UNIT 6 SCREENING AND ENUMERATION OF SPOILAGE MICRO-ORGANISMS IN FOOD

Structure

6.0 OBJECTIVES

After reading this Unit, we shall be able to:

- explain the role of food as carrier of spoilage organisms,
- describe the causes of spoilage of food,
- enlist major types of spoilage bacteria found in foods
- identify the common source of contamination by these pathogens
- specify types of media used and enumeration protocol of various spoilage bacteria

6.1 INTRODUCTION

It is of common knowledge that every **food** item we eat is biological in origin, so knowledge of biology is important in all aspects of initial food **production**, as well as **preparation** and **distribution**. In particular, we expect our food to be fresh and wholesome, and not to contain any undesired added impurities (adulterants).

Food can deteriorate as a result of two main factors:

1) Growth of spoilage micro-organisms - usually from surface contamination- especially important in processed food.
2) Enzymatic action - from within cells – by normal metabolic processes, e.g. respiration. 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.

Micro-organisms enter foods from various sources on its way from production to processing, packaging, distribution and even consumption. That's why we sometime use phrase such as "From pasture to fork" in context of total quality management. Common sources are soil, water, plant products, food utensils, gastrointestinal tract, food handlers, animal feeds, air and dust.

"Negative" aspects of microbial growth include food deterioration and spoilage by **decay**, and **food poisoning**, mainly caused by different and less widespread bacteria. As they grow, micro-organisms release their own extra cellular enzymes into the medium/ matrix surrounding them, and absorb the products of external breakdown. This is the main basis of microbial food spoilage, which lowers its nutritional value and decreases its shelf life. Bacteria and moulds may also produce waste products which act as poisons or toxins, thus causing the ill-effects. Growth of these organisms is facilitated by a number of intrinsic and extrinsic factors present in food such as presence of organic food (proteins, carbohydrates, fats), suitable temperature, moisture (water), animal feeds, animal hides, air, and suitable pH.

6.2 DETECTION AND ENUMERATION OF SPOILAGE MICRO-ORGANISMS

The spoilage micro-organisms on the basis of their biochemical characteristics and nutritional requirement (factors detrimental to their ability to spoil food in which they are present) can be broadly categorized into psychtrophic, thermoduric, lipolytic, proteolytic, pectinolytic, halophiles, osmophiles, acid producers etc.

6.2.1 Psychrotrophic Count

The term “Psychrotrophic” is used for the organism capable of growing at refrigerated temperatures irrespective of their optimum temperature of growth. Psychrotrophs commonly include genera of gram negative thin rods e.g. *Pseudomonas, Achromobacter, Flavobacterium* and *Alcaligenes*.

These organisms owing to their high proteolytic and lipolytic activity can cause taint production (discoloration & off-flavour production) in foods stored under refrigerated conditions for longer periods. The excessive growth of such organisms can be detrimental to the shelf-life of both raw and processed food products especially, during extended storage.

For enumeration of psychrotrophic organisms appropriate dilutions can be prepared and plated (pour or spread plate method) on non selective media such as plate count agar, tryptone glucose extract agar, Trypticase soy agar and subsequently incubated at refrigeration temperature at 7^{o}C for 10 days. These organisms are heat labile (with few exceptions) and hence can be possibly injured or inactivated if plates are poured with agar held above 45^{o}C. Hence due care should be taken.

6.2.2 Thermoduric Count

Thermoduric organisms differ from thermophiles in their ability only to survive heat treatment while latter can survive and grow at elevated temperature e.g. pasteurization. The species of genera viz. *Arthrobacter, Bacillus*, *Clostridium*, *Enterococcus*, *Micrococcus* and *Lactobacillus* belongs to these groups. Biochemical characteristics of these organisms include acid production, proteolysis and lipolysis and can cause spoilage of heat treated and canned products. These organisms are of special interest in dairy products where they are known to survive pasteurization.

These organisms along with psychrotrophs have limited ability to reduce dyes such methylene blue and resazurin and hence dye reduction tests do not correlate well with plate counts in such cases.

