Microbial Quality Analysis of Fish and Fishery Products

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2020

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Citation: Joseph, T.C., Jha, A.K., Renuka, V., Remya, S. and Anupama, T. K, 2020 Microbial Quality Analysis of Fish and Fishery Products, Veraval Research Centre of ICAR - Central Institute of Fisheries Technology, Gujarat, India. 123p.

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2020

Published by	**:**	**Dr. Ravishankar C N, Director, ICAR - CIFT, Cochin**
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Foreword

ICAR-Central Institute of Fisheries Technology is the leading institute in the field of harvest and post-harvest sector including microbial quality assurance of seafood. Fish and fishery products are not only nutritionally important but also important in global trade as foreign exchange earner. Hence, it is very important to maintain the quality of the fish for its acceptance in international trade as well as avoiding the health problems of consumers. The microbial quality of fish particularly is of major concern to the food processors, consumers and public health authorities. A number of microbiological tests of fish and fish products are used by authorities to check that the microbiological status is satisfactory. The purpose of these tests is to detect pathogenic bacteria or indicator organisms of faecal pollution or other types of general contamination or poor handling practices. Estimation of bacterial numbers in fish is frequently used to retrospectively assess microbiological quality or to assess the presumptive safety of the product. Skill development programmes on microbiology are essential to ensure that personnel are equipped with the knowledge and skills required to fully understand and address the related challenges in the seafood industry. I am very happy to note that Veraval Research Centre of ICAR-CIFT regularly impart training to the managers/ technologist/ college

students from different stakeholders of Gujarat who gets a hands on training experience in microbial quality analysis of fish and fishery products. To cater to the needs of stakeholders of seafood establishments in Gujarat, there is pressing need for a book that details all the microbial quality analysis in a systematic way. I am sure that the techniques described in this book will be a very useful guide for food technologists, students and teachers working in the field of food microbiology and support to the candidates preluding to the laboratory works.

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Preface

Fish is one of the most perishable food item, highly susceptible to bacterial and enzymatic spoilage unless handled, processed, packed and stored scientifically. Gujarat seafood industry plays a major role in Indian seafood export having 126 seafood processing plants with a capacity of 6596 MT. The seafood industry provides nutritious food to the masses and gives direct and indirect employment to millions of people. Seafood-associated infections have been reported worldwide. Ensuring safety of product for human consumption is the foremost priority that requires not only considerable resources but also a high level of expertise and knowledge. Safe and wholesome fish and fishery products can be provided by controlling contamination and is highly essential from the food safety point of view. It is necessary to carry out microbiological examination of fish to estimate the shelf-life and to ensure its suitability for human consumption. The Microbial Quality Analysis of Fish and Fishery Products is a compilation of microbiological validated methods of USFDA and AOAC. We are sure that this book will be of great help to the technologists in the seafood processing plants and also to students and scientists working in this field. We have great pleasure in dedicating this publication with the ardent hope that the book will be utilised by the developmental organizations and entrepreneurs for their profitable exploitation and to the betterment of the nation.

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Contents

An Introduction to Bacteriology

Toms C. Joseph

Microorganisms have different cellular and colony morphologies and are characteristic of a species.

Cellular morphology

Bacteria come in a number of shapes. The common shapes of bacteria are:

Cocci – Spherical shaped

Coccobacillus – Bacterial shape that is intermediate between cocci and bacilli

Bacillus – Rod shaped Filamentous – Cells that resemble filaments

Spirochete – Helically coiled, spiral-shaped cells

Vibrio – Curved rod or comma shape

Colony Morphology

Colony morphology can significantly vary between species and is often used in the initial identification of bacteria.

Terms that are commonly used to describe colony morphology are given below.

Shape - Form of the colony, or its overall appearance is described.

Common shapes are circular, rhizoid, irregular, filamentous, and spindle.

Margin - The margin refers to the edge of a colony and include entire, undulate (resembling waves), lobate (lobed structure), curled, rhizoid, filamentous, or erose (irregularly notched).

Elevation - Elevation describes the side-view of the colony. Elevation can be described as flat, raised, convex (curved surface), pulvinate (cushion-shaped), and umbonate (having a knobby protuberance).

Size - The size of a colony can be described as punctiform (shaped like a point), small, moderate, or large and is often used as a useful characteristic for identification.

Appearance - The appearance of the colony surface, often describes if the colony is glistening (glossy, shiny) or dull (cloudy).

Optical Property - This describes the opacity of the colonies. Colonies are frequently described as opaque (does not transmit light), translucent (lets light through diffusely), or transparent (allows light to pass through without disruption).

Texture - Describes the consistency of a colony. Rough colonies are often associated with loss of virulence and have a granular, flattened surface. Smooth colonies have a glistening, rounded surface.

The terms used to describe colony texture include mucoid (gummy, viscous), butyrous (buttery texture) or dry (brittle or powdery colonies).

Pigmentation **-** Pigments are produced by bacteria that can be either water soluble or soluble in fat. The production of pigments may vary depending on the environmental conditions or age of the colony.

Culturing techniques

A culture medium is a solution of nutrients that is required for microbial growth. Culture media can be categorized into several groups depending on their composition or use. These include defined, complex, selective and enrichment medium.

General - purpose media/Basal media

These media support the growth of wide variety of bacteria. Trypticase soy agar (TSA) and Nutrient agar (NA) are considered as basal medium. These media are generally used for the primary isolation of microorganisms.

Defined media

Defined media are those were the exact chemical composition is known. These media are composed of pure biochemicals and the nutrient requirement of a microorganism can be studied using these media.

Complex media

The chemical composition of a complex medium is not known. This media often contains reagents of a biological origin, such as yeast extract and peptone, where the exact chemical composition is unknown. Complex media has various components that usually provide a large range of growth factors that help in the cultivation of unknown and fastidious bacterial species.

Selective media

A selective medium is formulated to inhibit the growth of certain bacterial species and/or promote the growth of a specific species of bacteria. These media consist of additional selective reagents, such as high salt concentration to select for halophiles, or can be used under selective growth conditions. TCBS Agar is a selective media used for isolating *Vibrio. cholerae*, which has a pH of 8.5 - 8.6, and inhibits most other bacteria.

Enrichment media

An enrichment medium also allows for the growth of specific bacterial species; however, enrichment media are supplemented with a reagent that permits the growth of a particular species and does not inhibit the growth of a species. Alkaline peptone water (APW) is an enrichment media used for the enrichment of bacteria belonging to *Vibrio* species.

