.

27.3. INDUSTRIAL ALCOHOL

Ethyl alcohol, CH3 CH2 OH (synonyms: ethanol, methyl carbinol, grain alcohol, molasses alcohol, grain neutral spirits, cologne spirit, wine spirit), is a colorless, neutral, mobile flammable liquid. It is rarely found in nature, being found only in the unripe seeds of*Heracleum giganteun* and *H.* S*pondylium.*

27.3.1. Uses of Ethanol

(i) *Use as a chemical feed stock*: In the chemical industry, ethanol is an intermediate in many chemical processes because of its great reactivity as shown above. It is thus a very important chemical feed stock.

(ii) *Solvent use*: Ethanol is widely used in industry as a solvent for dyes, oils, waxes,

explosives, cosmetics etc.

(iii) *General utility*: Alcohol is used as a disinfectant in hospitals, for cleaning and

lighting in the home, and in the laboratory second only to water as a solvent.

(iv) *Fuel*: Ethanol is mixed with petrol or gasoline up to 10% and known as gasohol and

used in automobiles.

27.3.2. Manufacture of Ethanol

Ethanol may be produced by either synthetic chemical method or by fermentation. Due to the increase in price of crude petroleum, the source of ethylene used for alcohol production, attention has turned worldwide to the production of alcohol by fermentation. Fermentation alcohol has the potential to replace two important needs currently satisfied by petroleum, namely the provision of fuel and that of feedstock in the chemical industry.

The production of gasohol (gasoline – alcohol blend) appears to have received more attention than alcohol use as a feed stock.

27.3.3. Substrates

a). Fermentable Substrates

Following are the types of substrates used for alcohol production :

Sugary Materials: Examples of sugary materials are sugarcane and its by products/wastes (molasses, bagasse) and sugar beet, tapioca, sweet potatoes, fruit juice, sweet sorghum, etc. Sugar cane molasses is largely being used in many countries for alcohol production.

***Starchy Materials* :** Starchy materials used in ethanol production are tapioca, maize, wheat, barley, oat, sorghum, rice and potatoes. But tapioca and corns are the two major substrates of the interest. It has been estimated that 11.7 kg of corn starch can be converted into about 7 liters of ethanol.

Lignocellulosic Materials: The sources of cellulosic and lignocellulosic materials are the agricultural wastes and wood. However, yield of ethanol from lignocellulose is low because of lack of suitable technology and failure of conversion of pentoses into ethanol. On the basis of technology available today about 409 liters of ethanol can be produced from one tonne of lignocelluloses. Production of ethanol from lignocelluloses follows the following steps: (i) hydrolysis, (ii) fermentation, and (iii) recovery.

27.3.4. Fermentation

Alcohol-resistant yeasts, strains of *Saccharomyces cerevisiae* are used, and nutrients such as nitrogen and phosphate lacking in the broth are added.

a). Distillation

After fermentation the fermented liquor or ‘beer’ contains alcohol as well as low boiling point volatile compounds such as acetaldeydes, esters and the higher boiling, fuel oils. The alcohol is obtained by several operations. First, steam is passed through the beer which is said to be steam-stripped. The result is a dilute alcohol solution which still contains part of the undesirable volatile compounds. Secondly, the dilute alcohol solution is passed into the center of a multi-plate aldehyde column in which the following fractions are separated: esters and aldehydes, fusel oil, water, and an ethanol solution containing about 25% ethanol. Thirdly, the dilute alcohol solution is passed into a rectifying column where a constant boiling mixture, an azeotrope, distils off at 95.6% alcohol concentration.

To obtain 200° proof alcohol, such as is used in gasohol blending, the 96.58% alcohol is obtained by azeotropic distillation. The principle of this method is to add an organic solvent which will form a ternary (three-membered) azeotrope with most of the water, but

with only a small proportion of the alcohol. Benzene, carbon tetrachloride, chloroform, and cyclohezane may be used, but in practice, benzene is used. Azeotropes usually have lower boiling point than their individual components and that of benzene-ethanol-water is 64.6°C. On condensation, it separates into two layers. The upper layer, which has about 84% of the condensate, has the following percentage composition: benzene 85%, ethanol 18%, water 1%. The heavier, lower portion, constituting 16% of the condensate, has the following composition: benzene 11%, ethanol 53%, and water 36%. In practice, the condensate is not allowed to separate out, but the arrangement of plates within the columns enable separation of the alcohol. Four columns are usually used. The first and second columns remove aldehydes and fusel oils, respectively, while the last two towers are for the concentration of the alcohol.

A flow diagram of conventional absolute alcohol production from molasses is given in the Figure 27.1.

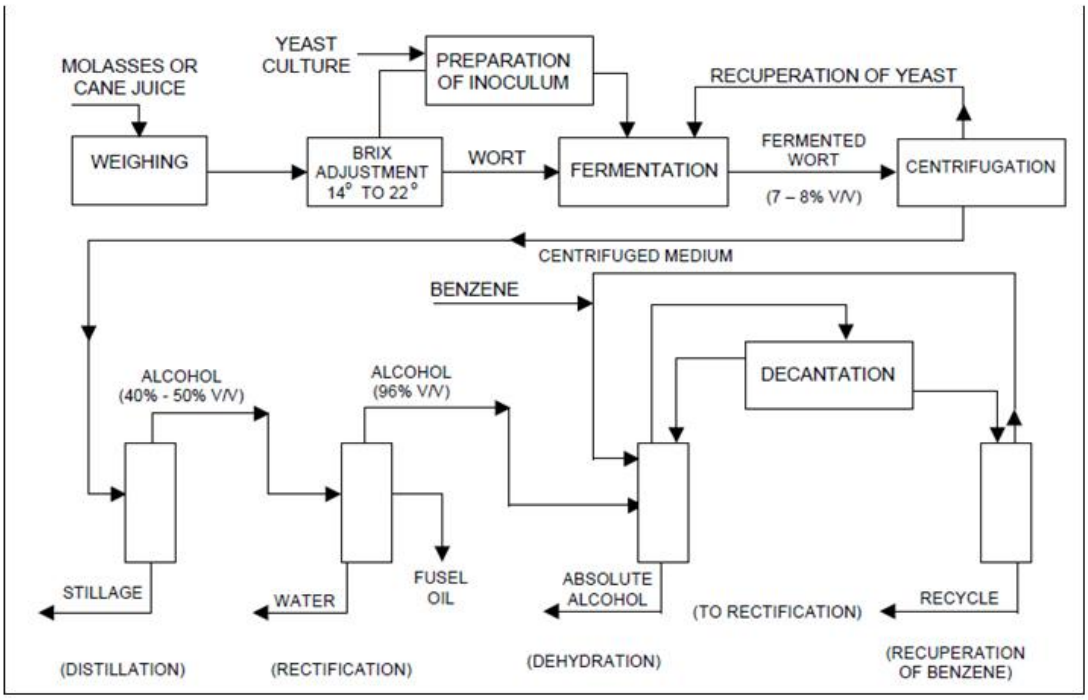

Fig. 27.1: Flow diagram of alcohol production from molasses (Okofer, 2007)

27.3.5. Developments in Alcohol Production

Due to the current interest in the potential of ethanol as a fuel and a chemical feedstock, research aimed at improving the conventional method of production has been undertaken, and more will, most certainly, be undertaken. Some of the techniques aimed at improving productivity are the following:

(i) Developments of new strains of yeast of *Saccharomyces uvarum* able to ferment sugar

rapidly, to tolerate high alcohol concentrations, flocculate rapidly, and whose regulatory system permits it to produce alcohol during growth.

(ii) The use of continuous fermentation with recycle using the rapidly flocculating yeasts.

(iii) Continuous vacuum fermentation in which alcohol is continuously evaporated under low pressure from the fermentation broth.

(iv) The use of immobilized *Saccharomyces cerevisiae* in a packed column, instead of in a conventional stirred tank fermentor. Higher productivity consequent on a higher cell concentration was said to be the advantage.

(v) In the 'Ex-ferm' process sugar cane chips are fermented directly with a yeast without first expressing the cane juice. The chips may be dried and used in the off season period of cane production. It is claimed that there is no need to add nutrients as would be the case with molasses, since these are derived from the cane itself.

(vi) The use of *Zymomonas mobilis*, a Gram-negative bacterium which is found in some tropical alcoholic beverages, rather than yeast is advocated. The advantages claimed for the use of *Zymomonas* are the following:

(a) Higher specific rates of glucose uptake and ethanol production than reported for yeasts.

(b) Higher ethanol yields and lower biomass than with yeasts.

(c) Ethanol tolerance is at least as high or even higher than with yeast.

(d) Tolerates high glucose concentration

(e) Grows anaerobically and, unlike yeasts, does not require the controlled addition of oxygen for viability at the high cell concentrations used in cell recycle.

(f) The many techniques for genetic engineering already worked out in bacteria can be easily applied to *Zymomonas* spp. for greater productivity

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Lesson-28

Organic Acids – Citric, Lactic and Vinegar

28.1. INTRODUCTION

A large number of organic acids with actual or potential uses are produced by microorganisms. Citric, itaconic, lactic, mallic, tartaric, gluconic, mevalonic, salicyclic, gibberelic, diamino-pimelic, and propionic acids are some of the acids produced using microorganisms.

28.2. PRODUCTION OF CITRIC ACID

Citric acid is a tribasic acid. It crystallizes with large rhombic crystals containing one molecule of water of crystallization, which is lost when it is heated to 130°C. At temperatures as high as 175°C it is converted to itaconic acid, aconitic acid, and other compounds.

28.2.1. Uses of Citric Acid

Citric acid is used in the food industry, in medicine, pharmacy and in various other industries.

Uses in the food industry

(i) Citric acid is the major food acidulant used in the manufacture of jellies, jams, sweets, and soft drinks.