For enumeration of thermoduric bacteria, a 5 ml volume of liquid sample in original or of decimal dilution is aseptically transferred to sterile test tube and a rack of such tubes is placed in a water bath maintained at 62.8^{o}C. After a period of 30 min (commonly termed as laboratory pasteurization), tubes are immediately cooled in an ice water bath. Now 1.0 ml of this sample can be analyzed by plate count method using plate count agar and incubation at 37 ^{o}C for 72 h.

6.2.3 Lipolytic Count

The fat rich food products are liable to be spoiled by lipolytic micro-organisms though there can be non-microbial origin of such defects. The known lipolytic organisms commonly encountered in foods include bacteria (*Pseudomonas, Achromobacter, Staphylococcus*), molds (*Rhizopus, Geotrichum, Aspergillus, Penicillium*), and yeast (*Candida, Rhodotorula, Hansenula*).

For lipolytic count a base layer medium which comprises fat, 50.0 g; victoria blue, 1:1500 solution, 200 ml; agar, 15 g is used. Fat may consist of tributyrin, corn oil, soybean oil, cooking oil. Out of these tributyrin is the most widely used substrate and hence commercially available tributyrin agar is commonly used for lipolytic count. To such agar victoria blue is not required to be added. The plates of lipolytic medium are streaked with the dilution of sample and incubated at 20 to 25 ^{o}C for 3 to 4 days. The presence of a clear zone of hydrolysis around the colony is taken as a positive indication and inferences are drawn accordingly.

6.2.4 Proteolytic Count

The proteolytic activity of micro-organisms can lead to a number of undesirable flavour defects in milk, meat, poultry and seafood. Such organisms include *Bacillus*, *Clostridium*, *Pseudomonas* and *Proteus*.

Milk agar medium

For enumeration of proteolytic bacteria especially from dairy products, this agar is commonly used. The hydrolysis of casein manifests into development of clear zone around the colony. The sample is poured or streaked on this medium and incubated at 21^{o}C for 72 h. Another medium, gelatin agar medium with double layer plating technique can also be used for this purpose. A common limitation of this method is the need to flood the medium with protein precipitant (1% HCl or 10 % acetic acid solution for 1 minute to ensure that the clear zones are formed as a result of proteolysis and not due to acid production from carbohydrates.

6.2.5 Pectinolytic Count

Pectinolytic enzymes capable of hydrolyzing pectic substances include pectin isomerase, pectin methoxylase, pectin methylesterase and pectate lyase. Sources of these enzymes are *Achromobacter, Aeromonas, Arthrobacter, Agrobacterium, Bacillus, Clostridium, Enterobacter, Flavobacterium, Pseudomonas, Xanthomonas,* yeasts, moulds, protozoa and nematodes. Medium used for detection of pectate lyase, polygalacturonase and fluorescent pectinolytic *Pseudomonads* are mineral pectin-7 medium (MP-7), mineral

pectin-5 medium (MP-5), fluorescent and pectinolytic agar (FPA) respectively.

A homogeneous suspension followed by appropriate dilutions of the food sample are prepared. A volume of not more than 0.25 ml is placed and spread on surface of prepoured plates. Incubation is carried at 30^{o}C for 48 h. Colonies which effect depressions in polypectate gel medium or are found to be encircled by a clear zone after flooding the MP-7, MP-5 or FPA plates with a pectin precipitant (1% solution of hexa-decyl tri-methyl ammonium bromide in water) are considered as pectinolytie colonies and are counted.

Check Your Progress Exercise 1

Note: a) Use the space below for your answer.
b) Compare your answers with those given at the end of the unit.

1) Incubation temperature for psychrotrophic, thermoduric and pectinolytic micro-organisms is ……, …… and ……^{o}C.

2) Most widely used substrate for lipophiles is ……..

3) Characteristic colony of proteolytic microbes on Milk Agar medium is ……..

4) List any three pectinases.

