Chapter 9
Indicator Organisms, Detection of Indicator Organisms, Microbiological Culture Methods and Immunological Methods Employed in Food Industry

Microbiological indicator organisms can be used to monitor hygienic conditions in food production. The presence of specific bacteria, yeasts or molds is an indicator of poor hygiene and a potential microbiological contamination.

Total aerobic count

The total viable count on surface describes the number of colony forming units (cfu) which exist on a defined area (eg. $1cm^2$) of the analyzed surface. Normally it will be determined using a total plate count agar by growing the colonies after incubation at 30-35^{0}C for approximately 48 hours. Counted the colonies. The total viable count is an indicator for the hygienic status of the food production and shows possible microbial loads and contamination sources. The aerobic mesophilic count indicates the number of colony forming units (cfu) formed on a plate count medium during a specified incubation time at mesophilic temperatures (30-37^{0}C). The aerobic count is an indicator for the microbial status of the production and environmental conditions.

Coliform bacteria

Coliform bacteria are considered to be indicators of fecal contaminations and are often used for monitoring water quality. Detection of coliform bacteria on surfaces in the production environment or solid foods indicates that the hygienic conditions in the food production process needs to be optimised. These bacteria can be easily identified using nutrient media which contain chromogenic substrates for their enzyme β- galactosidase (eg: X-GAL).

Enterobacteria

The Enterobacteriaceae are gram-negative, rod shaped bacteria which are

typically 1-5 μm in length. They are facultative anaerobes and most are motile, but non motile genera exist as well. Enterobacteriaceae cannot produce oxidase and can be distinguished from similar genera by this criterion. Enterobacteriaceae are a normal part of the gut flora which is found in the intestinal tract of humans and animals. They are also spread widely in the environment (eg: soil, water). Some genera are pathogenic and can cause serious diseases. Genera of Enterobacteriaceae are *Enterobacter*, *Escherichia*, *Klebsiella*, *Proteus*, *Salmonella*, *Serratia*, *Shigella*, *Yersinia*, *Morganella*, *Hafnia*, *Citrobacter* etc.

Enterococcus

Enterococci are gram-positive organisms which belong to the intestinal bacteria as well as the gram-negative Enteriobacteriacae. Enterococci may appear as contaminations in a variety of fermented foods. Their presence in food products has been considered as an indication of poor sanitary conditions during production and processing. On the other hand enterococci are specifically used as starter cultures for the fermentation processes of a variety of foods. It is claimed that enterococci play an important role in the development of the organoleptic properties of the fermented foods. For water, the presence of enterococci serves as an indicator of fecal contamination. Enterococci will only appear in water if they are inserted by contamination with human or animal feces.

Yeast and molds

Yeast and molds are able to contaminate foods and are responsible for quick spoilage of the infested food stuff. Due to their ability to produce toxic or allergenic substances molds are especially predestinated to be a potential health risk. As these organisms might be rapidly spread by dusts and aerosols, surfaces in the production environment will be consistently contaminated.

Yeasts are facultative anaerobe, mono cellular fungi fermenting sugar substrates to CO_2 and H_2O. Under anaerobic conditions yeasts ferment sugar to alcohol and CO_2. In terms of food spoilage genera of *candida* play an important role. This is located on the human and animal mucosa (nose, throat). The term ‘mold’ is commonly used for the visible part of the fungi present on the surface of contaminated food. Under the surface, the fungi forms mycelium which can’t be recognized with the naked eye. Specific molds as well as yeast are used for industrial purposes (eg: cheese production). Harmful genera of molds exist are able to produce toxins (mycotoxins). Almost all molds have an allergenic potential related to their spore form capabilities.

Culture methods

Indicator bacteria can be cultured on media which are specifically formulated to allow the growth of the species of interest and inhibit growth of other organisms. Typically, environmental water samples are filtered through membranes with small pore sizes and then the membrane is placed on to a selective agar. It is often necessary to vary the volume of water sample filtered in order to prevent too few or too many colonies from forming on a plate. Bacterial colonies can be counted after the 24-48 hours depending on the type of bacteria. Counts are reported as colony forming units per 100 ml (cfu /100 ml).

