

Methods for analysis for bacterial quality parameters, chemical parameters and filth

Fish and seafoods are major source of animal protein in the diet. Because of their health advantages over red meats, the consumption of fish and seafood has increased. Catches can be gathered from seas, rivers and lakes whose water can range from pristine to contaminate. Often contamination can occur from human and animal sources; thus, fish and seafood can be involved in the transmission of pathogenic microorganisms and toxins. Biochemical and microbiological testing methods may best be used in resolving issues regarding products of marginal quality. Such objective methods should however correlate with sensory quality evaluations and the chemical compound to be measured should increase or decrease with the level of microbial spoilage or autolysis. It is also important that the compounds to be measured must not be affected by processing (e.g., breakdown of amines or nucleotides in the canning process as a result of high temperatures).

A. chemical parameters

1) Chemical methods of assessing fish quality: Determination of hypoxanthine in seafoods

The quality deterioration of fresh fish is due to activity of autolytic enzymes and associated bacterial flora which bring about chemical changes leading to spoilage. The extent of spoilage is directly related to the accumulation of chemical substances such as ammonia, histamine, hypoxanthine, total volatile bases (TVB) and trimethyl amine (TMA). The levels for each these chemical substances can be established for fishes with different spoilage stages.

Hypoxanthine (Hx) is an important freshness index, which is formed as a result of breakdown of ATP by the enzymes. A protein free extract of fish muscle made using perchloric acid is neutralized and the perchlorate is removed as its insoluble potassium salt. The hypoxanthine present in an aliquot of the neutral solution is converted to uric acid by the enzyme, xanthine oxidase and its level determined by measuring absorbance at 290 nm using spectrophotometer.

Materials required

1. **0.6M Perchloric acid:** Dilute 200 ml of conc. 60-62% acid to 3L with distilled water.
2. **Potassium hydroxide – phosphate buffer:** Dissolve 27.2 g of KH_2PO_4 in about 250 ml water and add 170 ml of 1.0M NaOH and adjust pH to 7.6 ± 0.5 . Add 557 ml of 1M KOH to the buffer solution and make up to 1 L with distilled water.
3. **0.25M Phosphate Buffer (pH 7.6):** Dissolve 17.0 g of KH_2PO_4 in about 250ml distilled water. Add 107 ml of 1.0M NaOH and check the pH and adjust to $\text{pH} 7.6 \pm 0.5$, if necessary. Make the volume up to 500 ml.
4. **0.05M Phosphate buffer (pH 7.6):** Dilute 0.25M phosphate buffer (reagent 3) five fold with distilled water.
5. **Xanthine oxidase working solution:** To be freshly prepared each day. Commercially available stock enzyme solution having an activity of 3-4 EU/ml is diluted to 50-fold to prevent denaturation with chilled 0.05 M phosphate buffer.
6. **Hypoxanthine stock standard solution (100mg/ml):** Dissolve 10 mg of hypoxanthine in about 70 ml of boiling water, cool and make up to 100ml and store in a refrigerator.
7. **Hypoxanthine working standards:** Dilute the stock with distilled water to give working standards containing 5, 10, 15, 20, 25mg hypoxanthine in 1.0 ml. The concentration can be checked by measuring the optical density of the 10mg/1.0 ml solution at 250nm. It should be 0.786. (The molar extinction coefficient of hypoxanthine molecular weight 136.1 is 10.7×10^3).
8. Tissue blender
9. Spectrophotometer (UV-Visible)
10. Water bath at $350 \pm 2^\circ\text{C}$

Procedure

1. Weigh out accurately 5.00 ± 0.05 g of fish muscle free from skin, blood and dark muscle and blend with 50 ml of chilled 0.6M perchloric acid and filter it.
2. Take 5.0 ml of the filtrate and add 5.0 ml of KOH^- phosphate buffer.
3. Chill and filter. Keep the extract chilled, but store in a freezer if the analysis is not to be completed the same day.
4. Prepare reaction mixture: Pipette out 0.5, 1.0 and 2.0 ml of filtrate into a test tube and make up to 2.0 ml with water, if necessary. 1.0 ml of the filtrate is sufficient for most

analyses. Add 2.0 ml of 0.25 M phosphate buffer and 0.5 ml of enzyme solution. Treat this as solution A.