(ii) It is used for artificial flavoring in various foods including soft drinks.

(iii) Sodium citrate is employed in processed cheese manufacture.

Uses in medicine and pharmacy

(iv) Sodium citrate is used in blood transfusion and bacteriology for the prevention of blood clotting.

(v) The acid is used in effervescent powers which depend for their effervescence on the CO_2 produced from the reaction between citric acid and sodium bicarbonate.

(vi) Since it is almost universally present in living things, it is rapidly and completely metabolized in the human body and can therefore serve as a source of energy.

Uses in the cosmetic industry

(vii) It is used in astringent lotions such as aftershave lotions because of its low pH.

(viii) Citric acid is used in hair rinses and hair and wig setting fluids.

Miscellaneous uses in industry

(ix) In neutral or low pH conditions the acid has a strong tendency to form complexes hence it is widely used in electroplating, leather tanning, and in the removal of iron clogging the pores of the sand face in old oil wells.

(x) Citric acid has recently formed the basis of manufacture of detergents in place of phosphates, because the presence of the latter in effluents gives rise to eutrophication (an increase in nutrients which encourages aquatic flora development).

28.2.2. Biochemical basis of the production of citric acid

Citric acid is an intermediate in the citric acid cycle. The acid can therefore be caused to accumulate by one of the following methods:

(a) By mutation – giving rise to mutant organisms which may only use part of a metabolic pathway, or regulatory mutants; that is using a mutant lacking an enzyme of the cycle.

(b) By inhibiting the free-flow of the cycle through altering the environmental conditions, e.g. temperature, pH, medium composition (especially the elimination of ions and cofactors considered essential for particular enzymes). The following are some of such environmental conditions which are applied to increase citric acid production:

(i) The concentrations of iron, manganese, magnesium, zinc, and phosphate must be limited.

(i) The dehydrogenases, especially isocitrate dehydrogenase, are inhibited by anaerobiosis, hence limited aeration is done on the fermentation so as to increase the yield of citric acid.

(ii) Low pH and especially the presence of citric acid itself inhibits the TCA and hence encourages the production of more citric acid.

Citric acid can be caused to accumulate by using a mutant lacking an enzyme of the cycle or by inhibiting the flow of the cycle.

28.2.3. Fermentation for citric acid production

For a long time the production of citric acid has been based on the use of molasses and various strains of *Aspergillus niger* and occasionally *Asp. wenti*. Although several reports of citric acid production by *Penicillium* are available, in practice, organisms in this group are not used because of their low productivity. In recent times yeasts, especially *Candida* spp. (including *Candida quillermondi)* have been used to produce the acid from sugar.

(a) **Surface fermentation** : Surface fermentation using *Aspergillus niger* may be done on rice bran as is the case in Japan, or in liquid solution in flat aluminium or stainless steel pans. Special strains of *A. niger* which can produce citric acid despite the high content of trace metals in rice bran are used. The citric acid is extracted from the bran by leaching and is then precipitated from the resulting solution as calcium citrate.

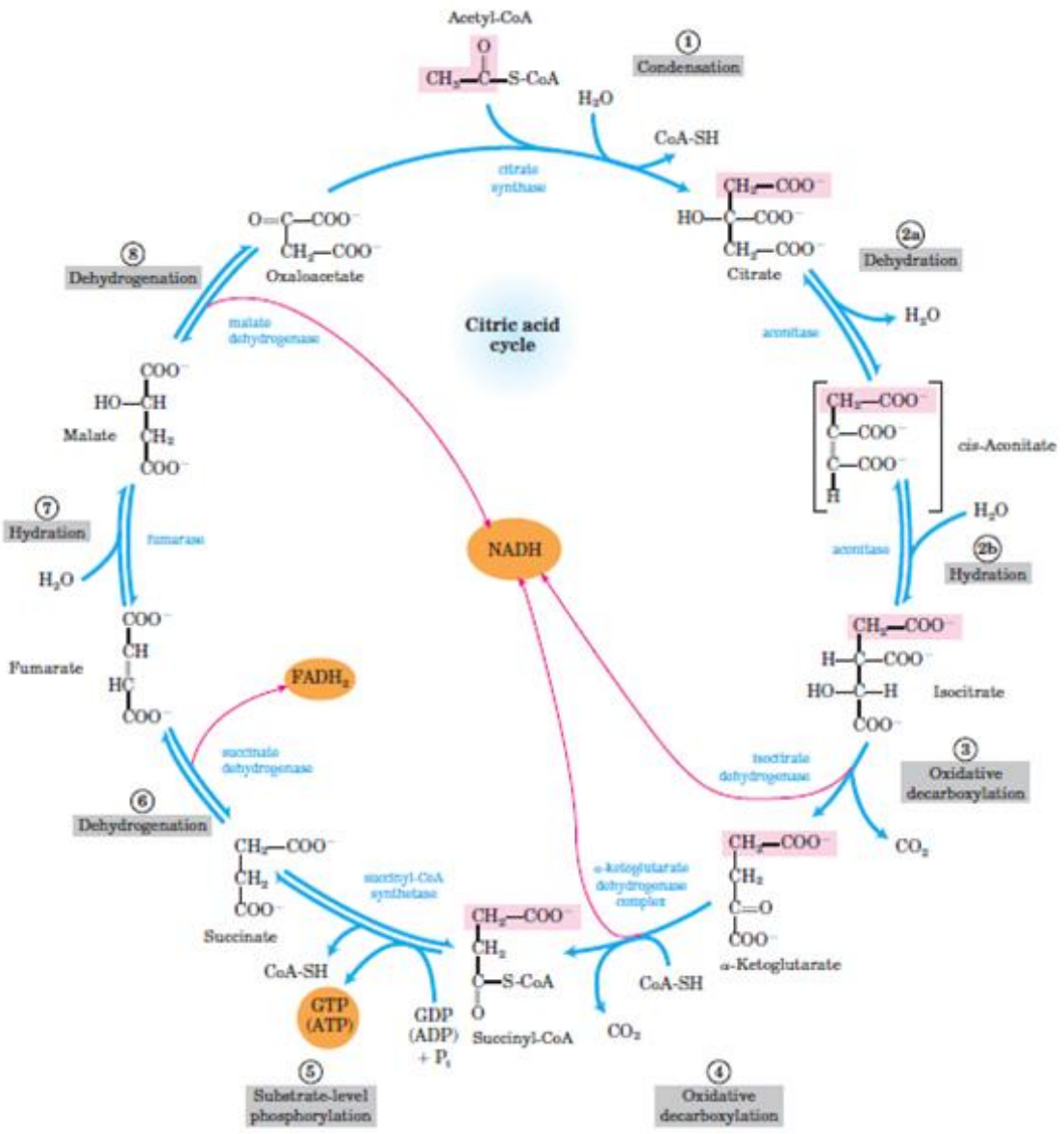

Fig-28.1: Citric acid cycle (Prescott, 2000)

(b) **Submerged fermentation** : As in all other processes where citric acid is made the fermentation the fermentor is made of acid-resistant materials such as stainless steel. The carbohydrate sources are molasses decationized by ion exchange, sucrose or glucose. The pH is never allowed higher than 3.5. Copper is used at up to 500 ppm as an antagonist of the enzyme aconitase which requires iron. 1-5% of methanol, isopranol or ethanol when added to fermentations containing unpurified materials increases the yield; the yields are reduced in media with purified materials. As high aeration is deleterious to citric acid production, mechanical agitation is not necessary and air may be bubbled through. Antifoam is added. The fungus occurs as a uniform dispersal of pellets in the medium. The fermentation lasts for five to fourteen days.

28.2.4. Extraction

The broth is filtered until clear. Calcium citrate is precipitated by the addition of magnesium-free $Ca(OH)_2$. Since magnesium is more soluble than calcium, some acid may be lost in the solution as magnesium citrate if magnesium is added. Calcium citrate is filtered and the filter cake is treated with sulfuric acid to precipitate the

calcium. The dilute solution containing citric acid is purified by treatment with activated carbon and passing through iron exchange beds. The purified dilute acid is evaporated to yield crystals of citric acid.

28.3. LACTIC ACID

Lactic acid is produced by many organisms: animals including man produce the acid in muscle during work.

28.3.1. Properties and chemical reactions of lactic acid

(i) Lactic acid is a three carbon organic acid: one terminal carbon atom is part of an acid or carboxyl group; the other terminal carbon atom is part of a methyl or hydrocarbon group; and a central carbon atom having an alcohol carbon group.

(ii) Lactic acid is soluble in water and water miscible organic solvents but insoluble in other organic solvents.

Technical grade lactic acid is used as an acidulant in vegetable and leather tanning industries. Various textile finishing operations and acid dyeing of food require low cost technical grade lactic acid to compete with cheaper inorganic acid. Lactic acid is being used in many small scale applications like pH adjustment, hardening baths for cellophanes used in food packaging, terminating agent for phenol formaldehyde resins, alkyl resin modifier, solder flux, lithographic and textile printing developers, adhesive formulations, electroplating and electropolishing baths, detergent builders. Lactic acid has many pharmaceutical and cosmetic applications and formulations in topical ointments, lotions, anti acne solutions, humectants, parenteral solutions and dialysis applications, and anti carries agents. Calcium lactate can be used for calcium deficiency therapy, and as an anti caries agent. Its biodegradable polymer has medical applications as sutures, orthopedic implants, controlled drug release, etc. Polymers of lactic acids are biodegradable thermoplastics. These polymers are transparent and their degradation can be controlled by adjusting the composition, and the molecular weight.Their properties approach those of petroleum derived plastics. Lactic acid esters like ethyl/butyl lactate can be used as green solvents. They are high boiling, non-toxic and degradable components. Poly L-lactic acid with low degree of polymerization can help in controlled release or degradable mulch films for large-scale agricultural applications. Lactic acid was among the earliest materials to be produced commercially by fermentation and the first organic acid to be produced by fermentation.