……

……

……

6.2.6 Halophilic Count

Halophiles are those organisms which need certain minimal salt concentrations for their growth. These organisms can be classified as slight halophiles (2 to 5% salt), moderate halophiles (5 to 20 % salt) and extreme halophiles (20 to 30 % salt).

a) Slight halophiles

Majority of slight halophiles are traced to marine environment and typical genera such as *Acinetobacter*, *Flavobacterium,* and *Pseudomonas* are known to spoil marine products and shellfish. For enumeration of these organisms, skins are removed from fish and six samples collected, three each from dorsal and ventral sides of the fish. Ten grams of skin is added to 90 ml of phosphate buffer. Sample is vigorously mixed and subsequent dilutions are prepared in phosphate buffer. A volume of 0.1 ml aliquots is dispensed on the trypticase soy agar plus 3% NaCl land evenly spread on plate using spread plate technique. Plates are incubated at 7^{o}C for 10 days. Results are reported as cfu per gram.

For light salted meat and vegetables, dilutions are prepared by blending 50 g samples with 450 ml diluent with added salt concentration equivalent to salt concentration of food sample. Rest of the procedure is same as above.

b) Moderate halophiles

This group includes bacteria belonging to *Bacillaceae* and *Micrococcaceae*. A 1: 10 dilution is prepared by mixing 50 g of food in 450 ml of sterile buffer

with added NaCl equivalent to the salt concentration prevalent in the food sample. Rest procedure for plating and incubation is same as for slight halophiles.

c) Extreme halophiles

They are usually found in aquatic environments of unusually high concentration and in solar evaporated sea salts. Genera like *Halobacterium* and *Halococcus* belong to this group. For isolation, the surface slime from salted fish is transferred to halophilic agar plate using a cotton or alginate swab. For enumeration 1: 10 dilution is prepared by mixing 50 g of sample with 450 ml of halophilic broth. A volume of 0.1 ml aliquot of each dilution is placed on halophilic agar and incubated at 33-35^{o}C for 5 to 12 days in a humid incubator. Results are given as halophilic count per ml or per gram.

6.2.7 Osmophilic Count

Yeasts capable of growing in high concentration of sugar happen to be the common osmophiles found in food. They are responsible for spoilage of honey, chocolate candy, jams, molasses, corn syrup, fruit juices and similar products. For their enumeration, appropriate dilutions are prepared in phosphate buffered dilution water as diluent, plated in agar (Potato dextrose agar, MY-40 agar i.e. malt yeast extract 40 % sucrose), and incubated at 30^{o}C for 4 to 5 days.

6.2.8 Acidophilic Count

Acid producing bacteria are found in various habitats e.g. soil, raw agricultural products, and processed foods. Lactic acid bacteria are well known in food industry for their ability to produce acids from sugar present in substratum and on this basis can be classified as homo-fermentative and hetero-fermentative. Besides, spore formers (*Bacillus* and *Clostridium*) and enteric bacteria (*Escherichia*, *Enterobacter*, *Salmonella*, *Shigella*) are also endowed with this ability.

For detection of such bacteria, determination of titratable acidity (acidity expressed as lactic or acetic acid) and indicator microbiological media (containing pH indicator e.g. bromo cresol purple) are required.

Lactic acid bacteria chiefly comprise lactic *Streptococci* and *Lactobacilli*. Lactic agar supports growth of both types of bacteria while M17 agar and MRS agar allows selective growth of former and later, respectively. Using pour plate method these media can be used for direct enumeration of lactic acid bacteria. Their identity can be further confirmed by gram staining and catalase test. These organisms are gram positive, non spore-forming, non motile and catalase negative.

Measurement of titratable acidity can be used as an indirect method of estimation of acid production. It can be performed by titration of an appropriate quantity of sample (say fermented milk) with 0.1 N NaOH to the phenolphthalein end point (pH 8.3).The % acid present as lactic acid is calculated as follows:

$$\% \text{ lactic acid} = \frac{V_2 \times 0.009}{V_1} \times 100$$

where, V_1= Volume of sample/milk (in ml)

V_2= Volume of NaOH used (in ml).

Check Your Progress Exercise 2

Note: a) Use the space below for your answer.
b) Compare your answers with those given at the end of the unit.

1) Spoilage micro-organisms in jams and pickles are called …… and …… respectively.

2) Media for plating halophiles is ……

3) Formula for percent acidity in milk ……

4) List any two media for growing yeast

……..