Water used in Microbiology

Only deionised and distilled water should be used for preparation of media and solutions. The water used should be free of nutrients, free of toxic substances and has low microbial content. All glass wares used in microbiology after washing with soap and water should be rinsed thoroughly with RO water.

Bacterial culture media generally contains a mixture of proteins, carbohydrates, amino acids salts and trace elements. The presence and quantity of these components can vary significantly among media depending on the macro- and micro-nutrient requirements of each bacteria.

How the bacterial strains are cultured also varies widely between bacteria. Liquid media are used for the growth and multiplication of pure batch cultures, while solid agar-based media are used for the isolation of pure cultures.

All culture media must be sterilized prior to its use. This is often accomplished by heating the media at high temperatures within an autoclave. This instrument provides 15 pounds per square inch of steam pressure, allowing for temperatures to reach and be maintained at 121 °C. All media must be loosely capped prior to sterilization to equalize the pressure of the autoclave and to prevent contamination upon removal from the autoclave.

Storage of prepared media

Agar plates containing media and tubes containing broths can be stored in refrigerator at 2 - 8 °C. Do not freeze the media. The agar plates should be stored in sealed polythene bags to avoid loss of moisture. It is always better to prepare fresh media than use stored media. A label may be put indicating the date of preparation of media. If the agar plates loses moisture, then the efficiency of media to support growth of media will be reduced.

Broth Culture

A broth culture is a nutritional medium in liquid form used to culture live microorganisms in either a test tube or a flask. This form of culture allows for the rapid growth of many microorganisms, and is often used to prepare specimens for cryopreservation or to propagate large volumes of microbial cultures.

Agar Slant

Slants are a form of solid media generated from the addition of agar to a broth. Agar slant is prepared by adding agar to the broth culture then autoclaved to dissolve the agar and sterilize the media. This medium is then solidified at 42 °C. Agar slants are used to temporarily store actively growing cultures. They provide a large surface area on which to grow microorganisms.

Agar Stab

The agar stab is used as an inoculation technique while inoculating semi-solid medium and can be used for the analysis of motility or oxygen usage. Agar stabing can be used to culture a microorganism with limited oxygen requirements.

Streak Plate

Streak plate technique is method used for the isolation of pure culture of bacteria from mixed population. An inoculum of bacterium is streaked over the agar surface so that the number of bacteria reduces after several streaking. Individual bacterial cells get separated and are well separated from each other. The number of organisms decreases as the original sample is diluted by streaking it over successive quadrants. Only a few organisms are transferred in the third or fourth quadrant and will give discrete colony forming units (CFUs).

Spread Plate

Similar to the streak plate technique, a spread plate is another form of inoculation that is used to isolate pure colonies from a culture. Spread plate method is primarily used to quantify the number of microorganisms in a sample.

Pour Plate

Pour plate method is the method used for finding out the microbial load in a sample. In this method, inoculum from a

broth/sample is placed in the centre of sterile Petri dish using a sterile pipette. Molten cooled agar (approx. 15 – 20 ml) is then poured into the Petri dish containing the inoculum and mixed well. The plate is inverted after solidification of the agar and incubated at 37 °C for 24 - 48 h.

Culturing Conditions

The optimal growth temperatures required for the growth of bacteria vary significantly between species since bacteria can grow and thrive in a variety of environments. Most of the pathogenic or commensal bacteria can grow well near the body temperature (37 °C), while the environmental strains thrive and grow at a range of 25 °C to 30 °C.

Based on the temperature required for growth, bacteria can be categorised as;

Psychrophiles (0 °C to 20 °C)

Mesophiles (25 °C to 40 °C)

Thermophiles (45 °C to 122 °C)

Though optimal temperatures are required for growth and reproduction of bacteria, most bacterial strains can survive with considerable drops in temperature and can survive several days at 4 °C. The bacterial growth and metabolism are significantly diminished at these low lower temperatures.

Other than the optimal growth temperatures required, bacteria also differ in their need of oxygen for respiration.

Oxygen is used as a terminal electron acceptor during respiration by aerobic organisms, such as Bacillus species.

Similarly, microaerophiles also require oxygen for their growth and multiplication, but at lower levels than naturally occurring in the environment.

While anaerobic microorganisms use electron acceptors such as nitrate or sulfate.

This inorganic compound has a lower reduction potential than oxygen and hence has less efficient respiration.

The need for oxygen and inorganic compounds by anaerobic organisms can vary greatly between species.

Obligate anaerobes, such as *Clostridium* species, can only survive and reproduce in the absence of oxygen. These organisms are often killed by the presence of oxygen. Similarly, aerotolerant anaerobes, such as Lactobacillus species, do not use oxygen during respiration; however, unlike strict anaerobes, these microorganisms can tolerate oxygen for short periods of time.

Facultative anaerobes can survive in both the presence and absence of oxygen e.g. *Escherichia coli* and *Staphylococcus* species. These organisms, if given a choice will prefer the use of

oxygen during respiration as it has the greatest reduction potential as compared to other electron acceptors. Exposure to oxygen may be avoided when working with anaerobic cultures.

Anaerobic bacteria are usually grown in an anaerobic gas mixture used for the growth of anaerobic bacteria is 80 % N_2, 10 % CO_2, and 10 % H_2.

Anaerobic conditions for growth of anaerobic bacteria are obtained by use of an anaerobic gas chamber.

The system consists of a polycarbonate jar, a lid to prevent air flow, a methylene blue strip, and a pouch that contains sodium borohydride, sodium bicarbonate, citric acid, and a palladium catalyst. When water is added to the pouch, the sodium borohydride, sodium bicarbonate, and citric acid react to form hydrogen and carbon dioxide. A reaction between the hydrogen and the oxygen is catalysed by Palladium within the jar. This reaction results in the formation of water, which condense on the inside of the jar. Methylene blue is colourless in an anaerobic environment but blue in the presence of oxygen. When the oxygen in the anaerobic jar is converted to water and condensation forms, the indicator strip will turn from blue to white.

Disposal of used media

The used media may have high concentrations of bacteria and are potentially hazardous and must be disposed of safely. All culture media that are inoculated should be handled only by qualified personnel who have been trained in microbiological procedures. It should be assumed that all used media contain pathogenic bacteria and hence should be autoclaved before disposing off.

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Microbiology Laboratory Practices and Safety

Toms C. Joseph

Special practices and facilities are required in a microbiology laboratory for containing the microorganisms and to properly protect persons working with microorganisms.