Fast detections using chromogenic substances

One technique for detecting indicator organisms is the use of chromogenic compounds, which are added to conventional or newly devised media used for isolation of the indicator bacteria. These chromogenic compounds are modified to change color or fluorescence by the addition of either enzymes or specific bacterial metabolites. This enables for easy detection for isolation of pure cultures and confirmatory tests.

Application of Antibodies

Immunological methods using Monoclonal Antibodies can be used to detect indicator bacteria in water samples. ELISA antibody technology has been developed to allow for readable detection by the naked eye for rapid identification of coliform micro colonies

Gene Sequence – based methods

Gene sequence based methods depend on the recognition of exclusive gene sequences particular to specific strains of organisms. Polymerase Chain Reaction (PCR) and Fluorescence In Situ Hybridization (FISH) are gene sequence – based methods currently being used to detect specific strains of indicator bacteria.

Test for metabolic products of Pathogens that indicate the health hazard

In certain cases, tests for metabolic products of pathogens are preferred to indicate the presence of pathogens or their toxins. Thermonuclease test for evidence of growth of *Staphylococci* and presence of enterotoxins. *S. aureus* produces thermostable deoxyribounclease (TNase), which has been used as a rapid and

inexpensive procedure for screening foods for indication of extensive staphylococcal growth and presence of enterotoxin. The TNase test has been recommended for testing foods such as cheeses and sausages. TNase can be a useful indicator because it can almost always be detected in foods whenever enterotoxins can be detected.

Aflatoxin detection by ultraviolet light

Long-wave ultraviolet (black) light has been used to detect the presence of *Aspergillus flavus* and *Aspergillus parasiticus* in corn. When corn viewed under U-V light displays a bright greenish- yellow fluorescence (BGYF). The examination of corn and other grains with U-V light as a rapid screening procedure has been adopted by industry.

Test for phosphatase

The phosphatase test is used for certain milk and milk products to determine whether the product was pasteurized properly and also to detect the possible addition of raw milk to pasteurized milk.

Microbial culture methods

The food material on which microorganisms are grown in the laboratory is known as a culture medium and the growth itself is called a culture. The most common growth media for microorganisms are nutrient broth and nutrient agar. But some microorganisms needs specialized media. Fastidious organisms require specialized environments due to complex nutritional requirements.

Classification of bacterial culture media on the basis of consistency

Solid medium:

Solid medium contains agar at a concentration of 1.5-2.0% or some other, mostly inert solidifying agent. Solid medium has physical structure and allows bacteria to grow in physically informative or useful ways (e.g. as colonies or in streaks). Solid medium is useful for **isolating bacteria** or for determining the colony characteristics of the isolate.

Semisolid medium:

Semisolid media are prepared with agar at concentrations of 0.5% or less. They have soft custard like consistency and are useful for the cultivation of microaerophilic bacteria or for determination of bacterial motility.

Liquid (Broth) medium:

These media contains specific amounts of nutrients but don't have trace of gelling agents such as gelatin or agar. Broth medium serves various purposes such as propagation of large number of organisms, fermentation studies, and various other tests. e.g. sugar fermentation tests, MR-VR broth**.**

Classification of culture media on the basis of composition

Synthetic or chemically defined medium

A chemically defined medium is one prepared from purified ingredients and therefore its exact composition is known.

Non synthetic or chemically undefined medium

Non-synthetic medium contains at least one component that is neither purified nor completely characterized nor even completely consistent from batch to batch. Often these are partially digested proteins from various organism sources. Nutrient broth, for example, is derived from cultures of yeasts.

Synthetic medium may be simple or complex depending up on the supplement incorporated in it. A simple non-synthetic medium is capable of meeting the nutrient requirements of organisms requiring relatively few growth factors whereas complex non-synthetic medium support the growth of more fastidious microorganisms.

Classification of Bacterial Culture media on the basis of purpose/ functional use/ application

Many special purpose media are needed to facilitate recognition, enumeration, and isolation of certain types of bacteria. To meet these needs, numerous media are available.