28.3.2. Fermentation for lactic acid

Although many organisms can produce lactic acid, the amount so produced is small: the organisms which produce adequate amounts and are therefore used in industry are the homofermentative lactic acid bacteria, *Lactobacillus* spp., especially *L. delbruckii*. In recent times *Rhizopus oryzae* has been used. Both organisms produce the L- form of the acid, but *Rhizopus* fermentation has the advantage of being much shorter in duration; further, the isolation of the acid is much easier when the fungus is used.

Lactic acid is very corrosive and the fermentor, which is usually between 25,000 and 110,000 liters in capacity, is made of wood. Alternatively special stainless steel (type 316) may be used. They are sterilized by steaming before the introduction of the broth as contamination with thermophilic clostridia yielding butanol and butyric acid is common. Such contamination drastically reduces the value of the product. During the step-wise preparation of the inoculum, which forms about 5% of the total beer, calcium carbonate is added to the medium to maintain the pH at around 5.5-6.5. The carbon source used in the broth has varied widely and has included whey, sugars in potato and corn hydrolysates, sulfite liquour, and molasses. However, because of the problems of recovery for high quality lactic acid, purified sugar and a minimum of other nutrients are used.

Lactobacillus requires the addition of vitamins and growth factors for growth. These requirements along with that of nitrogen are often met with ground vegetable materials such as ground malt sprouts or malt rootlets. To

aid recovery the initial sugar content of the broth is not more than 12% to enable its exhaustion at the end of 72 hours. Fermentation with *Lactobacillus delbruckii* is usually for 5 to 10 days whereas with *Rhizopus oryzae*, it is about two days. Although lactic fermentation is anaerobic, the organisms involved are facultative and while air is excluded as much as possible, complete anaerobiosis is not necessary. The temperature of the fermentation is high in comparison with other fermentation, and is around 45°C. Contamination is therefore not a problem, except by thermophilic*Clostridia*.

28.3.3. Extraction

The main problem in lactic acid production is not fermentation but the recovery of the acid. Lactic acid is crystallized with great difficulty and in low yield. The purest forms are usually colorless syrups which readily absorb water. At the end of the fermentation when the sugar content is about 0.1%, the beer is pumped into settling tanks. Calcium hydroxide at pH 10 is mixed in and the mixture is allowed to settle. The clear calcium lactate is decanted off and combined with the filtrate from the slurry. It is then treated with sodium sulfide, decolorized by adsorption with activated charcoal, acidified to pH 6.2 with lactic acid and filtered. The calcium lactate liquor may then be spray-dried.

For *technical grade* lactic acid the calcium is precipitated as CaSO4.2H2O which is filtered off. It is 44-45% total acidity. *Food grade*acid has a total acidity of about 50%. It is made from the fermentation of higher grade sugar and bleached with activated carbon. Metals especially iron and copper are removed by treatment with ferrocyanide. It is then filtered. *Plastic grade* is obtained by esterification with methanol after concentration. High-grade lactic acid is made by various methods: steam distillation under high vacuum, solvent extraction etc.

28.4. **VINEGAR**

Vinegar is a product resulting from the conversion of alcohol to acetic acid by acetic acid bacteria, *Acetobacter* spp. The name is derived from French (*Vin* = wine; *Aigre*-sour or sharp). Although acetic acid is the major component of vinegar, the material cannot be produced simply by dissolving acetic acid in water. When alcoholic fermentation occurs and later during acidifications many other compounds are produced, depending mostly on the nature of the material fermented and some of these find their way into vinegar. Furthermore, reactions also occur between these fermentation products. Ethyl acetate, for example, is formed from the reaction between acetic acid and ethanol. It is these other compounds which give the various vinegars their bouquets or organoleptic properties. The other compounds include non-volatile organic acids such as malic, citric, succinic and lactic acids; unfermented and unfermentable sugars; oxidized alcohol and acetaldelyde, acetoin, phosphate, chloride, and other ions.

28.4.1. Uses

(i) **Ancient uses** : The ancient uses of vinegar which can be seen from various records include a wide variety of uses including use as a food condiment, treatment of wounds, and a wide variety of illnesses such as plague, ringworms, burns, lameness, variocose veins. It was also used as a general cleansing agent. Finally, it was used as a cosmetic aid.

(ii) **Modern uses**: Vinegar is used today mainly in the food industry as; (a) a food condiment, sprinkled on certain foods such as fish at the table; (b) for pickling and preserving meats and vegetables; vinegar is particularly useful in this respect as it can reduce the pH of food below that which even sporeformers may not survive; (c) It is an important component of sauces especially renowned French sauces many of which contain vinegar; (d) Nearly 70% of the vinegar produced today is supplied to various arms of the food industry where it finds use in the manufacture of sauces, salad dressings, mayonnaise, tomato productions, cheese dressings, mustard, and soft drinks.

28.4.2. Substrate

The substrate for the alcoholic fermentation for vinegar productions varies from one locality to the other. Thus, while wine vinegar made from grapes is common in continental Europe and other vine growing countries, malt vinegar is common in the United Kingdom; the United States on account of its great variety of climatic regions uses both malt and wine vinegars. Rice vinegar is common in the far Eastern countries of Japan and China and pineapple vinegar is used in Malaysia. In some tropical countries vinegar has been manufactured from palm wine derived from oil or raffia palm.

28.4.3. Organisms involved

Although *Acetobacter* spp are responsible for vinegar production, pure cultures are hardly used, except in submerged fermentation because of the difficulty of isolating and maintaining the organisms. The only member of the genus which is not useful, if not positively harmful in vinegar production is *Acetobacter xylinum* which tends to produce slime. Recently a new species, *Acetobacter europaeus*, was described. Its distinguishing features are its strong tolerance of acetic acid and its absolute requirement of acetic acid for growth.

Strains of acetic acid bacteria to be used in industrial production should a) tolerate high concentrations of acetic acid; b) require small amounts of nutrient; c) not overoxidize the acetic acid formed; and d) be high yielding in terms of the acetic acid produced.

The biochemical processes are simple and are shown below:

28.4.5. Manufacture of vinegar

The three methods used for the production of vinegar are the Orleans Method (also known as the slow method), the Trickling (or quick) Method and Submerged Fermentation. The last two are the most widely used in modern times.

The common feature in all submerged vinegar production is that the aeration must be very vigorous as shortage of oxygen because of the highly acid conditions of submerged production, would result in the death of the bacteria within 30 seconds. Furthermore, because a lot of heat is released (over 30,000 calories are released per gallon of ethanol) an efficient cooling system must be provided. All submerged vinegar is turbid because of the high bacterial content and have to be filtered.

28.4.6. Frings acetator

First publicized in 1949, most of the world's vinegar is now produced with this fermentor. It consists of a stainless steel tank fitted with internal cooling coils and a highspeed agitator fitted through the bottom. Air is sucked in through an air-meter located at the top. It is then finely dispersed by the agitator and distributed throughout the liquid. Temperature is maintained at 30°C, although some strains can grow at a higher temperature. Foaming is interrupted with an automatic foam breaker. Essentially it is shaped like the typical aerated stirred tank fermentor. It is operated batchwise and the cycle time for producing 12% vinegar is about 35 hours. The Frings alkograph automatically monitors the alcohol content and signals the end of the batch when the alcohol content falls to 0.2% (v/v). At this stage about one third of the product is pumped out and fresh feed pumped to the original level. The aeration must continue throughout the period of the unloading and loading. A fermentation cycle takes 24 to 48 hours Since its first description, improvements and modifications have been made on the Frings acetator.

28.4.7. Processing of vinegar

(a) ***Clarification and bottling***: Irrespective of the method of manufacture, vinegar for retailing is clarified by careful filtration using a filter aid such as diatomaceous earth. Vinegar from trickling generators are however less turbid than those from submerged fermentations because a high proportion of the bacterial population responsible for the acetification is held back on the shavings. After clarification it is pasteurized at 60-65°C for 30 minutes.

(b) ***Concentration of vinegar:*** Vinegar can be concentrated by freezing; thereafter the resulting slurry is centrifuged to separate the ice and produce the concentrate. With this method 200° grain (i.e., 20% w/v) acetic acid can be produced.

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Lesson-29

Industrial Enzymes and Vitamin B_{12}

29.1. INTRODUCTION

Major industrial enzymes from bacteria, molds and yeasts are listed below

Table-29.1: Major Industrial Enzymes from Bacteria, Molds and Yeasts

Enzymes	Producer organisms
Asparaginase	*Aspergillus spp. and and Penicillium spp*
Amylase	*Aspergillus niger, Aspergillus. oryzae*
Catalase	*A. niger, Penicillium spp.*
Cellulase	*A. niger, Trichoderma reesei, T. viride, Penicillium finiculosum*
Dextranase	*Penicillium spp.*
ß-Glucanase	*A. niger, Penicillium emersonii, T. reesei, T. viride*
Glucoamylase	*A. niger, A. oryzae*
Glucose oxidase	*A. niger, Penicillium spp.*
Hemicellulase	*A. niger, A. oryzae, T. reesei, T. viride, P. emersonii*
Laccase	*Pyricularia oryzae*
Lipase	Several species including *A.niger, A. oryzae*
Pectinase	Several species including *A. niger, Rhizopus oryzae*
Protease	Several species including *A. niger, A. oryzae*
Rennet	*Mucor miehei, Endothia parasitica*
Tannase	*A.niger, A. oryzae*
Xylanase	*A. niger, T. reesei*

29.2. CHYMOSIN

Chymosin is also known as rennet or chymase and is used in the manufacture of cheese. Over 90% of the chymosin used today is produced by *E. coli*, and the fungi, *Kluyveromyces lactis* and *Aspergillus niger*.