- Wash hands with disinfectant soap on arrival at the laboratory and before leaving the laboratory.
- While in the laboratory, avoid touching face or eyes. Do not handle mobile phones, purse etc. when you are in the laboratory as they may become contaminated.
- Food, drinks or chewing gum are not allowed in the laboratory. Do not put anything in mouth such as pencils, pens or fingers.
- Do not store food, water for drinking and soft drinks in refrigerators where microorganisms are stored.
- Wear a lab coat in the laboratory. Leave lab coat in the lab and do not wear it in non-lab areas.
- Avoid loose clothing. Wear shoes in the laboratory. Sandals are not recommended in laboratory.
- Keep workspace free of all unnecessary materials. Backpacks, bags, purses, and coats should not be kept in the lab.

- Disinfect the work areas before and after use with 70 % ethanol or fresh 10 % bleach.
- If your eyes come in contact with microorganisms or harmful chemicals, wash your eyes for 15 minutes with water and seek medical attention immediately.
- Label all reagents, media, chemicals etc. clearly and indicate date of preparation.
- Do not open Petri dishes in the lab except near the flame of Bunsen burner or in a biosafety cabinet.
- Inoculating loops and needles should be flame sterilized in a Bunsen burner each time before it is kept down.
- Turn off Bunsen burners when not in use.
- Long hair must be restrained especially when Bunsen burners are in use.
- When you flame sterilize with alcohol, be sure that you do not have any papers or cotton near you.
- Treat all microorganisms as potential pathogens and hence give appropriate care.
- Wear disposable gloves when working with potentially infectious microbes or samples.
- Never pipette by mouth. Use a micropipette, pipetting aid or adjustable volume pipettors.

- Consider everything a biohazard. Do not pour anything down the sink. Autoclave liquids, petri dishes and broth cultures to sterilize them before discarding.
- All solid waste material may be disposed of in a biohazard bag and autoclaved before discarding in the regular trash.
- Spills and accidents may be reported immediately to your Laboratory in charge. Clean small spills with care.

Biological safety cabinets

Biosafety cabinets are workspaces that are designed to protect laboratory personnel and materials from contamination during laboratory procedures. High-efficiency particulate air (HEPA) filters are used in biosafety cabinet which removes harmful microbes from the air.

Biological safety cabinet

Class I:

These biological safety cabinets are open-front systems with HEPA filtration systems. Class I biosafety cabinets provide protection for laboratory personnel and the environment, but will not provide protection to the product. These cabinets are used to keep specific equipment or used for procedures that may generate potentially hazardous aerosols.

Class II:

These biological safety cabinets are open-front, ventilated, laminar-flow cabinets. HEPA-filtered, recirculated airflow is provided by these cabinets within the work space. Class II cabinets provide protection to laboratory personnel, the environment, and to products by drawing a curtain of sterile air over the products that are being handled. These cabinets are commonly used in microbiology laboratories working with potentially infectious agents (Biosafety Level 1 or 2) as they protect the contained materials from extraneous airborne contaminants.

Class III:

The class III biological safety cabinets are totally enclosed, ventilated systems with gas-tight construction. These cabinets are used through attached rubber gloves. The air supply is drawn into the cabinet through HEPA filters, and the exhaust air is filtered by two HEPA filters installed in a series. This biosafety cabinet system is commonly used with high-risk infectious agents (Biosafety Level 3 or 4) to prevent the escape of aerosols. Tips to properly use Class I or Class II biosafety cabinet when working with non-infectious materials:

- Clean work surfaces with an appropriate disinfectant before and after use.

- If the biosafety cabinet is equipped with germicidal UV lights, decontaminate work surfaces before and after use by turning on the UV light. Never use the UV light while the biosafety cabinet is in use.
- Routinely remove any biohazard waste including used tips, pipettes etc. from the biosafety cabinet.
- Wipe down the outer surface of all pipettes, pipette tip boxes, media, materials, etc. using an appropriate disinfectant, prior to placing them in the biosafety cabinet.
- Clean lab coat and sterile gloves may be always worn when working in a biosafety cabinet.

How to properly use a Biosafety Cabinet

- Turn on the biosafety cabinet 15 minutes prior to use.
- Raise the biosafety cabinet sash only to the recommended level, this will reduce disruption to the air flow as well as assist in the prevention of airborne contaminant entry.
- The amount of movement around the cabinet may be limited. Additionally, limit the access to the area around the biosafety cabinet. This will reduce disruption to the airflow.

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Sterilization and Disinfection Techniques

Toms C. Joseph

Disinfection and sterilization are both decontamination processes. However there are distinct differences between sterilization and disinfection. Disinfection eliminates or reduces harmful microorganisms from inanimate objects and surfaces, while sterilization kills all microorganisms, including bacterial spores, which are highly resistant. Disinfection is usually carried out with phenolic disinfectants, halogens (eg chlorine), heavy metals, alcohols, bleach, hydrogen peroxide, detergents. Heating and pasteurization are used for disinfection, while heat, chemicals, irradiation, high pressure, and filtration are used for sterilisation.

There are 3 methods of Sterilization:

A. Physical B. Mechanical C. Chemical

A. Physical:

I. Heat

Heat sterilization is carried out by destruction of enzymes and other essential cell constituents of microorganisms and is the most widely used and reliable method of sterilization. It is the most practical and dependable method of sterilization and can be carried out by:

1. Dry heat:

a. Red heat or flaming (Incineration)

Red heat can be used for sterilisation of points of forceps, spatulas, wire loop, needles, mouth of culture tubes, glass slide etc..

b. Hot air oven

Hot air oven can be used for the sterilisation of glass petri dishes, glass pipettes, glass flasks, dry glass ware, measuring cylinders, all glass syringes. Metal instruments and oils and grease can also be sterilized by this method.

Hot air oven consists of (i) An insulated chamber containing electric heaters. (ii) fan (iii) shelves (iv) thermocouples (v) temperature sensor.

The temperature used for sterilisation in hot air oven is 160 °C – 180 °C. Conditions of sterilization by hot air oven: 160 °C for 60 minutes or 180 °C for 30 minutes.

2. Moist heat:

Moist heat is the most effective method for sterilisation. Moist heat is used in the form of steam under pressure. Heat transfer is rapid when moisture is present. There are different methods for sterilisation by steam.

1. Intermittent exposure at 100 °C for several times (Tyndallization): This method is applied in the microbiology

laboratory for killing heat resistant endospores. Tyndallization consists of heating the material to boiling point and holding it for 15 minutes for three days in succession. After each heating, the spores that have survived will germinate into bacterial cells. These cells will be killed by the next day's heating.