5. Prepare extract blank: Pipette the same volume of extract as used for A into a test tube and make up to 2.0 ml as before. Add 2.0 ml of 0.25 M buffer and 0.5 ml of water. Treat this as solution B.
6. Prepare enzyme blank: For each set of determinations, prepare a blank containing 2.0 ml of buffer and 0.5 ml of enzyme solution. Treat this as solution C.
7. Prepare buffer blank: For each set of determinations, prepare a blank containing 2.0 ml of buffer and 2.5 ml of water. Treat this as solution D.
8. Incubate all the mixtures at 35°C for 30 min.
9. Measure the absorbance at 290 nm in a 10mm silica cell with water in the reference cell.
10. Calculate the net absorbance due to uric acid after correcting for the blanks as follows.

$$AB = (A-D) - (B-D) - (C-D) = (A-B) - (C-D)$$

Calculation

1. The molar extinction coefficient of uric acid at 290 nm in a 10 mm cell is 12.4×10^3 . One mole of uric acid is equivalent to one mole of hypoxanthine, in 4.5 ml of the reaction mixture is:

$$AB \times 136.1 \times 10^6 \times 4.5 / 12.4 \times 10^3 \times 10^3 = OD \times 49.4 \text{ mg}$$

2. Assuming that fish muscle contains 80% water, all the hypoxanthine is extracted into 54 ml of 0.6M perchloric acid. This is diluted 2 fold in step 2 of the procedure.
3. The conc. Of hypoxanthine in a 5 g sample of fish is

$$AB \times 49.4 \times 54 \times 2 \times 100 / V \times 1000 \times 5 = OD \times 119 / V \text{ mg /100g}$$

Where V is the volume of extract taken to prepare the reaction.

Enzyme action

Submitting 2.0 ml aliquots of the hypoxanthine standards to analysis should check the activity of the enzyme working solution. A plot of absorbance against mg hypoxanthine in the test solution should be linear, and the line should pass through the origin. The gradient of the line, absorbance / mg hypoxanthine, should be close to $1/49.4$ is 0.0202. If the conditions are not met, the enzyme is probably inactive or the wavelength setting of the spectrophotometer is incorrect. The full calibration curve should be determined for each new consignment of enzyme but to test the working solution, reagent No.5, made up on a daily basis only the most concentrated standard need be used.

2) Chemical methods of assessing fish quality: Determination of histamine in seafoods by fluorometric method

Histamine is formed in seafoods due to the degradation of amino acid, histamine by the action of spoilage bacteria possessing the enzyme histidine decarboxylase. Histamine poisoning is commonly associated with scombrid fishes and the level of histamine increases as the bacterial spoilage progresses. The estimation of histamine involves extraction by perchloric acid, followed by n-butanol under alkaline condition and finally collecting it in acid- aqueous phase. The histamine in the acid-aqueous phase on condensation with o-phthalaldehyde under alkaline condition emits fluorescence, which is read at an excitation wavelength of 340 nm and emission wavelength of 425 nm in a spectrophotometer.

Materials required

1. *0.4N Perchloric acid (HClO₄) Solution*: Dissolve 10 ml of conc. HClO₄ in 100 ml of distilled water
2. *0.1N HCl Solution* : 10 ml of 1N HCl is made up to 100 ml with distilled water in a volumetric flask (1N HCl is prepared by taking 11 ml of conc. HCl in a volumetric flask and making up to 100 ml with distilled water)
3. *1N NaOH Solution* : Dissolve 4 g of NaOH in 100 ml of distilled water
4. *0.1N NaOH Solution* : Dilute 10 ml of 0.1N NaOH in 100 ml of distilled water
5. *Sodium Chloride salt*
6. *Solvents* : n-Butanol and Petroleum ether

7. *1 % ortho-Ophthaldehyde solution (OPT)*: Dissolve 0.05 g of OPT in 5 ml of methanol
8. *0.2M Citric acid*: Dissolve 4.20 g of citric acid in 100 ml of distilled water
9. *Histamine standard*:

Stock solution (1000 ppm): Dissolve 16.55 mg of histamine dichloride in 10 ml of 0.1N HCl solution

Working solution (10 ppm): Pipette out 1.0 ml of the stock and make up the volume to 100 ml with 0.1N HCl solution in volumetric flask

Procedure

Preparation of standard

Take 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the histamine working standard along with blank (2.0 ml of 0.1N HCL) in separate test tubes and make up the volume to 5ml with 0.1N HCl.

Preparation of sample

Homogenize 5 g of fish sample with 25 ml of 0.4 N HClO₄ in a homogeniser for 1 minute and centrifuge the homogenate for 10 min at 3000 rpm. Filter the supernatant through Whatman No. 1 filter paper under slight suction and make up to 50 ml with 0.4 N HClO₄.