Genetically engineered chymosin is preferred by manufacturers because while it behaves in exactly the same way as calf chymosin, it is purer than calf chymosin and is more predictable. Furthermore, it is preferred by vegetarians and some religious organizations.

29.2.1. General principle of chymosin production using rDNA technology

In higher eukaryotes, most chromosomal genes have intron sequences which interrupt the coding sequences for translation to proteins. When these sequences are transcribed in eukaryotic cells, the introns are spliced out of the mRNA transcript and subsequent translation gives proteins with correct amino acid sequences. Because bacterial cells have no such splicing mechanism, genes obtained from eukaryotic chromosomes (genomic genes) cannot be correctly expressed in bacteria. Therefore, the general procedure for cloning any eukaryotic gene in a bacterial host is to synthesize complementary DNA (cDNA) by reverse transcription of mRNA from which introns have been spliced out.

Highly specialized cells within tissues (for example, the mucosal layer of abomasum), frequently contain large amounts of mRNA that codes for pre prochymosin. This provides the basis for isolation of the specific mRNA to make cloning easier. The total RNA from the mucosal layer of abomasum is isolated and the mRNA is fractionated by oligo(dT) cellulose affinity chromatography. The mRNA is reverse transcribed to cDNA and subsequently cloned in a suitable vector and expressed in a suitable bacterial or yeast host.

The gene for chymosin has been successfully cloned and expressed using a number of plasmid or modified plasmid vectors and host organisms such as *E. coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Aspergillus* spp, baby hamster kidney cells, and so forth. Several forms of chymosins have been expressed, including preprochymosin, met-prochymosin, and met-chymosin as heterologous proteins made in *E. coli*. Expression as fusion protein is often more efficient than direct expression. Chymosin is especially well suited for expression as a fusion protein which is autocatalytically reactivated to chymosin during product recovery, ensuring that the final product has the proper amino terminus.

The method for Chymosin A produced from *Escherichia coli* K-12 containing calf prochymosin A gene is explained below **29.2.1.1. Construction of production strain** *E. coli* K12 JA198 strain was subjected to several genetic manipulations to construct the recipient strain for the expression plasmid carrying the prochymosin A gene. The expression plasmid was derived from the widely used cloning vector pBR322. cDNA coding for bovine chymosin A was previously cloned and characterized. The prochymosin gene was divided into three sections, each terminated by a unique restriction endonuclease recognition site. Each section was assembled from several synthetic oligonucleotides synthesized in an automated DNA synthesizer. Each assembled section was subcloned into a pBR322 vector, transformed into *E. coli*, and amplified. All three subcloned sections were assembled together in the correct order to reconstruct the prochymosin gene, which was inserted into pBR322 vector. The gene was attached to the vector DNA through the ribosomal binding site and the *E. coli* tryptophan (trp) promoter. The created expression vector was transformed into the recipient strain GE81. The plasmid carries the ampicillin resistance gene as a selective marker for bacterial transformants carrying the prochymosin gene. **29.2.1.2. Fermentation** The production strain is grown in an aqueous solution containing carbohydrates, nitrogen, mineral salts and miscellaneous inorganic and organic compounds. **29.2.1.3. Recovery** The solid prochymosin is liberated from the producing organism by cell disruption and harvesting of "inclusion bodies" by centrifugation or membrane concentration. The harvested inclusion bodies are washed with phosphate buffer solution containing 1-4 M urea. The residual *E. coli* are inactivated by holding at pH less than 2.0 for at least one hour. The inclusion bodies are then dissolved by addition of urea to a concentration of 7-9 M and pH adjustment to 10.0 - 11.0. Subsequently, the solution containing prochymosin is diluted with a buffer, and the pH is reduced to 8.5- 9.5, followed by a 2-hour period to allow renaturing of the prochymosin, which is subsequently activated to chymosin by adjusting the pH to 1.8 - 2.2 and holding for one hour. Following readjustment of the pH to 5.5 - 6.0, the chymosin is purified via absorption on a suitable anion-exchange resin followed by elution with a buffer containing 1 M sodium

chloride. Increasing effort is being devoted to improve the expression of chymosin in bacteria and yeasts; better yeast expression systems are particularly needed. Protein engineering of chymosin is still another area of interest. Protein engineering can potentially enhance activity of chymosin and beneficially modify the pH optima.

29.3. VITAMIN B_{12}

Vitamin B_{12} is needed to help maintain healthy red blood cells and nerve cells, as well as aid in the production of DNA. As the body is capable of storing large amounts of B12 in the body, deficiencies occur infrequently, but they do happen. A person with a B12 deficiency is susceptible to several different types of anemia, low blood pressure, dementia and muscle weakness. Other disorders that have been tied to low vitamin B12 levels are Alzheimer's disease, breast cancer, fatigue and heart disease.

It seems probable that the only primary source of vitamin B12 in nature is the metabolic activity of the microorganisms. It is synthesized by a wide range of bacteria and Streptomycetes, though not to any extent by yeasts and fungi. While over 100 fermentation processes have been described for the production of vitamin Bl2 only half a dozen have apparently been used on a commercial scale.

Fermentation processes using *Bacillus megatherium*, *Streptomyces olivaceus* and other species, *Propionibacterium freundreichii*, and *P. shermanii*. The processes using the *Propionibacterium* species are the most productive and are now widely used commercially. Both batch and continuous processes have been described.
It is important to select microbial species which make the 5, 6-dimethyl benzimidazolylcobamide exclusively. Several manufacturers have been led astray by organisms that gave high yields of the related cobamides including pseudo-vitamin B_{12}*Streptomyces* cells. The vitamin B12 activity is released from the cells by acid, heating, cyanide or other treatments. Addition of cyanide solutions decomposes the coenzyme form of the vitamin in and results in the formation of the cyanocobalamin. (adeninylcobamide). The natural form of the vitamin is Barker's Coenzyme where a deoxyadenosyl residue replaces the cyano group found in the commercial vitamin. Practically all of the cobamides formed in the fermentation are retained in the cells, and the first step is the separation of the cells from the fermentation medium. Large high speed centrifuges are used to concentrate the bacteria to a cream, while filters are used to remove

The cyanocobalamin is adsorbed on ion exchange resin IRC-50 or charcoal, and is eluted. It is then purified further by partition between Phenolic solvents and water. The vitamin is finally crystallized from aqueous-acetone solutions. The crystalline product often contains some water of crystallization.

The most commercial sound procedure produces B12 produced industrially by microbial fermentation, using almost exclusively*Pseudomonas denitrificans* and *Propionibacterium* spp. Contrary to *Pseudomonas*, Propionibacteria are food-grade. Processes using*Propionibacterium* species thus have the advantage that they allow to formulate natural vitamin B12 together with the biomass in which it is produced. Such processes avoid the conversion of natural vitamin B12 into the cyanocobalamin form by chemical processes including cyanidisation followed by extraction and purification steps using organic solvents. The chemical conversion step and any subsequent purification steps cause this production process to be expensive, unsafe to the operators and environmentally unfriendly.

Several *Propionibacterium* species are capable to produce vitamin B_{12} in large scale fermentation processes. The process is described as a two-stage fermentation with a 72-88 hours anaerobic fermentation followed by a 72-88 hours aerobic phase. The vitamin B_{12}concentration in the cells rapidly increases in the aerobic phase, with typical values of 25-40 mg vitamin B_{12}/ l . Anaerobic growth followed by an aerobic phase with limited growth is important for economic production of vitamin B_{12} using *Propionibacterium*species. This requirement, however, limits the amount of biomass to 25-35 g/l as described above. Several attempts have been made to

overcome the barrier of propionic acid toxicity in order to increase biomass and thereby the yield of vitamin B_{12}.

Alternated anaerobic-aerobic phases are e.g. suggested to reduce the amount of acids. In the aerobic phase the propionic acid is converted to less toxic acetic acid, with simultaneous formation of vitamin B_{12}. The relative yield of vitamin B12 has been increased, but the final titre is rather low. This is probably due to inhibition early in the synthesis of vitamin B_{12} and/or other oxygen related products limiting the synthesis of vitamin B_{12}. The final vitamin B_{12} produced with this method is 9 mg/l compared to 4.5 mg/l with the fully separated anaerobic and aerobic phases. Both values are rather low for vitamin B_{12} production with *Propionibacterum.*

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Lesson-30

Bacteriocins and Antibiotics

30.1. Penicillin fermentation

Organisms

In the early days of penicillin production, when the surface culture method was used, a variant of the original culture of *Penicillium notatum* discovered by Sir Alexander Fleming was employed. When however the production shifted to submerged cultivation, a strain of *Penicillium chrysogenum* designated NRRL 1951 (after Northern Regional Research Laboratory of the United States Department of Agriculture) discovered in 1943, was introduced. A 'super strain' was produced from a variant of NRRL 1951 and designated X 1612. By ultraviolet irradiation of X-1612, a strain resulted and was named WISQ 176 after the University of Wisconsin where much of the stain development work was done. On further ultra violet irradiation of WISQ 176, BL3-D10 was produced. Present-day penicillin producing *P. chrysogenum* strains are far more highly productive than their parents. They were produced through natural selection, and mutation using ultra violet irradiation, x-irradiation or nitrogen mustard treatment. It was soon recognized that there were several naturally occurring penicillins, *viz.*, Penicillins G, X, F, and K. Penicillin G (benzyl penicillin) was selected because it was markedly more effective against pyogenic cocci. Furthermore, higher yields were achieved by supplementing the medium with phenylacetic acid, analogues (phenylalanine and phenethylaninie) of which are present in corn steep liquor used to grow penicillin in the United States.