2. Pasteurization is used to eliminate pathogens and extend shelf life of certain foods such as milk and fruit juice by treating it with mild heat, usually less than 100 °C. Pasteurization is not a method of sterilization. Rather it is a process used principally in food industries for the preservation of milk and other dairy products. This process consists of exposing the product to temperature of 63 °C for 30 min. or 72 °C for 15 sec and then cooling it immediately.

3. At temperature above 100 °C were saturated steam under increased pressure is used for sterilization by autoclaves. This procedure is suitable for culture media, lab coats, and aqueous solution. The temperature for autoclaving is 121 °C for 15 min at 15 psi (pound/inch)

Pressurised steam is used in autoclaves to destroy microorganisms, and is used for the decontamination of laboratory media and waste and the sterilization of laboratory glassware, media, and reagents. For efficient heat transfer, air must be flushed out of the autoclave chamber. The efficiency of

the autoclave can be tested periodically with biological indicators like spores of *Bacillus stearothermophilus*. Autoclaves can be used for sterilisation of many metal and glass items but cannot be used for rubber, plastics, and equipment that would be damaged by high temperatures.

II. Radiation

Radiation including electromagnetic radiation (e.g. gamma rays and UV light), particulate radiation (e.g. accelerated electrons) are used for sterilization. The radiation target the microbial DNA. While gamma rays and electrons cause free radical production and ionization, UV light causes excitation. Radiation sterilization with high energy gamma rays or accelerated electrons is used for the industrial sterilization of heat sensitive products. Radiation sterilization used for sterilisation of plastic syringes and dry pharmaceutical products. UV light of 260 nm can be used for the sterilization of air and for surface sterilization of work areas. UV light has low energy and poor penetrability. It can be used for treatment of manufacturing grade water, but is not suitable for sterilization of pharmaceutical dosage forms. Cobalt - 60 is the source of gamma rays for sterilization.

B. Mechanical methods (Filtration)

Solutions and fluids can be made free of microorganisms by filtration. These solutions are then sterile. Filtration is used for sterilisation of solutions and fluids that cannot be exposed to heat or chemicals. Filtration physically separates the microorganisms as the fluid passes through and does not kill them. Filters with pore size of 0.2 µm in diameter will remove bacteria and microorganisms but cannot remove viruses. Filters of porosity of 10 nm are recommended for the removal of viruses. Filtration is used to remove the microorganisms from biologic fluid such as normal sera, various sugar solutions, antisera, enzymes containing solution, microbial toxins and antibiotic solutions that cannot be sterilized by other methods.

C. Chemical methods

Chemical method of sterilization is used when the material to be sterilised is sensitive to the high heat used in steam sterilization. Disinfectants are chemicals that are used to destroy pathogenic bacteria from inanimate surfaces. Some chemicals that can be safely applied over skin and mucus membranes are called antiseptics. A chemical agent kills pathogenic and non-pathogenic microorganisms but not spores. Disinfection is process of reducing the number of pathogenic bacteria to a level low enough that disease is unlikely to occur. The concentrations of disinfectants are as given: ethyl alcohol (50-70 %), phenol group (2-5 %), Chlorine compounds 5 %, Formaldehyde (gas) 37 %.

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Gram Staining of Bacteria

Ashish Kumar Jha

Staining is a technique used to enhance the clarity of the image in a microscopic. Different stains and dyes are used to enhance the visibility of microscopic structure of cells, tissues or microorganisms.

In microbiological examinations the most commonly used staining procedure is Gram staining named after Danish physician Hans Christian Joachim Gram who discovered it in 1884. Gram staining is a differential staining system which differentiates the bacteria into two groups i.e gram positives and gram negatives.The procedure is based on the ability of the bacteria to retain the colour of the stains used during staining procedure. Gram positive bacteria retain the purple colour of the stain and do not get decolorize during the alcohol wash while gram negative bacteria looses the colour and get decolorized with alcohol wash.

Significance of Gram Staining

1. Gram staining plays an important role in initial characterization and classification of bacteria.
2. It helps in identification of bacteria based on their staining characteristics, enabling us to examine the bacteria under light microscope. The bacteria present

in an unstained smear are invisible under light microscope.

3. It helps in understanding morphology and arrangement of the bacteria.

Basic steps of Gram Staining

1. Smear preparation
2. Fixation of smear
3. Application of primary stain
4. Application of mordant
5. Decolourization
6. Counter staining

Smear preparation

A thin layer of bacteria on the surface of slide for staining is called bacteria smear. While preparing smear, slides should be clean and free from dust and grease. The slide should be cleaned with soap and clean water and thoroughly dried with lint free cloth or paper. Usually young bacterial cultures (16-24 hr old) shall be used for staining.

a) From broth: Place a loopful of broth on to a clean slide and spread in about 1 cm diameter.
b) From plated media: Put a drop of sterile water or normal saline on to the slide, select the target bacterial colony and collect the bacterial cell from

top center of the colony. Make emulsion of water and bacterial cell using loop and spread the cell in thin and uniform layer on glass slide.

c) Dry in the air

d) Fix the smear by passing the slide 3-4 times through the blue flame of Bunsen burner, with the smeared side on the top.

The thickness of smear determines the degree of decolourization and ultimately affect the result of gram staining.

Fixation of smear

Bacterial culture are fixed on to the microscopic slides either by heat or using chemicals like methanol. Methanol fixation preserves the morphology of host cells as well as bacteria.

Application of primary stain

Crystal violet (CV) is the primary stain used in gram staining. In aqueous solutions crystal violet (CV) dissociates into CV^+ and Cl^- ions. These ions then penetrate through the cell memberane of both Gram-positive and Gram-negative bacteria. These CV^+ then interacts with the negatively charged components of bacterial cell walls and stains purple.

Application of mordant

Mordant is a substance that enhances the affinity of the bacterial cell wall for a stain. In gram staining Iodine is used as mordant which forms crystal violet-Iodine complex (CV-I) and gets trapped in the bacterial cell wall and ultimately impart purple colour to the bacteria.

Decolourization

This step actually distinguishes the gram-positive and gram-negative bacteria. Alcohol or acetone are used for the decolourization during the staining process. Alcohol or acetone dissolves the thick lipid layer of gram negative bacteria exposing the peptidoglycan layer consequently CV-I complex is then washed away from the peptidoglyan ultimately rendering the gram negative bacteria colourless. On the other hand organic solvents like alcohol and acetone dehydrate the cell of gram positive bacteria leading to the shrinkage of peptidoglycan layer which inturn retains CV-I complex and retains the purple colour.