1. General purpose media/ Basic media: Basal media are basically simple media that supports most non-fastidious bacteria. Peptone water, nutrient broth and nutrient agar (NA) are considered as basal medium. These media are generally used for the primary isolation of microorganisms.
2. Enriched medium (Added growth factors): Addition of extra nutrients in the form of blood, serum, egg yolk etc, to basal medium makes enriched media. Enriched media are used to grow nutritionally exacting (fastidious) bacteria. Blood agar, chocolate agar, Loeffler's serum slope etc are few of the enriched media. Blood agar is prepared by adding 5-10% (by volume) blood to a blood agar base. Chocolate agar is also known as heated blood

agar or lysed blood agar**.**

3. Selective and enrichment media: Are designed to inhibit unwanted commensal or contaminating bacteria and help to recover pathogen from a mixture of bacteria. While selective media are agar based, enrichment media are liquid in consistency. Both these media serve the same purpose. Any agar media can be made selective by addition of certain inhibitory agents that don't affect the pathogen of interest. Various approaches to make a medium selective include addition of antibiotics, dyes, chemicals, alteration of pH or a combination of these.

a. Selective medium: Selective medium is designed to suppress the growth of some microorganisms while allowing the growth of others. Selective medium are agar based (solid) medium so that individual colonies may be isolated. Examples of selective media include:

1. Thayer Martin Agar used to recover *Neisseria gonorrhoeae* contains antibiotics; vancomycin, colistin and nystatin.
2. Mannitol Salt Agar and Salt Milk Agar used to recover *S.aureus* contains 10% NaCl.
3. Potassium tellurite medium used to recover *C.diphtheriae* contains 0.04% potassium tellurite.
4. Mac Conkey Agar used for Enterobacteriaceae members contains bile salt that inhibits most gram positive bacteria.
5. Pseudosel Agar (Cetrimide Agar) used to recover *P. aeruginosa* contains cetrimide (antiseptic agent).
6. Crystal Violet Blood Agar used to recover *S. pyogenes* contains 0.0002% crystal violet.
7. Lowenstein Jensen Medium used to recover *M.tuberculosis* is made selective by incorporating malachite green.
8. Wilson and Blair's Agar for recovering *S. typhi* is rendered selective by the addition of dye brilliant green.
9. Selective media such as TCBS Agar used for isolating *V. cholerae* from faecal specimens have elevated pH (8.5-8.6), which inhibits most other bacteria.

b. Enrichment culture medium: Enrichment medium is used to increase the relative concentration of certain microorganisms in the culture prior to plating on solid selective medium. Unlike selective media, enrichment culture is typically used as broth medium. Enrichment media are liquid media that also serves to inhibit commensals in the clinical specimen. Selenite F broth, tetrathionate broth and alkaline peptone water (APW) are used to

recover pathogens from faecal specimens.

4. Differential/indicator media: Certain media are designed in such a way that different bacteria can be recognized on the basis of their colony color. Various approaches include incorporation of dyes, metabolic substrates etc, so that those bacteria that utilize them appear as differently colored colonies. Such media are called differential media or indicator media. Differential media allow the growth of more than one microorganism of interest but with morphologically distinguishable colonies. Examples of differential media include

    1. Mannitol Salt Agar (Mannitol fermentation- yellow).
    2. Blood Agar (Various kinds of hemolysis i.e. α, β & γ hemolysis).
    3. Mac Conkey Agar (Lactose fermenters, pink colonies whereas non-lactose fermenter produces pale or colorless colonies).
    4. TCBS (*Vibrio cholerae* produces yellow colonies due to fermentation of sucrose).

5. Transport media: Clinical specimens must be transported to the laboratory immediately after collection to prevent overgrowth of contaminating organisms or commensals. This can be achieved by using transport media. Such media prevent drying (desiccation) of specimen, maintain the pathogen to commensal ratio and inhibit overgrowth of unwanted bacteria. Some of these media (Stuart's & Amie's) are semi-solid in consistency. Addition of charcoal serves to neutralize inhibitory factors.

    1. **Cary Blair transport medium** and Venkatraman Ramakrishnan (VR) medium are used to transport feces from suspected cholera patients.
    2. Sach's buffered glycerol saline is used to transport feces from patients suspected to be suffering from bacillary dysentery.
    3. Pike's medium is used to transport *streptococci* from throat specimens.