Penicillin has since been shown to be produced by a wide range of organisms including the fungi *Aspergillus, Malbranchea, Cephalosporium, Emericellopsis, Paecilomyces, Trichophyton, Anixiopsis, Epidermophyton, Scopulariopsis, Spiroidium* and the actionomycete, *Streptomyces.*

Fermentation for penicillin production

The inoculum is usually built up from lyophilized spores or a frozen culture and developed through vessels of increasing size to a final 5-10% of the fermentation tank. As the antibiotic concentration in the fermentation beer is usually dilute the tanks are generally large for penicillin and most other antibiotic production. The fermentors vary from 38,000 to 380,000 liters in capacity and in modern establishments are worked by computerized automation, which monitor various parameters including oxygen content, Beta-lactam content, pH, etc.

The medium for penicillin production now usually has as carbohydrate source glucose, beet molasses or lactose. The nitrogen is supplied by corn steep liquor. Cotton nitrogen source is sometimes exhausted towards the end of the fermentation and it must then therefore be replenished. Calcium carbonate or phosphates may be added as a buffer. Sulfur compounds are sometimes added for additional yields since penicillin contains sulfur. The practice nowadays is to add the carbohydrate source intermittently, i.e. using fed-batch fermentation. Lactose is more slowly utilized and need not be added intermittently. Glucose suppresses secondary metabolism and excess of it therefore limits penicillin production. The pH is maintained at between 6.8 and 7.4 by the automatic addition of H_2SO_4 or NaOH as necessary. Precursors of the appropriate side-chain are added to the fermentation. Thus if benzyl penicillin is desired, phenylacetic acid is added. High yielding strains of *P. chrysogenum* resistant to the precursors have therefore been developed. 30-32°C was found suitable for the

trophophase and 24°C for the idiophase. Aeration and agitation are vigorous in order to keep the components of the medium in suspension and to maintain yield in the highly aerobic fungus.

Penicillin fermentation can be divided into three phases.

The first phase (trophophase) during which rapid growth occurs, lasts for about 30 hours during which mycelia are produced.

The second phase (idiophase) lasts for five to seven days; growth is reduced and penicillin is produced.

In the third phase, carbon and nitrogen sources are depleted, antibiotic production ceases, the mycelia lyse releasing ammonia and the pH rises.

Extraction of penicillin after fermentation

At the end of the fermentation the broth is transferred to a settling tank. Penicillin is highly reactive and is easily destroyed by alkali conditions (pH 7.5-8.0) or by enzymes. It is therefore cooled rapidly to 5-10°C. A reduction of the pH to 6 with mineral acids sometimes accompanied by cooling helps also to preserve the antibiotic. The fermentation broth contains a large number of other materials and the method used for the separation of penicillin from them is based on the solubility, adsorption and ionic properties of penicillin. Since penicillins are monobasic carboxylic acids they are easily separated by solvent extraction as described below.

The fermentation beer or broth is filtered with a rotary vacuum filter to remove mycelia and other solids and the resulting broth is adjusted to about pH 2 using a mineral acid. It is then extracted with a smaller volume of an organic solvent such as amyl acetate or butyl acetate, keeping it at this very low pH for as short a time as possible. The aqueous phase is separated from the organic solvent usually by centrifugation. The organic solvent containing the penicillin is then typically passed through charcoal to remove impurities, after which it is back extracted with a 2% phosphate buffer at pH 7.5. The buffer solution containing the penicillin is then acidified once again with mineral acid (phosphoric acid) and the penicillin is again extracted into an organic solvent (e.g. amyl acetate). The product is transferred into smaller and smaller volumes of the organic solvent with each successive extraction process and in this way, the penicillin becomes concentrated several times over, up to 80-100 times.

When it is sufficiently concentrated the penicillin may be converted to a stable salt form in one of several ways which employ the fact that penicillin is an acid: (a) it can be reacted with a calcium carbonate slurry to give the calcium salt which may be filtered, lyophilized or spray dried. (b) it may be reacted with sodium or potassium buffers to give the salts of these metals which can also be freeze or spray dried; (c) it may be precipitated with an organic base such as triethylamine. When benzyl penicillin is administered intramuscularly it is given either as the sodium (or potassium) salt or as procaine penicillin. The former gives high blood levels but it quickly excreted. Procaine penicillin gives lower blood levels, but it lasts longer in

the body because it is only slowly removed from the blood. It is produced by dissolving

sodium or penicillin in procaine hydrochloride.

Nisin

Biopreservation systems in foods are of increasing interest for industry and consumers. Bacteriocinogenic lactic acid bacteria and/or their isolated bacteriocins are considered safe additives (GRAS), useful to control the frequent development of pathogens and spoiling microorganisms in foods and feed. The spreading of bacterial

antibiotic resistance and the demand for products with fewer chemicals create the necessity of exploring new alternatives, in order to reduce the abusive use of therapeutic antibiotics. In this context, bacteriocins are

indicated to prevent the growth of undesirable bacteria in a food-grade and more natural way, which is convenient for health and accepted by the community. According to their properties, structure, molecular weight (MW), and antimicrobial spectrum, bacteriocins are classified in three different groups: lantibiotics and nonlantibiotics of low MW, and those of higher MW. Several strategies for isolation and purification of bacteriocins from complex cultivation broths to final products were described. Biotechnological procedures including salting out, solvent extraction, ultrafiltration, adsorption-desortion, ion-exchange, and size exclusion chromatography are among the most usual methods. The best known and most characterized bacteriocin is nisin. There are 11 genes making the nisin cluster that code for nisin production, immunity, and externalization.

Nisin is a 34-amino acid antimicrobial polypeptide produced during a fermentation of Lactococcus lactis subsp. lactis. Due to its antimicrobial activity against a wide range of Gram-positive bacteria, including several major foodborne pathogens such as Clostridium and Listeria, nisin has been used extensively in the food industry as a natural food preservative. It is the only bacteriocin that is approved for food applications by FDA and produced commercially.

Nisin production is affected by several cultural factors such as producer strain, nutrient composition of media, pH, temperature, agitation and aeration, as well as the unique characteristic of nisin production, such as substrate and product inhibition, adsorption of nisin onto the producer cells, and enzymatic degradation. A dramatic decrease in nisin level after reaching the peak value was suspected to be a result of proteolytic degradation and/or adsorption of nisin by producer cells. At pH 6.80 (controlled fermentation), more than 80% of the nisin synthesized was bound to the cells, whereas at a pH below 6.0, more than 80% of the nisin was in the culture fluid.

Removal of nisin during fermentation would reduce the chance of the nisin being degraded or adsorbed and thus increase the amount of nisin that can be recovered. Although nisin production is auto-regulated with the nisin itself acting as an inducer molecule or peptide, the presence of product inhibition caused by nisin and removal of nisin from culture broth during fermentation significantly enhanced nisin production.

Purification steps of large-scale nisin production are commercially sensitive but are suspected to include foam precipitation (frothing), sodium chloride precipitation, centrifugation or ultrafiltration, and spray or drum drying. On the other hand, laboratory scale purification of nisin includes an ammonium sulphate precipication step, followed by various combinations of ion-exchange and hydrophobic interaction chromatography, with a final reverse phase-high pressure liquid chromatography purification step. immunological-based techniques have also been developed and tested like a one-step purification of nisin A using immunoaffinity purification with the specific monoclonal antibody against nisin A.

Nisin is manufactured by controlled fermentation of *L. lactis* in a milk-based medium at pH 2.0. Above pH 3.0 nisin adsorbs to the producer cells, but is completely desorbed at pH 3.0 or below. Consequently, at the pH of the fermentation all nisin is released into the medium, from which it can be extracted at the end of the fermentation. Solvent extraction methods have been used and a one-step immunoaffinity chromatography method is highly efficient. However, in the industrial-scale process it is concentrated and separated by a simple low-cost foaming process. This involves merely bubbling nitrogen or air through a column of the completed fermentation medium. As nisin is a surface-active agent it accumulates in the foam at the air–aqueous interface. The foam is collected, broken mechanically and the nisin recovered. It is then spray-dried before being milled into fine particles and finally standardized by the addition of sodium chloride.

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Lesson- 31

Fermented foods

31.1. INTRODUCTION

Traditional fermented food is not only the staple food for most of developing countries but also the key healthy food for developed countries. As the healthy function of these foods are gradually discovered, more and more high throughput biotechnologies are being used to promote the old and new industry. As a result, the microflora, manufacturing processes and product healthy function of these foods were pushed forward. The application and progress of the high throughput biotechnologies into traditional fermented food industries like fermented milk products (yogurt, cheese), fermented sausages, fermented vegetables (kimchi, sauerkraut), fermented cereals (sourdough) and fermented beans (tempeh, natto) are gaining momentum. Given the further promotion by high throughput biotechnologies, the middle and/or down-stream process of traditional fermented foods would be optimized and the process of industrialization of local traditional fermented food having many functional factors but in small quantity would be accelerated. Fermented foods may be defined as foods which are processed through the activities of microorganisms but in which the weight of the microorganisms in the food is usually small. The influence of microbial activity on the nature of the food, especially in terms of flavor and other organoleptic properties is profound. A few fermented foods of economic and nutritional importance are listed in Table 31.1.

Table- 31.1: Some fermented foods of economic and nutritional importance

Raw material	Fermented food product
Wheat	Bread
Milk	Cheese, yoghurt
Maize	Ogi, Akamu, Kokonte
Cassava	Garri, Foo-foo, Akpu, Lafun
Vegetables	Sauerkraut, pickled cucumbers
Stimulant beverages	Coffee, Tea and Cocoa
Legumes and oil seeds	Soy sauce, Miso, Sufu , Oncom. Idli, Ogili, Dawa dawa, Ugba
Fish	Fish sauce

31.2. FERMENTED VEGETABLES

Like the fermentation of other foods, vegetables have been preserved by fermentation from time immemorial by lactic bacterial action. A wide range of vegetables and fruits including cabbages, olives, cucumber, onions, peppers, green tomatoes, carrots, okra, celery, and cauliflower have been preserved. Only sauerkraut and cucumbers will be discussed, as the same general principles apply to the fermentation of all vegetables and fruits. In general they are fermented in brine, which eliminates other organisms and encourages the lactic acid bacteria.