Counter staining

The counterstain is used to impart colour to the colourless cells of gram negative bacteria which otherwise could not

be visible. Safranin is the commonly used counterstain. Sometimes basic fuschin is also used instead of safranin. Safranin stains the bacterial cell to pink but in gram positive bacteria the pink colour is masked by the purple colour of the crystal violet, hence in gram staining gram negative bacteria will take pink colour and gram positive bacteria remains purple.

Working protocol of gram staining

- Stain the air dried heat fixed smear of bacterial cells with abundant quantity of crystal violet staining reagent for one minute.
- Wash the excess of stain on the slide in a gentle indirect stream of tap water.
- Flood with Gram's iodine to the slide and wait for a minute.
- Wash the slide in a gentle indirect stream of tap water.
- Wash the slide with decolourizing agent (95% ethanol) until decolourizing solution from the slide runs clear.
- Flood the slides with counterstain, safranin. Wait for almost a minute.

- Wash the slide in a running water till the solution from slide runs clear, blot the slide with absorbent paper or air dry .
- Observe the slide under oil immersion (100X) using bright field microscope.

Things to remember:

- Gram staining is the differential staining procedure which separates bacteria into two groups and the separation is based on the composition of cell wall.
- Gram positive bacteria is having thick cell wall, mainly composed of peptidogycan layers (90%) - It stains purple.
- Gram negative bacteria is having thin cell wall,mainly contains lipid layer and very thin peptidoglycan layer (10%) - It stains pink to red.
- Almost all the bacteria can be visualized by using gram staining but there are few exceptions
  - i) The bacteria which exclusively lives in the host cells (intracellular bacteria) cannot be seen e.g Chlamydia.
  - ii) The bacteria without the cell wall cannot be seen by grams staining eg. Mycoplasma
  - iii) Bacteria which are too small to be resolved by light microscope eg. Spirochetes

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Motility Test of Bacterial Cultures

Ashish Kumar Jha

The ability of the organism or bacterial cell to move by itself is called motility. Most of the bacteria moves by locomotor appendage known as flagella unique to the bacteria or by the special fibrils that produces a gliding form of motility. Bacterial flagella are thread like appendages extending outward from the plasma membrane and cell wall. Bacteria may have single or multiple flagella. Motility test is performed for taxonomic classification, species differentiation and pathogenic characterization of bacteria. Since early days in the field of microbiology, the motility of bacteria has been used as a means of differentiation and classification of organisms.

Based on the number and position of flagella, bacteria are classified in 4 major groups:

1. **Monotrichous:** A single flagellum extending from one end of the cell. (*Vibrio cholerae, Campylobacter* spp.)
2. **Amphitrichous**: A single flagellum extending from both the ends of the cell. (*Alcaligens faecalis*)
3. **Lophotrichous**: Several tuft like flagella can extend from one or both the ends of the cell (*Spirilla* spp).

4. **Peritrichous:** Multiple flagella may be spread all over the bacterial cell (*Salmonella typhi, Escherchia coli, Proteus* spp.)

Motility test method

Broadly there are two different methods used for motility determination:

1) **Slide method**
    a) Wet mount method
    b) Hanging drop method
2) **Soft agar stabing**

Slide method is generally used for non pathogenic organisms where as agar method is used for pathogenic organisms.

Wet mount slide method is the simplest method for motility determination. In this method a few loopful of the culture is placed on the clean slide and covered with a coverslip. Apply a small quantity of petroleum jelly on the four side of a coverslip, place a droplet of culture in the center of the coverslip, gently place a microscopic slide over the coverslip and tight seal all the four edges without pressing the center. Carefuly turn the slide upside down and observe the slide under microscope.

Precautions: Brownian motion should not be confused with the motility of the bacterial cell. Brownian movement is a

random dancing and bouncing of the organism caused by the bombardment of the surrounding water.

Advantage

- It is simple and the shape and cellular arrangement is preserved to the extent.

Disadvantage

- The method is risky hence preferably used for non pathogenic bacteria only.

Hanging drop method

This method is useful in observing general shape and the arrangement of bacterial cell when they associate together.

Procedure

- Take a coverslip, apply thin layer of petroleum jelly on the edge of the coverslip.
- Place a loopful of culture to the center of coverslip add a drop of sterile water.
- Take a depression slide put it over the drop in a way that the concave portion of slide lie over the drop.
- Fix the slide over coverslip by gently pressing the edges.
- Invert the slide gently and quickly without disturbing the droplets.
- Place the slide in the microscope and observe it under lowest magnification to oil immersion objective.

Advantages

- In this method the shape and arrangement of the bacterial cells are preserved.
- Due to sealing from all the sides the drying process also gets slowed down.

Disadvantage

- This method is risky to use for pathogenic organism.

Soft agar stabing (Tube method)

This is comparatively safer method of motility testing. In this method motility of the bacterium is determined in semisolid agar. The medium used for this method is Motility test medium. Tryptose in the medium serves as a source of essential growth nutrients required for bacterial metabolism. Sodium chloride maintains the osmotic equilibrium of the medium. Small amount of agar helps to create a semisolid medium. Bacterial motility can be observed directly by examination of the tubes following incubation. Inoculation is done by stabbing through the centre of the medium. Incubate at appropriate temperature for 18 - 40 hours. Non-motile organisms grow only along the line of inoculation whereas motile organisms grow away from the line of inoculation or may show growth even throughout the medium. All weak or equivocal motility results should be confirmed by flagellum stain or by hanging drop method.

Limitations of the method

- If the bacterial flagella gets damaged due to heat or physical mishandling, the test will give false negative result.
- Weakly motile organisms may also give false negative result.
- In semisolid media it is important to remove innoculating loop exactly vertical i.e in the same line of innoculation otherwise a fanning motion may result in growth along the stabline which give the false positive result.

Swarming of the bacteria

Swarming type of bacterial movement is exhibited by the extremely motile bacteria. These bacteria can move even on solid agar to find nutrient. If this type of bacterial culture is innoculated at the center of the agar plate and the plate is incubated, the bacteria start moving towards the perimeter. During this movement each bacteria absorbs nutrient and increase the size and after moving to the certain distance they divide and then the progeny starts moving out. This type of movement causes the formation of concentric rings on the agar plate, otherwise known as swarms. This method can also be used to estimate the generation time. *Proteus spp.* is the classical example of swarming type of bacteria.

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Sampling of Seafood for Microbial Parameters

Remya. S

Introduction

The adequacy and condition of the sample or specimen received for examination are of primary importance. If samples are improperly collected and mishandled or are not representative of the sampled lot, the laboratory results will be meaningless. Because interpretations about a large consignment of food are based on a relatively small sample of the lot, established sampling procedures must be applied uniformly. A representative sample is essential when pathogens or toxins are sparsely distributed within the food or when disposal of a food shipment depends on the demonstrated bacterial content in relation to a legal standard.

