Study of microorganisms by alternate and rapid methods

The types and number of microorganisms present in food sample can be determined either by measuring the metabolites released by the microorganisms and constituents of microbial cells employing physical /chemical methods, or by using rapid methods such as enzyme linked immunosorbant assay (ELISA) or polymerase chain reaction (PCR).

Alternative methods (physical and chemical methods)

1. Dye reduction test (DRT)

DRT is mainly used in dairy industry for assessing overall microbial quality of raw milk. The number of viable organisms in a sample is determined by their ability to reduce the redox dyes. The redox dyes take up electrons from active biological systems and are coloured when oxidized and colorless when reduced.

Commonly used redox dyes

Methylene blue: appears blue when oxidized, and colorless when reduced.

Resazurin: appears blue when oxidized, and pink or white when reduced.

Triphenyltetrazolium salt: appers different from above two dyes in reduced/ oxidized stste. It is colorless when oxidized, and coloured (red or maroon) when reduced due to formation of formzan.

Procedure

Food supernatant is added to standard dye solution, incubated (Ex. 10 min in resazurin dye) and color observed. Extent of reduction is related to bacterial load. Time for reduction to occur is inversely proportional to number of organisms present in the sample.

Advantages

- Simple, rapid and inexpensive method.
- Suitable for assessing quality of raw material at farm or dairy.

Disadvantages

- Not all organisms are able to reduce dye equally.
- Not suitable for food that contain reductive enzymes.

2. Electrical methods

Electrical method is a physical method and is one of the most widely used alternative methods for microbiological analysis of foods.

Principle

Growth of microorganisms in a liquid medium changes the chemical composition of the medium, which leads to changes in its electrical properties. Measuring changes in electrical properties forms the basis of determining the microbial load in a sample. This is done by measuring the electrical impendence.

When microorganisms grow in a culture media, metabolite substances of low conductivity in to the products of higher conductivity, thereby decrease the impendence of the media. In broth culture, measurement of independence over time gives reproducible results for species and strains of microorganisms. It is capable of detecting organisms in the range of 10 to 100 cells. Generally, cell populations of 105-106 /ml are detectable in 3-5 hours, and 104-105/ml in 5-7 hours. The times noted are required for the organisms to attain the threshold of 106-107 cells/ml.

Application

- Useful in assessing the quality of vegetables: 90-95% agreement has been found between impedence measurement and TPC, requires 5 hours to analyse and suitable for ground meat and other foods.
- Microbiological quality of pasteurized milk was assessed by using impedence detection time of 7 hour or less, which is equivqlent to TPC of 104 /ml or more bacteria.

3. ATP measurement

Adenosine triphosphate (ATP) is the primary source of energy in all living cells and universal agent for the transfer of free energy from catabolic process to anabolic process. ATP generally disappears within 2h after cell death. The amount of ATP per cell is generally constant (10-18 – 10-17 mole per bacterial cell which is equivalent to 4 x 104 M ATP/105 cfu of bacteria) . ATP in exponentially growing bacterial cells is 2-6 nanomole ATP/mg dry weight (about 0.40 % of dry weight of bacteria). Thus the measurement of cellular ATP can be equated to individual groups of microorganisms. A linear relation is observed between microbial ATP and bacterial numbers.

Procedure

ATP measurement is done by using firefly luciferin- luciferase system. In the presence of ATP, luciferase emits light which is measured by luminometer. Amount of light produced is directly proportional to the amount ATP added. Luciferase produces one photon of light on hydrolysis of 1 ATP molecule. ATP facilitates the formation of enzyme – substrate complex which is oxidized by molecular oxygen.

Application

- ATP assay is proved to be suitable in foods for assessing microbial quality, and involves complex sample preparation procedures. One of the problem in ATP assay in foods is contribution from non-microbial ATP mainly from foods which can be removed by sample processing procedures. In meat non-microbial ATP removal involves centrifugation, use of cation exchange resin and filtration.

- Though this is a rapid and sensitive method not used for routine monitoring of microbial contamination in foods. However, suitable for monitoring hygiene in food processing plants. On the spot monitoring of food handling surfaces/ equipments can be done by swabbing a designated area and reading the relative light units (RLU) using lumonometer. The amount of ATP measured is of both microbial and non-microbial origin and the presence of both indicates poor hygiene. Thus it is valuable for monitoring purpose but not for indicating numbers of microorganisms.

4. Thermostable nuclease test

It is a chemical method used to detect presence of Staphylococcus aureus, a food poisoning organism in foods by detecting thermostable nuclease. S. aureus involve in food poisoning by producing enterotoxin which is a neurotoxin.

The enterotoxin producing *S. aureus* produce coagulate and nuclease enzyme, and a high correlation is observed between these two enzymes. Nuclease of S. aureus is more heat stable than nuclease of other *Staphylococcus sp* and other bacteria. Coagulase is not heat stable and hence it is not used. Increase in cell numbers increases the extractable thermonuclease of staphylococcal origin. Presence of 0.34 units of nuclease corresponds to 9.5 x 10-3µg of enterotoxin by *S. aureus*.

Thermostable nuclease assay was found to be as good as coagulase assay for toxigenic strains. All the foods that are contaminated with enterotoxin were found to contain thermostable nuclease at S. aureus level of 106/g of food. Nuclease is detectable in sample at S. aureus numbers of 105-106/ml. And enterotoxins is detectable at cell number >106/ml.