Extraction of histamine from sample:

Transfer 5 ml of the extract into 250 ml separating funnel, add 10 ml of distilled water and make alkaline with 5 ml of 1N NaOH. Then, add 1.5 – 2.0 g of NaCl (to saturation) and extract two times with 25 ml of n-butanol. Collect all the butanolic phases. Then, wash the butanolic phases with 10 ml of 1N NaOH and 2.0 g NaCl. Remove the aqueous phase and add 25 ml of petroleum ether and shake well. Discard the aqueous phase. Then, extract again the butanolic phases five times with 10 ml of 0.1N HCl. Collect the aqueous-acid phases (0.1N HCl) in a 50 ml volumetric flask and make up to 50 ml with 0.1N HCl.

Condensation reaction

Transfer 2.0 ml of each standard solution, sample and blank in separate test tubes, add 4.0 ml of 0.1N NaOH and shake well. Then, add 0.2 ml of OPT solution immediately. Shake to mix thoroughly and let stand for 5 min. Then, add 2.0 ml of 0.2 M Citric acid to the tubes and shake well. Finally, measure the fluorescence at the excitation wavelength of 340 nm and emission wavelength of 425 nm in a spectrofluorometer.

Calculation:

Draw the standard graph by plotting the concentration of the histamine (mg) in the X axis and the fluorescent intensity (mV) on the Y axis. Compute the concentration present in the sample extract from the standard graph and express as mg per gram of sample.

B. Bacterial quality parameters

1) Enumeration of microorganisms in foods

Introduction

The raw, semi-processed and processed foods as well as the ingredients used in the preparation of processed foods may contain several microorganisms. The microbial examination of foods is generally done to know the presence or absence of specific microorganisms, types of microorganisms, number of microorganisms and their products in foods. The information on the microorganisms associated with food will help in; Estimating shelf life (suitability for human consumption) by determining microbiological quality of a food or constituent of food. Identifying the cause of spoilage or presence of pathogens, when food is implicated in food borne illness.

Methods of enumeration

Several methods are available for the enumeration of microorganisms from food. These can be broadly divided in to direct methods or indirect enumeration methods based on whether the microorganisms are counted directly or the products released by them is estimated.

I. Direct enumeration methods

a. Direct counting methods

Direct microscopic count (DMC)

Direct counting on membrane filters

b. Culture based methods

Plate count method

Pour plate method

Spread plate method

I. Most probable number (MPN) technique

II. Indirect enumeration methods

a. Alternative Methods (chemical and physical methods)

Dye reduction test

Electrical methods

ATP determination

b. Rapid methods

Immunological methods

DNA/RNA methodology

A. Study of microorganisms in foods by conventional methods Enumeration of microorganisms in foods is traditionally performed by directly counting all microorganisms present in foods or by allowing them to develop in to colonies and then counting.

a) Direct counting methods Microorganisms present in the food can be counted by observing the food sample directly or retaining the microorganisms on a filter paper by filtering the sample and then observing under microscope.

A. Direct microscopic count (DMC) DMC involves detecting the presence of microorganisms in food by microscopic observation. It is a simple method and easy to perform. DMC is performed by making a smear of food specimen/cultures on to microscopic slides, staining with appropriate dye and viewing and counting all cells using microscope under oil immersion objective. This method can be used only when microorganisms are present in large numbers (10⁶/ml). It is commonly used in dairy industry for assessing microbial quality of raw milk and other dairy products.

Advantages

Rapid and easy enumeration

Can be employed to any foods (Ex: dried/frozen foods)

Simple to perform

Cell morphology can be assessed

Efficiency can be increased by using florescent probes

Disadvantages

Requires tiresome counting under microscope causing fatigue to analyst

Counts both viable and non-viable cells

Food particles may interfere with counting and mistaken for microorganisms

Cells may not be distributed uniformly (single cells/ clumps)

Some cells may not take up stain and missed while counting

DMC counts are always higher than standard plate count

Requires dilution of sample

B. Direct counting on membrane filters Membrane filters with pore size smaller (0.45 μm) than bacteria retain bacteria and the retained bacteria can be counted using microscope.

Procedure involved

Concentrating/collecting bacteria on polycarbonate filters by filtering known volume of homogenized sample.

Staining and counting of retained bacteria.