The mandatory requirements on microbiological criteria, including samples, plans and methods of analysis, are laid down in Commission Regulation (EC) No. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs (2073/2005).

Sampling

- In case there are no specific international or legal standards for sampling, it is recommended that the laboratory and parties concerned come to an agreement in advance.

- In such cases, relevant standards of ISO and the guidelines of the Codex Alimentarius should be used as reference methods, such as Codex Alimentarius: General guidelines on sampling, CAC/GL 50-2004; ISO 7218, also NMKL (Nordic Committee on Food Analysis) Procedure No. 12: Guide on Sampling for Analysis of Foods (www.nmkl.org). ISO 18593 is the EU reference sampling method for sampling of processing areas and equipment.

Sampling methods

- Samples must be handled and labelled in such a way as to guarantee both their legal and analytical validity.
- For official control, it is important that the laboratory receives a sample truly representative of the product that has not been damaged or changed during transport or storage.
- Incorrect sampling can lead to false negative or false positive results.
- Whenever possible, samples are submitted to the laboratory in the original unopened containers, or representative portions are transferred to sterile containers under aseptic conditions.

- Sample taking is always by use of sterile sampling equipment and use of aseptic technique.
- Containers used in sampling must be clean, dry, leak-proof, wide-mouthed, sterile, and of a size suitable for samples of the product.
- Sterile plastic bags (for dry, unfrozen materials only) or plastic bottles, are useful containers for line samples.
- Each sample unit should be identified with a properly marked strip of masking tape.
- Whenever possible, at least 100 g for each sample unit should be obtained.
- Do not use a marker pen on plastic because the ink might penetrate the container.
- The samples should be delivered promptly to the laboratory, with the original storage conditions maintained as nearly as possible.
- For transport of samples, they should be kept under conditions, which prevent alteration in the number of microorganism present.
- The fastest means of transport should be preferred.
- Frozen or refrigerated products are transported in approved insulated containers of rigid construction, so that they will arrive at the laboratory unchanged.

- Frozen samples shall be collected in pre-chilled containers.
- Refrigerated samples should be cooled in ice at 0 - 4°C, and transported in a sample chest with suitable refrigerant, capable of maintaining the sample at 0 - 4°C until arrival at the laboratory.
- When collecting liquid samples, an additional sample as a temperature control should be taken.
- The temperature of the control sample should be checked at the time of collection, and on receipt at the laboratory.
- The times and dates of collection and of arrival of all samples at the laboratory should be recorded.
- Dry or canned foods, that are not perishable and are collected at ambient temperatures need not be refrigerated.
- The following storage temperature should be observed:
  - fresh and refrigerated products between 0 and 4 °C,
  - frozen or deep-frozen products below -18 °C,
  - fresh fish and sensitive products between 0 and 2 °C,
  - spoiled stable units between 0 and 4° C and transport in closed packaging.

Sample reception and handling

- The conditions of the sample on receipt in the laboratory should be checked.
- If the samples are insufficient or their conditions is unsatisfactory, the laboratory should refuse the samples.
- Accepted samples are documented.
- Samples awaiting storage should be stored in a way to prevent any alteration in the number of microorganism present.
- It is important to have appropriate storage temperatures, and to keep examination deadlines, e.g. for fresh and refrigerated products within 24 h after receipt; for longer storage periods immediately freeze the sample at -18 °C.
- Upon receipt at the laboratory, the analyst should note the general physical condition of the sample.
- If the sample cannot be analysed immediately, it should be stored.
- Frozen samples should be stored at -20 °C until examination.
- Refrigerate unfrozen perishable samples at 0 - 4 °C not longer than 36 h.
- Non-perishable, canned, or low-moisture foods are stored at room temperature until analysis.

- Place containers in a freezer long enough to chill them thoroughly.
- Keep frozen samples solidly frozen at all times.
- Do not freeze refrigerated products.
- Unless otherwise specified, refrigerated samples should be analysed within 36 h after collection.

Sample preparation

- Sample preparation is to be performed according to appropriate parts (applicable to fish and seafood) of:
    - **EN ISO 6887-1**: Microbiology of food and animal feeding stuffs - Preparation of the test samples, of initial suspension and of decimal dilutions for microbiological examination - Part 1: General rules for the preparation of the initial suspension and decimal dilutions
    - **ISO 6887-3**: Microbiology of food and animal feeding stuffs - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination - Part 3: Specific rules for the preparation of fish and fishery products

- **ISO 7218**: Microbiology of food and animal feeding stuffs - General requirements and guidance for microbiological examinations.

- When handling product, aseptic technique is always used.
- Before handling or analysis of the sample, the immediate and surrounding work areas must be cleaned.
- In addition, the immediate work area should be swabbed with commercial germicidal agent.
- Preferably, frozen samples should not be thawed before analysis.
- If necessary, to temper a frozen sample to obtain an analytical portion, thaw it in the original container, or in the container in which it was received in the laboratory.
- Whenever possible, transferring the sample to a second container for thawing should be avoided.
- Normally, a frozen sample will get thawed at 2 - 5 °C within 18 hours.
- Various degrees of non-uniform distribution of microorganisms are to be expected in any food sample.
- To ensure more even distribution, liquid samples must be shaken thoroughly and, if practical, dried samples should be mixed using sterile spoons or other utensils

before withdrawing the analytical unit from a sample of 100 g or greater.

- An analytical unit of 50 g of liquid or dry food is used to determine aerobic plate count value and MPN of coliforms.
- Other analytical unit sizes (e.g., 225 g for *Salmonella*) may be recommended, depending on the specific analysis to be performed.
- Use an analytical unit size and diluent volume as recommended by the method in use.
- If contents of package are obviously not homogeneous, macerate the entire contents of the package and withdraw the analytical unit, or, preferably, analyse each different food portion separately, depending on the purpose of test.
- Samples can be prepared by using a stomacher blender.
- If the entire sample weighs less than the required amount, weigh a portion equivalent to one-half of the sample and adjust the amount of diluent or broth accordingly.

References

1. COMMISSION REGULATION (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs.
2. Wallace H. Andrews and Thomas S. Hammack. 2003. Chapter 1. Food Sampling and Preparation of Sample Homogenate. Bacteriological Analytical Manual (BAM). USFDA (Food and Drug Administration).