Advantages

- Thermostable nuclease is heat stable hence persists in food even after the bacteria are destroyed by heat/chemicals etc.
- Thermostable nuclease is detectable faster (within 3 hrs) than enterotoxin.
- Nucleases are produced by enterotoxignic stains before the appearance of enterotoxin.
- Nuclease estimation does not require concentration of cultures in food, but enterotoxin detection requires concentration of samples.
- Nucleases are heat stable, like enterotoxins.

5. Limulus lysate test

Limulus lysate test is a chemical method used to detect the presence of endotoxin in foods. Endotoxin is produced by pathogenic Gram negative bacteria which consist of lipopolysaccharide (LPS) layer and lipid A in their cell wall.

LPS is pyrogenic and responsible for ssymptoms associated with infection by Gram negative bacteria. Limulus amaebocyte lysate (LAL) test uses lysate protein obtained from the blood (haemolymph) cells (amaebocytes) of horse shoe crab, Limulus polyphemous. The lysate protein is most sensitive substance known for endotoxin. LAL test is performed by adding aliquots of food suspensions to

small quantities of lysate preparation and incubated at 370c for 1 hour. Presence of endotoxin causes gel formation of lysate material. LAL reagents can detect endotoxin as low as 1 pg of LPS.

Incorporation of chromogenic substrate (p-nitroaniline), to endotoxin activated enzyme cleaves the substrate and releases free p- nitroaniline that can be read at 405 nm. The amount of chromogenic compound released is proportional to the quantity of endotoxin in the sample. By knowing the amount of endotoxin per cell of Gram negaticve bacteria (which is fairly constant), the total bacterial load can be determined from the quantity of endotoxin measured.

Application

LAL test is a good and rapid indicator of total number of Gram negative bacteria in refrigerated foods (fish/meat) which are spoiled mainly by Gram negative bacteria.

Advantages

- LAL test detects both viable and non-viable Gram negative bacteria in food sample.
- Found suitable for assessing microbial quality of milk/ milk products and raw fish.
- Gives quick result.

Food of high LAL value need further tests by other methods. Foods with low LAL titre can be categorized as low risk relative to Gram negative bacteria

Enumeration of microorganisms by rapid methods

The conventional cultured based methods often fail to detect the presence of microorganisms in foods and also take long time for laboratory analysis. This may be due to their low cell numbers and loss of viability due to damage to cells. This can be overcome by applying culture independ methods involving the detection of bacterial cell components and metabolites by immunological and molecular based approaches. The commonly employed rapid tests in food microbiology are;

- Enzyme linked immunosorbant assay
- Polymerase chain reaction

1. Enzyme Linked Immunosorbent Assay (ELISA)

- ELISA or enzyme immunoassay (EIA) is used in food microbiology to identify the specific pathogens or toxins released by them. Detection of specific microorganism among the mixture of organisms is made easier by employing ELISA.
- This method based on the antigen (cell or toxin) – antibody reaction is highly specific and helps to detect when antigens are present in very low levels. Thus, ELISA involves specific reaction between the antigen, antibody and an enzyme. This reaction complex produces a colour in the presence of chromogenic substrate which is proportional to the amount of antigen present. The enzymes such as horse radish peroxidase and alkaline phosphotase are commonly used which release a dye (chromogen) when exposed to their substrate.

Procedure

- The antigen to be detected is taken in a test tube or microtitre plate and incubated with the antiserum.
- The excess antiserum is washed and the enzyme labelled with anti-immunoglobulin is added.
- After washing, the enzyme remaining in the tube or microtitre well is assayed to determine the amount of specific antibodies in the initial serum.
- The amount of peroxidase enzyme is measured by adding enzyme specific substrate and the colour developed is measured colorimetrically.

Polymerase chain reaction: (PCR)

Polymerase chain reaction (PCR) is a molecular biology technique employed in food microbiology to identify the presence of specific microorganism of interest especially when present in very low numbers by targeting the specific gene sequence. As the foods may contain pathogenic microorganisms which may be present in low numbers or injured by conditions of food processing, the recovery of such organisms by routine plating techniques is not possible. In such situations PCR based methods become very useful in identifying the target organism. The PCR for the detection of pathogens generally targets virulence associated genes such as ctx gene encoding cholera toxin for *Vibrio cholerae* 01 and 0139; tdh gene coding thermostable direct hemolysin for *V. parahaemolyticus*; hns and invE gene for *Salmonella*; stx1, stx2 and eae gene for enterohaemorrhagic *E. coli*.

Principle of PCR

The method is based on the amplification of target DNA sequences in the genomic DNA in the presence of specific primers, oligonucleotides, buffers and polymerase enzyme. Under the conditions of repeated heating and cooling cycles millions of copies of target DNA will be synthesized within a short period of time. The amplified product is detected after performing electrophoresis on agarose gel and visualized under UV light after staining with ethydium bromide.

Advantages

- PCR is highly specific as it can amplify a target DNA fragment of pathogen of interest against DNA of other organisms.
- PCR is highly sensitive as it is possible to amplify target sequence from samples having very few microbial cells of interest.
- PCR is a rapid test since the results can be obtained within a few hours.
- It facilitataes use of food samples directly or after enrichment. Sample lysate or enrichment lysate can be use for extracting DNA for further PCR amplification.