31.2.1. Sauerkraut

Sauerkraut is produced by the fermentation of cabbages, *Brassica oleracea*, and has been known for a long time. Specially selected varieties which are mild-flavored are used. The cabbage is sliced into thin pieces known as slaw and preserved in salt water or brine containing about 2.5% salt. The slaw must be completely immersed in brine to prevent it from darkening. Kraut fermentation is initiated by *Leuconostoc mesenteroides*, a heterofermentative lactic acid bacterium (i.e., it produces lactic acid as well as acetic acid and CO_2). It grows over a wide range of pH and temperature conditions. CO_2 creates anaerobic conditions and eliminates organisms which might produce enzymes which can cause the softening of the slaw and also encourages the growth of other lactic acid bacteria. Gram negative coliforms and pseudomonads soon disappear, and give way to a rapid proliferation of other lactic acid bacteria, including *Lactobacillus brevis*, which is heterofermentative, and the homofermentative *Lactobacillus plantarum*; sometimes*Pediococcus cerevisiae* also occurs. Compounds which contribute to the flavor of sauerkraut begin to appear with the increasing growth of the lactics. These compounds include lactic and acetic acids, ethanol, and volatile compounds such as diacetyl, acetaldehyde, acetal, isoamyl alcohol, n-hexanol, ethyl lactate, ethyl butarate, and isoamyl acetate. Besides the 2.5% salt, it is important that a temperature of about 15°C be used. Higher temperatures cause a deterioration of the kraut.

31.2.2. Cucumbers (pickling)

Cucumber (*Cucumis sativus*) is eaten raw as well after fermentation or pickling. Cucumbers for pickling are best harvested before they are mature. Mature cucumbers are too large, ripen easily and are full of mature seeds. Cucumbers may be pickled by dry salting or by brine salting .

Dry salting is also generally used for cauliflower, peppers, okra, and carrots. It consists of adding 10 to 12% salt to the water before the cucumbers are placed in the tank. This prevents bruising or other damage to the vegetables.

Brine salting is more widely used. A lower amount of salt is added, between 5 and 8% salt being used. Higher amounts were previously used to prevent spoilage. During the primary fermentation lasting two or three days, most of the unwanted bacteria disappear allowing the lactics and yeasts to proliferate. In the final stages, after 10 to 14 days, *Lactobacillus plantarum* and *L. brevis*, followed by *Pediococcus*, are the major organisms.

31.3. FERMENTED FOODS FROM CEREALS AND BEANS

31.3.1. Idli

Idli is a popular fermented breakfast and hospital food which has been eaten in South India for many years. It is prepared from rice grains and the seeds of the leguminous mung grain, *Phaeseolus mungo*, or from black gram (*udad* dhal), *Vigna mungo*, which are also known as dal. When the material contains Bengal grain, *Circer orientium*, the product is known as khaman. It has a spongy texture and a pleasant sour taste due to the lactic acid in the food. It is often embellished with flavoring ingredients such as cashew nuts, pepper and ginger.

31.3.1.1. Production of Idli

The seeds of the dahl (black gram) are soaked in water for 1-3 hours to soften them and to facilitate decortication, after which the seeds are mixed and pounded with rice in a proportion of three parts of the beans and one of rice (Figure 31.1). The mixture is allowed to ferment overnight (20-22 hours). In the traditional system the fermentation is spontaneous and the mixture is leavened up to approximately 2 or 3 times. The organisms involved in the acidification have been identified as *Streptococcus faecalis*, and*Pediococcus* spp. The leavening is brought about by *Leuconostoc mesenteroides*, although the yeasts, *Torulopsis*

candida and*Trichosporon pulluloma* have also been found in traditional Idli. The fermented batter is steamed and served hot. Idli is highly nutritious, being rich in nicotinic acid, thiamine, riboflavin, and methionine.

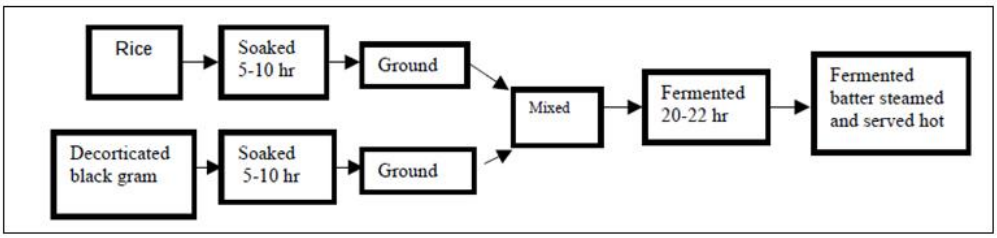

Fig.31.1: Flow diagram for production of idli (Okofer et al., 2007)

31.3.2. Production of beer

Barley beers can be divided into two broad groups: top-fermented beers and bottom fermented beers. This distinction is based on whether the yeast remains at the top of brew (top-fermented beers) or sediments to the bottom (bottom-fermented beers) at the end of the fermentation.

31.3.2.1. Raw Materials for Brewing

The raw materials used in brewing are: barley, malt, adjuncts, yeasts, hops, and water.

a) Brewer's yeasts

Yeasts in general will produce alcohol from sugars under anaerobic conditions, but not all yeasts are necessarily suitable for brewing. Brewing yeasts besides producing alcohol, are able to produce from wort sugars and proteins in a balanced proportion of esters, acids, higher alcohols, and ketones which contribute to the peculiar flavor of beer.

b) Brewery Processes

The processes involved in the conversion of barley malt to beer may be divided into the

following:

1. Malting

2. Cleaning and milling of the malt

3. Mashing

4. Mash operation

5. Wort boiling treatment

6. Fermentation

7. Storage or lagering

8. Packaging

31.3.2.2. Malting

The purpose of malting is to develop amylases and proteases in the grain. These enzymes are produced by the germinated barley to enable it to break down the carbohydrates and proteins in the grain to nourish the germinated seedling before its photosynthetic systems are developed enough to support the plant.

a. Cleaning and milling of malt

The purpose of milling is to expose particles of the malt to the hydrolytic effects of malt enzymes during the mashing process. The finer the particles, greater the extract from the malt.

b. Mashing

Mashing is the central part of brewing. It determines the nature of the wort, hence the nature of the nutrients available to the yeasts and therefore the type of beer produced. The purpose of mashing is to extract as much as possible the soluble portion of the malt and to enzymatically hydrolyze insoluble portions of the malt and adjuncts. The aqueous solution resulting from mashing is known as wort.The wort is boiled for 1-1½ hours in a stainless steel kettle. When corn syrup or sucrose is used as an adjunct it is added at the beginning of the boiling. Hops are also added, some before and some at the end of the boiling. Hops are the dried cone-shaped female flower of hop-plant *Humulus lupulus*. The importance of hops in brewing lies in its resins which provide the precursors of the bitter principles in beer and the essential (volatile) oils which provide the hop aroma.

The purpose of boiling is as follows.

(a) To *concentrate* the wort,

(b) To *sterilize* the wort

(c) To *inactivate* any enzymes

(d) To *extract* soluble materials from the hops

(e) To precipitate protein, which forms large flocs because of heat denaturation and complexing with tannins extracted from the hops and malt husks. Unprecipitated proteins form hazes in the beer, but too little protein leads to poor foam head formation.

(f) To develop color in the beer; some of the color in beer comes from malting but the bulk develops during wort boiling. Color is formed by several chemical reactions including caramelization of sugars, oxidation of phenolic compounds, and reactions between amino acids and reducing sugars.

(g) Removal of volatile compounds: volatile compounds such as fatty acids which could lead to rancidity in the beer are removed.

c. Fermentation

The cooled wort is pumped or allowed to flow by gravity into fermentation tanks and yeast is inoculated or 'pitched in' at a rate of 7-15 x 10^6 yeast cells/ml, usually collected from a previous brew. The progress of fermentation is followed by wort specific gravity. During fermentation the gravity of the wort gradually decreases because yeasts are using up the extract. However, alcohol is also being formed. As alcohol has a lower gravity than wort the reading of the special hydrometer (known as a saccharometer) is even lower. °Brix

is used in the sugar industry, whereas Balling (United States) and °Plato (continental Europe) are used in the brewing industry.

d. Lagering

During lagering secondary fermentation occurs. Yeasts are sometimes added to induce this secondary fermentation, utilizing some sugars in the green beer. The secondary fermentation saturates the beer with CO_2.

31.3.2.3. Packaging

The beer is transferred to pressure tanks from where it is distributed to cans, bottles and other containers. The beer is not allowed to come in contact with oxygen during this operation; it is also not allowed to lose CO_2 or to become contaminated with microorganisms. To achieve these objectives, the beer is added to the tanks under a CO_2, atmosphere, bottled under a counter pressure of CO_2 and all the equipment is cleaned and disinfected regularly.

31.3.2.4. Beer Defects

The most important beer defect is the presence of haze or turbidity, which can be of biological or physico-chemical origin. Biological turbidities are caused by spoilage organisms and arise because of poor brewery hygiene (i.e. poorly washed pipes) and poor pasteurization. Spoilage organisms in beer must be able to survive the following stringent conditions found in beer: low pH, the antiseptic substances in hops, pasteurization of beer, and anaerobic conditions.