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Enumeration of Microorganisms by Plating Technique

Ashish Kumar Jha

Hygiene plays a key role in food or fish production as well as processing system. Microorganisms like bacteria, viruses, yeasts, molds etc. can spoil the food and cause risk to the human health. The presence of specific bacteria and their concentration in food or fish must be determined to assess and control safety hazards, their spoilage potential and ensure the desired quality of the products.

In food microbiology, bacteria can be divided into infectious agents, causes of food born intoxication, spoilage and processing aids. Total number of bacteria in any food item is the key to the quality management. The total number of bacteria indicates the freshness and also determine the shelf life of the food item.

There are different methods of enumeration of bacteria, that include,

1. Direct count of cells
    a) Direct count using counting chamber
    b) Direct count using fluorescence
2. Indirect count
    a) Viable count/total plate count

b) Most probable number (MPN)

3. Direct measurement of microbial mass
4. Indirect measurement of microbial mass

Different plating methods are used for isolation and enumeration of microorganisms. The commonly used plating methods are as follows

1. Streak plate procedure
2. Pour plate procedure
3. Spread plate procedure
4. Soft agar overlay procedure
5. Replica plate procedure

Aerobic plate count (APC) or the standard plate count also known as total viable count, total mesophilic count or total bacterial count is one of the most common test method applied to indicate the microbiological quality of food.

Most comonly used methods for total plate count are

a) Spread plate method
b) Pour plate method

Spread Plate Method

This method is used to separate bacteria contained in small volume by spreading over the surface of an agar plate. When

appropriate concentration of bacteria is plated, evenly distributed descrete colonies are formed.

Equipment and materials

a. Laminar Flow/Sterile working area.
b. Micropipettes of 1ml and 10ml capacity and sterile pipette tips or glass pipettes of similar capacity
c. Sterile knives, forks, spatulas, forceps, scissors, tablespoons
d. Mortar and pestle or Stomacher blender and bags
e. Sterile diluents (Butterfield's phosphate-buffered dilution water)
f. Sterile plate count agar (PCA)
g. Sterile (L-shaped) Spreader
h. Incubator, 35 ± 1°C
i. Electronic balance (sensitivity of 0.1g)

Preparation of diluent (Butterfield's Phosphate-Buffered Dilution Water)

- Potassium dihydrogen phosphate (KH_2PO_4): 34 g
- Distilled water: 500 ml
- Adjust pH to 7.2 with 1 N NaOH.
- Bring volume to 1 L with distilled water.
- Sterilize for 15 min at 121°C.
- Store in refrigerator.

- Take 1.25 ml of above stock solution and bring volume to 1 liter with distilled water.
- Dispense into bottles/flasks to 90 ± 1 ml.
- Sterilize 15 min at 121°C.

Preparation of PCA agar plates

- Melt two flasks of PCA in boiling waterbath and cool to 45-50ºC
- Pour into 10 - 12 petriplates (approximately 15 - 18 ml per petriplates).
- Allow PCA to set
- Dry the surface of the agar by placing the Petri plate open in a 56 ºC incubator in an inverted position or upright in a laminar flow chamber for 45 minutes
- Cool the plates to room temperature.

Sampling and preparation of sample homogenate

1. Aseptically weigh 50 g of fish/shrimp in to a sterile sample dish.
2. Transfer 50 g of sample to a stomacher bag and add 450 ml of sterile diluent (butterfields phosphate-buffered dilution water) and blend for 2 min in the stomacher. This results in a dilution of 10^{-1}.

3. Using separate sterile pipette, transfer 10 ml from the above 10^{-1} dilution to 90 ml of sterile diluent and mix well. This gives 10^{-2} dilution.
4. Then transfer 10 ml from the above 10^{-2} dilution to 90 ml of sterile diluent and mix well.This gives 10^{-3} dilution.
5. Similarly prepare further dilutions (10^{-4} and 10^{-5} etc.) depending on the microbial load of the sample.

Method

Arrange six pre-set PCA plates in 3 rows in duplicate

Label the plates appropriately (name of sample, media, dilution and date)

Pipette out 0.5ml from the desired dilutions (10^{-3}, 10^{-4} and 10^{-5}) on to the centre of the surface of agar plate in duplicates. Take a sterile L-shaped glass spreader dipped in alcohol and flame it over bunsen burner. Allow the spreader to cool in air. Do not shake the spreader for cooling. Spread the sample evenly over the surface of agar using the sterile glass spreader by rotating the petridish. Incubate the plate at 35 °C for 48 ± 2 h. Count the number of colonies using Qubec colony counter. The number of colonies in the duplicate plates should not vary more than 10%.

Note: If the sample taken is 0.5ml, then during calculation of number of colonies, the average count of the colony has to be doubled.

Limitations of Spread plate method

1) Strict aerobes are favoured where as microaerophilic grows slower.
2) Sometimes crowding of the colonies make the enumeration difficult.

Pour Plate Method

In this method fixed amount of innoculum (generally 1ml) from a broth/ sample is placed in the center of sterile petridish using sterile pipette. Molten cooled agar (at 45 ^{o}C) approximately 15 -18 ml is then poured into the petridish containing innoculum and the media is mixed well by rotating the plates. Make sure the media doesn't spill over the plate. Allow the agar to solidify and incubate at 35 °C for 48 ± 2 h.

Method

1) Arrange 6 petridishes in 3 rows in duplicates and label appropriately
2) Pipette 1 ml of the desired dilution (10^{-3}, 10^{-4} and 10^{-5}) in the centre of the sterile petriplate.

3) Pour 15-18 ml of molten cooled agar (45 ± 1^0C) into the petridish containing samples.
4) Following the addition of the molten agar, place the cover and mix sample dilutions and agar medium thoroughly and uniformly by alternate rotation and back and forth motion of plates on flat surface.
5) Allow the agar to solidify. Invert the solidify petridishes, and incubate for 48 ± 2 h at 35 ± 2^0C.

Limitations

1) Use of compartively hotter agar may kill sensitive bacteria.
2) Smaller colonies may be overlooked.
3) Reduced growth rate of obligate aerobes in the depth of the agar.

Precautions

Take the plate with 25 - 250 colonies. Select spreader free plate(s). Count all colony forming units (CFU), including those of pinpoint size. Record dilution used and total number of colonies counted.

Rounding of the digit(s)

In order to avoid any false impression about precision and accuracy while computing plate counts (APC/TPC), it is customary to report first two significant digits, and the

second digit is rounded by raising next highest number when the third digit is 6,7,8,or 9 and use zero for each successive digit towards the right from the second digit. Round down when the third digit is 1, 2, 3 or 4. In case the third digit is 5, it is rounded up if the second digit is odd and rounded down when the second digit is even.