Placing the membrane on a nutrient agar media or absorbent pad saturated with culture media of choice, and incubating

Following growth, colonies are counted

Advantages

Well suited for samples containing low numbers of bacteria

Facilities concentrating bacteria by filtering large volume of sample

Only small volume of food samples can be used for a single membrane

Efficiency of membrane filter method can be increased by staining with fluorescent dyes (Ex. acridine orange) and observing under epifluorescence microscope (DEFT: Direct Epifluorescence Filter technique). Viable cells fluoresce green and are counted. Non viable cells appear orange. Acridine orange is a metachromatic fluorochrome which binds to double stranded DNA of viable bacterial cells .

Can be used to enumerate microorganisms from a variety of foods (fresh fish, meat, fish/meat products, water samples etc).

Culture based methods Culture methods involve examination of microorganisms in food by encouraging them to multiply in a liquid or solid media. On solid agar media bacteria develop as colonies and counting such viable colonies gives microbial load in foods. Enumerating microorganisms by culture based methods can be done by using plate count methods or MPN technique.

Culture media

A wide variety of media with varied composition capable of supporting the growth are available for the cultivation of different microorganisms. The composition of the media varies depending on,

Group/type of microorganism to be studied

Overall purpose of the study

Whether to grow wide range of microorganisms or specific types

Resuscitation of damaged but viable cells

Type of diagnostic information required

Ex: General purpose media:

Plate count agar Lactose broth: For *Escherichia coli* Selective media: Baird parker agar for *Staphylococcus* Bismuth sulphite agar for *Salmonella* TCBS for *Vibrios*

A. Plate count method

This method is variously referred to as total plate count (TPC), standard plate count (SPC) or aerobic plate count (APC). It is most widely used conventional method for determining viable

cells or colony forming units (CFU) in foods. SPC involves blending/ homogenizing the sample, serially diluting in appropriate diluent, plating in or on suitable agar media, incubating at appropriate temperature for a given time, and counting visible colonies as CFU. Principle involved SPC is based on the principle that each viable bacterial cells multiples and grows in to a visible colony. Thus, counting number of colonies gives an idea about bacterial cells present in a sample. Counts determined by taking average of replicate plates showing 30-300 colonies.

Factors affecting SPC

Sampling method employed.

Distribution of microorganisms in food.

Nature of food biota

Nutritional adequacy of plating media

Incubation temperature and time

Type of diluent used

Presence of other competing organisms etc.

Plating on selective media for specific organisms is limited by degree of inhibition and effectiveness of selective/ differential agents employed.

SPC can be performed by pour plate method or spread plate method (surface plating method).

a) Pour plate method

Appropriate dilution of the sample (1 ml/ 1000 µl) is mixed with agar medium, allowed to set, incubated at appropriate temperature and colonies developed are counted. Here colonies develop both on surface and subsurface of agar plate.

Proper mixing of sample with agar medium is necessary so as to get isolated colonies which can be done by 2 ways:

1 ml of appropriate sample dilution is added to petri-plate and about 15 ml of agar medium is added and mixed by rotating the plate in clockwise and anticlockwise direction.

1 ml of appropriate sample dilution is added to test tube containing about 15 ml of molten agar medium, mixed by rolling the tube between the palm and poured to petri-plates, allowed to set and incubated.

b) Spread plate method

Diluted sample (0.1 ml) is spread on the surface of pre-poured, hardened agar plates using glass rod, incubated at appropriate temperature and colony developing on surface counted.

Advantages

Suitable for heat sensitive psychrotrophs in food as they do not come in contact with molten agar.

Enables providing colony features useful in presumptive identification especially on selective media.

Favours strict aerobes on surface, but microaerophils grow slowly

Disadvantage

Problem of spreaders and colony crowding makes the enumeration difficult.

B. Most probable number (MPN) technique

MPN is suited for enumerating the presence of low numbers of microorganisms in foods.

This involves inoculating replicate tubes of appropriate liquid media (3 or 5 tube) with three

different sample sizes/ dilutions of the material to be studied and incubating at appropriate temperature. Then the absence or presence of growth is observed and MPN table consulted to get probable number of organisms in the sample. MPN numbers are generally higher than

SPC. Advantages

Relatively a simple method and assay to perform

Results are comparable from one laboratory to another

Specific group of organism determined by use of specific media.

Suitable for detecting organisms present in low numbers

Method of choice for coliform detection

Disadvantages

Requires use of large number of glassware and large volume of sample

Can not observe colony morphology

Lack of precision