Yeasts and certain bacteria are responsible for biological spoilage because they can withstand these. Wild or unwanted yeasts which have been identified in beer spoilage are spread into many genera including *Kloeckera*, *Hansenula*, and *Brettanomyces*, but*Saccharomyces* spp appear to be commonest, particularly in top-fermented beers. These include *Sacch. cerevisiae* var. *turblidans,*and *Sacch. diastaticus.* The latter is important because of its ability to grow on dextrins in beer, thereby causing hazes and off flavors. Among the bacteria, *Acetobacter*, and the lactic acid bacteria, *Lactobacillus* and *Streptococcus* are the most important. The latter are tolerant of low pH and hop antiseptics and are microaerophilic hence they grow well in beer. *Acetobacter* is an acetic acid bacterium and produces acetic acid from alcohol thereby giving rise to sourness in beer. *Lactobacillus pastorianus* is the typical beer spoiling lactobacilli, in top-fermented beers, where it produces sourness and a silky type of turbidity. *Streptococcus damnosus* (*Pediococcus damnosus*, *Pediococcus cerevisiae*) is known as 'beer sarcina' and gives rise to 'sarcina sickness' of beer which is characterized by a honey-like odor.

31.3.3. WINES

Wine is by common usage defined as a product of the "normal alcoholic fermentation of the juice of sound ripe grapes". Nevertheless any fruit with a good proportion of sugar may be used for wine production. If the term is not qualified then it is regarded as being derived from grapes, *Vitis vinifera.* The production of wine is simpler than that of beer in that no need exists for malting since sugars are already present in the fruit juice being used. This however exposes wine making to greater contamination hazards

31.3.3.1. Processes in Wine Making

a. Crushing of Grapes

Selected ripe grapes are crushed to release the juice which is known as 'must', after the stalks which support the fruits have been removed. These stalks contain tannins which would give the wine a harsh taste if left in the

must. The skin contains most of the materials which give wine its aroma and color. Grape juice has an acidity of 0.60-0.65% and a pH of 3.0-4.0 due mainly to malic and tartaric acids with a little citric acid.

b. Fermentation

(i) **Yeast used**: The grapes themselves harbor a natural flora of microorganisms (the bloom) which in previous times brought about the fermentation and contributed to the special characters of various wines. Yeasts are then inoculated into the must. The yeast which is used is *Saccaromyces cerevisiae* var, *ellipsoideus* (synonyms: *Sacch. cerevisiae, Sacch. ellipsoideus, Sacch, vini.).*

Wine yeasts have the following characteristics: (a) growth at the relatively high acidity (i.e., low pH) of grape juice; (b) resistance to high alcohol content (higher than 10%); (c) resistance to sulfite.

c. Control of fermentation

(a) **Temperature**: Heat is released during the fermentations. It has been calculated the temperature of a must containing 22% sugar would rise 52°F (11°C) if all the heat were stopped from escaping. If the initial temperature were 60°F (16°C) the temperature would be 100°F (38°C) and fermentation would halt while only 5% alcohol has been accumulated. For this reason the fermentation is cooled and the temperature is maintained at around 24°C with cooling coils mounted in the fermentor.

(b) **Yeast Nutrition**: Yeasts normally ferment the glucose preferentially although some yeasts e.g. *Sacch. elegans* prefer fructose. Most nutrients including macro- and micro-nutrients are usually abundant in must; occasionally, however, nitrogeneous compounds are limiting. They are then made adequate with small amounts of $(NH_4)_2 SO_4$.

(c) **Oxygen**: As with beer, oxygen is required in the earlier stage of fermentation when yeast multiplication is occurring. In the second stage when alcohol is produced the growth is anaerobic and this forces the yeasts to utilize such intermediate products as acetaldehydes as hydrogen acceptors and hence encourage alcohol production.

(iii) **Flavor development**: Although some flavor materials come from the grape most of it come from yeast action and has been shown to be due to alcohols, esters, fatty acids, and carbonyl compounds, the esters being the most important. Diacetyl, acetoin, fuel oils, volatile esters, and hydrogen sulfide have received special attention.

d. Ageing and Storage

The fermentation is usually over in three to five days. At this time ‘pomace’ formed from grape skins (in red wines) will have risen to the top of the brew. At the end of this fermentation the wine is allowed to flow through a perforated bottom if pomace had been allowed. When the pomace has been separated from wine and the fermentation is complete or stopped, the next stage is ‘racking’. The wine is allowed to stand until a major portion of the yeast cells and other fine suspended materials have collected at the bottom of the container as sediment or ‘lees’. It is then ‘racked’, during which process the clear wine is carefully pumped. The wine is then transferred to wooden casks (100-1,000 gallons), barrels (about 50 gallons) or tanks (several thousand gallons). The wood allows the wine only slow access to oxygen. Water and ethanol evaporate slowly leading to air pockets which permit the growth of aerobic wine spoilers e.g. acetic acid bacteria and some yeasts. The casks are, therefore regularly topped up to prevent the pockets. In modern tanks made of stainless steel the problem of air pockets is tackled by filling the airspace with an inert gas such as carbon dioxide or nitrogen. During ageing desirable changes occur in the wine. These changes are due to a number of factors:

e. Clarification

The wine is allowed to age in a period ranging from two years to five years, depending on the type of wine. At the end of the period some will have cleared naturally. For others artificial clarification may be necessary. The addition of a fining agent is often practiced to help clarification. Fining agents react with the tannin, acid, protein or with some added substance to give heavy quick-settling coagulums. The usual fining agents for wine are gelatin, casein, tannin, egg albumin, and bentonite.

f. Packaging

Before packing in bottles the wine from various sources is sometimes blended and then pasteurized. In some wineries, the wine is not pasteurized, rather it is sterilized by filtration. In many countries the wine is packaged and distributed in casks.

g. Wine Defects

The most important cause of wine spoilage is microbial; less important defects are acidity and cloudiness. Factors which influence spoilage by bacteria and yeasts include the following (a) wine composition, specifically the sugar, alcohol, and sulfur dioxide content; (b) storage conditions e.g. high temperature and the amount of air space in the container; (c) the extent of the initial contamination by microorganism during the bottling

process. When proper hygiene is practiced bacterial spoilage is rare. When it does occur the microorganisms concerned are acetic acid bacteria which cause sourness in the wine. Lactic acid bacteria especially *Leuconostoc*, and sometimes *Lactobacillus* also spoil wines. Various spoilage yeasts may also grow in wine. The most prevalent is *Brettanomyces*, slow growing yeasts which grow in wine causing turbidities and off-flavors. Other wine spoilage yeasts are *Saccharomyces oviformis*, which may use up residual sugars in a sweet wine and *Saccharomyces bayanus* which may cause turbidity and sedimentation in dry wines with some residual sugar. *Pichia membanaefaciens* is an aerobic yeast which grows especially in young wines with sufficient oxygen. Other defects of wine include cloudiness and acidity.

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Module 10. Dairy waste management

Lesson-32

Treatment and Disposal of Waste Water and Effluents

32.1. INTRODUCTION

With increase in demand for milk and milk products, many dairies of different capacities have come up in different places. These dairies collect milk from the producers and then either simply bottle it for marketing, or produce different milk products according to their capacities. Large quantities of waste water originate due to their different operations. The organic substances in the wastes comes either in the form in which they were present in milk or in a degraded form due to their processing. As such, the dairy waste, though biodegradable, are very strong in nature.

Dairy plants process a wide variety of products including milk, cheese, butter, ice cream, yogurt, nonfat dry milk, whey and lactose. The volume and composition of dairy wastes from each plant depends on the types of products produced, waste minimization practices, types of cleaners used and water management in the plant. Because most dairy plants process several milk products, waste streams may vary widely from day to day.

32.2. SOURCES OF DAIRY WASTES

The liquid waste from a large dairy originates from the following sections or plants: receiving stations, bottling plant, cheese plant, casein plant, condensed milk plant, dried milk plant, and ice cream plant. The main sources of dairy effluents are those arising from the following:

1. Spills and leaks of products or by-products

2. Residual milk or milk products in piping and equipment before cleaning

3. Wash solutions from equipment and floors

4. Condensate from evaporation processes

5. Pressings and brines from cheese manufacture

Dairy plant operators may choose from a wide variety of methods for treating dairy wastes from their plants. This may range from land application for small plants to operation of biological waste water treatment systems for larger plants. Some dairy plants may pretreat the effluents and discharge them to a municipal waste water treatment plant.

In addition to the wastes from all the above milk processing units, some amount of uncontaminated cooling water comes as waste; these are very often re-circulated.

32.3. OBJECTIVES OF TREATING DAIRY WASTES

The objectives of treating dairy wastes are to

a. Reduce the organic content of the waste water,

b. Remove or reduce nutrients that could cause pollution of receiving surface waters or ground water, and

c. Remove or inactivate potential pathogenic microorganisms or parasites.

The level of treatment needed for dairy waste water for each plant is dictated by the environmental regulations applicable to the location of the dairy plant. The Environmental Protection Agency (EPA) establishes general regulations concerning discharges to surface waters and ground water. Each state environmental regulatory agency is responsible for ensuring compliance with those regulations. Each plant must have a discharge permit for each outfall discharging to surface waters. The limits within that permit depend on the flow and type of surface water into which the treated waste water is discharged. If a plant discharges waste water to municipal sewers for treatment, the municipal treatment system may require pretreatment of high-strength wastes to bring the waste load down to domestic sewage strength. This allows for proper treatment of waste water before it is discharged to surface water. For land applications, state regulatory agencies dictate hydraulic loadings and maximum levels of toxic substances that can be spread on each unit of land.