6. Anaerobic media: Anaerobic bacteria needs special media for the growth because they need low oxygen content, reduced oxidation-reduction potential and extra nutrients. Media for anaerobes may have to be supplemented with nutrients like hemin and vitamin K. Such media may also have to be reduced by physical or chemical means. Boiling the medium serves to expel any dissolved oxygen. Addition of 1% glucose, 0.1% thioglycollate, 0.1% ascorbic acid, 0.05% cysteine can render a medium reduced. Before using the medium it must be boiled in waterbath to expel any dissolved oxygen and then sealed with sterile liquid paraffin.

    1. Robertson Cooked Meat (RCM) medium that is commomly used to grow *Clostridium* spp. contains a 2.5 cm column of bullock heart meat and 15

ml of Nutrient broth.

2. Thioglycollate broth contains sodium thioglycollate, glucose, cystine, yeast extract and casein hydrolysate. Methylene blue or resazurin is an oxidation-reduction potential indicator that is incorporated in the medium. Under reduced condition, methylene blue is colorless.

7. Assay media: These media are used for the assay of vitamins, aminoacids and antibiotics. Example- antibiotic assay media are used for determining antibiotic potency by the microbiological assay technique.

   Other types of media include:

   - Media for enumeration of Bacteria
   - Media for Characterization of Bacteria
   - Maintenance media etc.

Culture Techniques

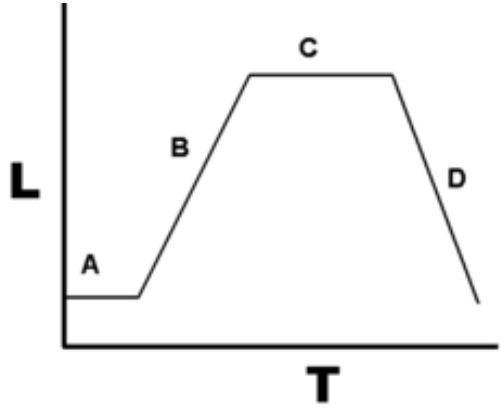
Batch culture is the most common laboratory-growth method in which bacterial growth is studied, but it is only one of many. The bacterial culture is incubated in a closed vessel with a single batch of medium.

In some experimental regimes, some of the bacterial culture is periodically removed and added to fresh sterile medium. In the extreme case, this leads to the continual renewal of the nutrients. This is a chemostat, also known as an open or continuous culture: a steady state defined by the rates of nutrient supply and bacterial growth. In comparison to batch culture, bacteria are maintained in exponential growth phase, and the growth rate of the bacteria is known. Related devices include turbidostats and auxostats. Bacterial growth can be suppressed with bacteriostats, without necessarily killing the bacteria.

In a synecological culture, a true-to-nature situation in which more than one bacterial species is present, the growth of microbes is more dynamic and continual.

Bacterial growth curve

The growth of bacteria in closed culture systems, such as a batch culture in LB broth, where no additional nutrients are added and waste products are not removed, the bacterial growth will follow a predicted growth curve and can be modeled. Growth is shown as L= log (numbers) where numbers is the number of colony forming units per ml, versus T (time).



Bacterial growth curve: Bacterial growth in batch culture can be modeled with four different phases: lag phase (A), exponential or log phase (B), stationary phase (C), and death phase (D).

Growth phases

During lag phase, bacteria adapt themselves to growth conditions. It is the period where the individual bacteria are maturing and not yet able to divide. During the lag phase of the bacterial growth cycle, synthesis of RNA, enzymes and other molecules occurs.

Exponential phase (sometimes called the log or logarithmic phase) is a period characterized by cell doubling. The number of new bacteria appearing per unit time is proportional to the present population. Under controlled conditions, cyanobacteria can double their population four times a day. Exponential growth cannot continue indefinitely, however, because the medium is soon depleted of nutrients and enriched with wastes.

The stationary phase is due to a growth-limiting factor; this is mostly depletion of a nutrient, and/or the formation of inhibitory products such as organic acids.

At death phase, bacteria run out of nutrients and die.