……..

6.3 LET US SUM UP

Microbes and their enzymes are major causative agents of spoilage of food. Growth and resultant biochemical activity of micro-organisms is supported by a number of intrinsic and extrinsic factors which exist in food and environment. Based on their biochemical attributes and nutritional requirement the spoilage causing microflora can be broadly grouped in to psychrotrophic, thermoduric, lipolytic, proteolytic, pectinolytic, halophiles, osmophiles, acid producers etc. P Psychrotrophic organisms can be enumerated by using media such as plate count agar, tryptone glucose extract agar, Trypticase soy agar and subsequently, incubation at refrigeration temperature at 7^{o}C for 10 days. For thermoduric bacteria count, laboratory pasteurized sample is plated on plate count agar and incubated at 37^{o} C for 3 days. For lipolytic and proteolytic count, tributyrin and milk agar are used respectively and plates are incubated at 20 to 25^{o}C for 3 to 4 days. The formation of clear zone of hydrolysis around the colony is interpreted as a positive sign and conclusions are drawn accordingly. In case of pectinolytic bacteria, selective media used for assesment of activity of pectinolytic enzymes such as pectate lyase, polygalacturonase and fluorescent pectinolytic *Pseudomonads* (FPA medium) are mineral pectin-7 medium, mineral pectin-5 medium, fluorescent and pectinolytic agar, respectively. An appropriate dilution is placed on halophilic agar and incubated at 33-35^{o}C for 5 to 12 days in a humid incubator for halophilic bacterial count. Paotato dextrose agar or MY-40 agar and incubation at 30 ^{o}C for 4 to 5 days are used for enumeration of osmophiles in food. Lactic acid bacteria happen to be the major acidophiles in foods and lactic agar, M17 agar and MRS agar can be used for their selective growth and enumeration.

6.4 KEY WORDS

Psychrotrophs : Organisms capable of growing at refrigerated temperature

Taint production : Discoloration & off-flavour production

Thermoduric : Organisms able to survive pasteurization

Laboratory pasteurization : Heating at 62.8^{o}C for 30 minutes

Tributyrin agar : Used for enumeration of lipolytic bacteria

Milk agar : Used for enumeration of proteolytic bacteria

FPA Medium : Fluorescent pectinolytic *Pseudomonads* medium

Halophilic Agar : Used for enumeration of halophiles

MY-40 Agar : Malt yeast extract 40 % sucrose agar

6.5 SUGGESTED FURTHER READING

American Public Health Association (APHA). (2001). Compendium of Methods for the Microbiological Examination of Foods (4th edition), F.P. Downes and K. Ito (Editors), Washington, D.C.

James M. Jay. (2000). Modern Food Microbiology (6th. Edition), Aspen Publisher, USA.

Frazier W. (1995). Food Microbiology (4th edition), Tata McGraw Hill, India

6.6 TERMINAL QUESTIONS

1. Describe the protocol for detection and enumeration of the following group of micro-organisms from the food mentioned against their name as follows:

S. No.	Microorganism	Food
1.	Psychrotrophic	Ice cream
2.	Thermoduric	Canned peas
3.	Lipolytic	Cooking oil
4.	Proteolytic	Nugget
5.	Pectinolytic	Frozen vegetables rot
6.	Halophilic	Canned fish
7.	Osmophilic	Rasogulla
8.	Acidophilic	Curd

2. Give typical examples of each group of microorganism mentioned above.
3. Prepare a list of defects caused by these organisms in various foods with the help of literature.
4. Suggest remedial measures to control the microbial spoilage of foods.

6.7 ANSWERS TO CHECK YOUR PROGRESS EXERCISES

Check Your Progress Exercise 1

Your answer should include following points:

1) 7, 32 and 30^{o}C

2) tributyrin

3) clear zone around the colony

4) pectin methoxylase, pectin methylesterase and pectate lyase

Check Your Progress Exercise 2

Your answer should include following points:

1) halophilic and osmophilic
2) trypticase soy agar plus 3% NaCl
3) % lactic acid= V_2 x 0.009/V_1 X 100
4) Potato dextrose agar, MY-40 agar