32.4. COMPOSITION OF DAIRY WASTES

Because more than 95% of the waste load from dairy plants comes from milk or milk products, it is of value to know the average composition of these products. Milk solids are primarily composed of fats, proteins, and carbohydrates. Other constituents in dairy waste water may include sweeteners, gums, flavoring, salt, cleaners, and sanitizers. Biochemical oxygen demand (BOD) is the amount of dissolved oxygen (DO) consumed by microorganisms for biochemical oxidation of organic solids in waste water. The analytical procedure for determining BOD measures dissolved oxygen consumed by a seeded, diluted waste water sample incubated at 20°C for 5 days. One gram of milk fat has a BOD of 0.89 g, whereas milk protein, lactose, and lactic acid have BOD value of 1.03, 0.65, and 0.63 g, respectively. Roughly, 1 kg of BOD in dairy wastewater represents 9 kg of whole milk. Chemical oxygen demand (COD) is the amount of oxygen necessary to oxidize the organic carbon completely to CO_2, H_2O, and ammonia. The COD is measured calorimetrically after refluxing a sample of wastewater in a mixture of chromic and sulfuric acid. If the BOD/COD ratio of waste water is less than 0.5, then the organic solids in the waste are not easily biodegraded. The BOD/COD ratio for dairy wastes has been reported to range from 0.50 to 0.78.

Some minor constituents, such as phosphorus and chloride, are also very important in the treatment of dairy wastes. Phosphorus is the element that limits plant and algal growth in surface waters. Discharge of any significant levels of phosphorus in waste effluents to surface waters can lead to decreased water quality in lakes and streams. Milk and milk by-products can contribute significant quantities of phosphorus to dairy wastes. The phosphorus content of milk is approximately 1000 mg/L, whereas whey contains 450 to 575 mg/L. Salty whey and brines can contribute significant levels of chloride to dairy waste water. Chloride concentrations in excess of 400 mg/L in effluents discharged to streams can result in chronic toxicity. Reported BOD values and percentage contribution of milk sensitive water insects such as Daphnia magna. Because chloride cannot be removed with biological or chemical treatments, waste minimization is the only method for reducing chloride in dairy wastes. The BOD values of various dairy products are shown in Table 32.1.

Table-32.1: BOD values and percentage contribution of milk components to product BOD

Product	% contribution to BOD_5 by			
	BOD_5 (mg/lt)	Milk fat	Milk protein	Lactose
Skim milk	67,000	6.3	49.3	44.5
Whole milk	104,000	17.8	43.3	39.0
Half and half	156,000	62.4	19.7	17.9
Heavy cream	399,000	89.2	5.7	5.0
Churned buttermilk	68,000	4.2	48.2	46.7
Evaporated milk	208,000	34.6	35.0	30.6
Ice cream	292,000	30.7	15.9	15.2
Whey	34,000	5.9	20.6	70.8

Source: Harper and Blaisdell (1971).

The dairy wastes are very often discharged intermittently the nature and composition of wastes also depend on the types of products produced, and the size of the plants. The Table 32.2 gives the characteristics of the wastes of a typical Indian dairy, handling about 300000 to 400000 lts of milk in a day.

Table 32.2 Composition of waste water of a typical dairy

Properties	Value
pH	7.2
Alkalinity	600mg/lt as CaCo3
Total dissolved solids	1060mg/lt
Suspended solids	760mg/lt
BOD	1320mg/lt
COD	84mg/lt
Total nitrogen	84mg/lt
Phosphorous	11.7mg/lt
Oil and grease	290mg/lt
Chloride	105mg/lt

32.5. TREATMENT OF MILK WASTE

Wastes from processing milk products are almost entirely composed of organic material in solution or colloidal suspension, although some larger suspended solids may be present in waste water from cheese or casein manufacturing plants. Sand and other foreign material is present in limited amounts as a result of floor or truck washes. Because milk waste contains very little suspended matter, preliminary settling of solids does not result in any appreciable reduction of BOD.

However, a screen and grit chamber with 0.95-cm mesh wire screen is recommended to remove large particles to prevent clogging of pipes and pumps in the treatment system. This is especially important, if the waste is to be pumped with high-pressure pumps, as in spray irrigation. After preliminary treatment in the screen and grit chamber, the waste should be pumped to an equalization tank. With wide variations in wastewater flow, strength, temperature, and pH, some reaction time is required to allow neutralization of acid and alkaline cleaning compounds and to allow for complete reaction of residual oxidants from cleaning solutions with organic solids of dairy waste. Ideally, a minimum of 6–12 h of equalization should be provided to allow for waste stabilization. The equilibrated waste can then be treated with one of the following systems or a combination of treatment systems: (a) land application, (b) treatment ponds or lagoons, (c) activated sludge, (d) biological filtration, or (e) anaerobic digestion.

32.6. TREATMENT PONDS OR LAGOONS

Dairy plants in rural areas with insufficient farmland available for land application may be able to use ponds or lagoons for economical treatment of dairy wastes. A pond or lagoon normally consists of a shallow basin designed for treatment of dairy wastewater without extensive equipment and controls. The three types of ponds used are aerobic, facultative, and anaerobic.

32.7. AEROBIC PONDS

Aerobic ponds are generally 0.5–2.0 m deep, and contents are mechanically mixed and aerated to allow penetration of sunlight necessary for growth of algae. The algae produce oxygen through photosynthesis and use waste products from the bacteria involved in the biological breakdown of milk wastes. At 20°C, a BOD removal of 85% can be experienced with an aeration period of 5 days.

32.8. ANAEROBIC PONDS

Anaerobic ponds are generally used to pretreat dairy wastes with high protein and fat levels or for stabilizing settled solids. Organic matter is biodegraded and gases such as CH4, CO2, and H2S are produced. To reduce effectively the BOD in anaerobic effluent, an aerobic process must follow to allow aerobic microorganisms to use up the residual breakdown products. The typical retention time for anaerobic treatment ponds ranges from 20 to 50 days.

32.9. ACTIVATED SLUDGE

Activated sludge is one of the most popular methods for treating dairy wastes. The process consists of aerobic oxidation of organic matter to CO_2, H_2O, NH_3, and cell biomass followed by sedimentation of activated sludge. A portion of the activated sludge is returned to the aeration tank to continue the treatment cycle (Figure.32.1.).

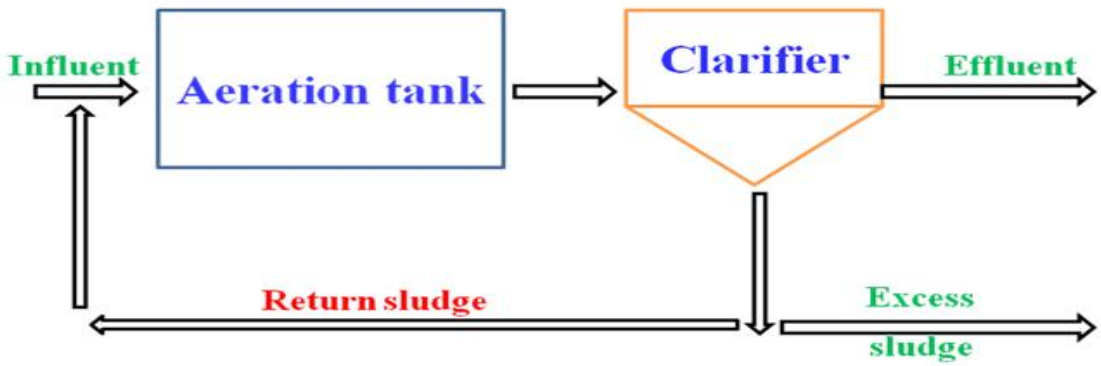

Fig. 32.1 Activated sludge process

Activated sludge contains a large mass of various microorganisms plus organic and inorganic particles. The concentration of biomass in the aeration or contact tank is normally called the mixed liquor suspended solids (MLSS). Bacteria make up the largest portion of activated sludge in the aeration process. Bacteria are primarily responsible for oxidation of organic matter and formation of polysaccharides and other polymeric materials that aid in flocculation of the microbial biomass. Table 32.3 lists the bacterial genera found in activated sludge. Estimates of aerobic bacterial counts in activated sludge are approximately 1010/g of MLSS or 10^7–10^8/mL. The active fraction of bacteria in activated sludge flocs represents only 1%–3% of total bacteria present. This indicates that the major portion of activated sludge is actually dead cells and extracellular material. Activated sludge does not normally favor growth of yeast, algae, or fungi. Protozoa may represent up to 5% of the MLSS. Protozoa are predators of bacteria in activated sludge; they help reduce effluent suspended solids and soluble BOD.

Table 32.3 Bacterial genera found in activated sludge

Major genera	Minor genera
Zoogloea	*Aeromonas*
Pseudomonas	*Aerobacter/Enterobacter*
Comomonas	*Micrococcus*
Flavobacterium	*Spirillum*
Alcaligenes	*Acinetobacter*
Brevibacterium	*Gluconobacter*
Bacillus	*Hyphomicrobium*
Achromobacter	*Cytophaga*
Corynebacterium	
Sphaerotilus	

Source: Sterritt and Lester (1988).

32.10. CONVENTIONAL PROCESS

In the conventional activated sludge process, dairy waste water is introduced into the aeration tank along with a portion of activated sludge from the clarifier. Air is incorporated into the waste mixture with diffusers or mechanical aerators. The air serves two purposes in the aeration tank: first, to supply oxygen to aerobic microorganisms and, second, to keep the activated sludge floc thoroughly mixed with incoming waste water to allow maximal efficiency in oxidation of organic matter. Key parameters controlling operation of the activated sludge process are rate of (a) aeration in the tank, (b) return of activated sludge to the aeration tank, and (c) waste or excess sludge discharged from the treatment system. Normal detention time for conventional activated sludge treatment of municipal or low strength waste water is 4–8 h. However, dairy waste waters may require longer detention times, 15–40 h, to reduce BODs to an acceptable level. This type of process is called an extended aeration system.