Immunological Methods employed in food industry

Immunoassay

- Immunoassay means a method to measure any particular substance in a

mixture using its specific binding antibody.

- One of the merits of immunoassay is that we can measure a substance that is present in a mixture of various contaminants.
- Immunoassays have become very popular in view of their high sensitivity, safety, economy and simple instrument requirements.
- Immunoassay technique in their most simple forms provide excellent screening tools to detect adulteration and contaminations qualitatively.
- Immunoassay techniques using the highly specific and sensitive nature of immunological reactions have been developed and applied in the food industry for detecting the naturally occurring constituents, antibiotics, pesticide residues, microorganisms, fragments of microbial constituents related to food analysis.

Radio Immuno Assay (RIA)

- RIA is an immunoassay that uses radiolabelled molecules in a step wise formation of immune complexes.
- RIA is a very sensitive in vitro assay technique used to measure concentrations of substances, usually measuring antigen concentrations by use of antibodies.
- It combines the principle of radioactivity of isotopes and immunological reaction hence the name Radio immuno assay.
- It is highly sensitive and specific analytical tool
- RIA can be used in evaluating the quality and wholesomeness of food. The method is advantageous for its speed, specificity, high sensitivity, relative ease of performance and the possibility of performing a great number of parallel determinations using automation and computer evaluation.
- It can be used in determining macromolecules of proteins and enzymes. Other possibilities of the methods include the determination of microbial toxins of the peptide nature, vitamins, hormones, antibiotics, pesticides and their residues, alkaloids and carcinogenic materials.

RAST

Radioallergosorbent Test is a way of testing a person's blood to see if they have any allergies.

ELISA

- ELISA is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample.

- In this method the antigen or antibody is conjugated to an enzyme
- First screening test widely used for HIV because of its high sensitivity
- It is a plate based assays designed for detecting and quantifying substances such as peptides, proteins, antibodies, antigens and hormones.
- The test can be done in polystyrene tubes (Macro-ELISA) or polyvinyl microtiter plates (Micro-ELISA).
- It has found applications in the food industry in detecting potential food allergens such as milk, peanuts, walnuts, almonds and eggs.
- Detection of enterotoxin of *E. coli* in feces
- Detection of HIV antibody in blood samples
- Detection of rotavirus in feces

ELISA and PCR in Food Industry

In food industry, the two most common and preferred methods for the detection of allergens are ELISA and PCR. Testing methods have been developed that can now detect these allergens in finished products at very low levels.

Techniques such as ELISA and PCR can detect levels of these contaminants at concentrations in the low parts per million (ppm) range. These techniques detect the food allergen at the molecular level and provide a quick and definitive result that allows manufacturers to dispose of or re-label contaminated products before they are released. It also allerts them to areas of their processing facilities that need to be decontaminated or to production lines that need to be used for other products. The ELISA methods detect the actual allergen protein molecule by binding antibodies to the allergen and then using an enzyme-linked conjugate to create a colorimetric change that can be measured. There are certain instances though, that ELISA methods should not be used. Some matrices can interfere with the ELISA method, such as chocolate can cause cross reactivity as seen between different types of nuts. This method is also not the most suitable for cooked or heated products because the protein molecules are denatured or broken down and the allergen is no longer detectable, but may still cause problems to sensitive individuals.

The PCR methods, which are more sensitive and detect the DNA molecules of these allergens can be used in raw and cooked products and are not affected by the heating process because DNA typically remains intact after exposed to the cooking temperatures of most foods. PCR methods are also not subject to the typical interferences that inhibit ELISA-based methods because the DNA is purified away from these inhibitors before analysis begins. PCR, however can't be used on all products. Oils and other products such as milk or egg whites can't be tested by PCR

because they do not contain DNA. These products must instead be tested using ELISA- based methods for detection. Using a PCR, a single copy of a DNA sequence is exponentially amplified to generate thousands to millions of more copies of that particular DNA segment. PCR have been used in the detection of numerous foodborne pathogens like *Listeria monocytogenes*, *E.coli* 0157 : H7, *Staphylococcus aureus*, *Campylobacter jejuni*, *Salmonella*, *Shigella* spp. and other important targets in food.

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