Calculated count	TPC/ APC
1,57,000	1,60,000
1,54,000	1,50,000
1,55,000	1,60,000
1,45,000	1,40,000

Calculation

1. When counts of duplicate plate falls within the range 25-250

APC/ g sample is calculated using the formula

APC/g sample (N)=

$$\frac{\Sigma C}{[(1 X n_1) + (0.1 X n_2) X (d)]}$$

Where

N= Number of colony forming units per gram /ml of sample

ΣC= Sum of all the colonies on all the plates

n_1= Number of plates in first dilution

n_2= Number of plates in second dilution

d= Dilution from which the first counts were obtained

Example

1:100	1:1000	1:10000
TNTC	220	30
TNTC	230	35

ΣC= 220+230+30+35=515; $\mathbf{n_1}$= 2; $\mathbf{n_2}$= 2; **d**= 1:1000 (10^{-3})

$$\frac{515}{[(1X2) + (0.1X\ 2)\ X\ (10^{-3})} = \mathbf{2,34,090 \approx 2,30,000}$$

2. When all the plates count are fewer than 25 CFU

When plates from both the dilution yield less than 25 CFU each then record actual plate count but record the count as less than 25x1/d where d is the dilution from which the first counts were obtained.

Example

1:100	1:1000	Estimated aerobic plate count (EAPC)
12	4	**< 250**
16	2	

3. When all the plates count are higher than 250 CFU

When plates from all the dilutions yield more than 250 CFU each (but fewer than 100/cm^2) Then the plate count (EAPC) should be estimated from the plate nearest to 250 and multiply by the dilution.

Example

1:100	1:1000	1:10000	Estimated aerobic plate count (EAPC)
TNTC	700	290	**280,0000**
TNTC	650	280	

4. **When all the plates are with spreader and or laboratory accident**

   Report it as Spreader (SPR), or Laboratory accident as the case may be.

5. **When all the plates with an average of 100 or more CFU per sq cm.**

   In case of all the plates counting more than 100 cfu /sq cm then plate count should be 100 times greater than the highest dilution multiplied by the area of plate.

   Example: **Plates showing an average count of 120 no of colony per sq cm**

1:100	1:1000	Estimated aerobic plate count (EAPC)
TNTC	7800[(a)]	>6500000
TNTC	7080[(b)]	>5900000

[a]Based on plate area of 65 Sq cm; [b]Based on plate area of 59 Sq cm

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Isolation and Identification of *Escherichia coli,* Total Enterobacteriaceae and Faecal *Streptococci* from Seafood

Anupama T.K

1. Escherichia Coli

Escherichia coli, originally known as Bacterium *coli* commune, was identified in 1885 by the German pediatrician, Theodor Escherich. *E. coli* is widely distributed in the intestine of humans and warm-blooded animals. *E. coli* is a member of the family *Enterobacteriaceae*, which includes many known pathogens such as *Salmonella*, *Shigella* and *Yersinia*. Most strains of *E. coli* are not regarded as pathogens; they can be opportunistic pathogens that cause infections in immunocompromised hosts. There are also pathogenic strains of *E. coli* that when ingested, causes gastrointestinal illness in healthy humans. *E. coli* is abundant in human and animal faeces and is not usually found in other niches. Therefore, presence of *E. coli* in food is considered as **indicator of faecal contamination.**

a) Preparation of dilutions of the shrimp/fish sample

- Weigh 25 grams of fish/shrimp into sterile sample dish aseptically near the flame in a laminar chamber.

- Transfer 25 g of sample to a stomacher bag and homogenize with 225 ml of sterile diluent (Butterfield's phosphate-buffered dilution water) using a stomacher blender. The resultant homogenized material is 1:10 dilution.
- Using separate sterile pipettes, prepare decimal dilutions of 10^{-2}, $10^{-3,}$ 10^{-4} and others as appropriate, of food homogenate by transferring 1 ml of previous dilution to 9 ml of diluents.

b) **Step1: Presumptive test for *E. coli***

Media used: Tergitol-7 Agar.

- Melt one 100 ml of Tergitol-7 agar (T7) in flask in a water bath and cool to 50 º C.
- Aseptically add 0.3 ml of 1% sterile (2,3,5-Triphenyl Tetrazolium Chloride (TTC) solution to the T7 agar.
- Pour into sterile petri dishes (15 - 20 ml each).
- Allow to set and dry at 56 ºC for 45 min and cool to room temperature.
- Pipette 0.5 ml each of the appropriate dilutions on the surface of T7 agar at different places.
- Using a sterile bent glass rod spread on the surface of agar.
- Incubate at 37 ºC for 18 - 24 h.

- Typical colonies of *E. coli* on T7 plates appear as lime yellow, occasionally with rust brown centre and a yellow zone around (Note: yellow slimy, raised or convex colonies are not to be considered as *E. coli* colonies). Take average of duplicate plates.

Calculation:

E. coli/g= average count x dilution factor x 2

2) **Step 2: Confirmation test for *E. coli***

For confirmation of *E. coli*, the following procedure has to be followed

1. Streak on Eosine-Methylene Blue (EMB) agar

- Melt one 100 ml flask of EMB agar, cool to 50 ºC, pour into sterile petri dishes and allow to set, dry at 56 ºC for 45 min. Cool to room temperature.
- Pick typical yellow colonies from T7 plates with a sterile loop and streak on to EMB plates, by the streak-dilution method; incubate at 35 °C ± 0.5 °C for 18-24 h.
- Colonies on EMB agar appear as 2 - 3 mm diameter with a greenish metallic sheen by reflected light and dark purple centre by transmitted light.
- Pick 5 suspicious colonies from each EMB plate to PCA slants and incubate at 35 °C ± 0.5 °C for 18 - 24 h.

1. IMViC tests

From the PCA slants above, inoculate to the following media.

(a) Tryptone broth: Inoculate culture to tryptone broth and incubate at 24 ± 2 h at 35 °C ± 0.5 °C

(b) MRVP Medium: Inoculate each culture into 2 tubes of MRVP medium and incubate at 48 ± 2 h at 35 °C± 0.5 °C.

(c) Simmon's Citrate agar: Streak culture to Simmon's Citrate agar slants and incubate at 35 °C ± 0.5 °C for 48 – 96 h.

Results

(a) Tryptone broth

Test for indole production by adding 0.2 - 0.3 ml of Kovac's indole reagent. A red or pink colour at top indicates positive test. Indole forms a red dye with p-dimethyl amino benzaldehyde of the Kovac's reagent.

(b) MRVP medium

MR Test: Into one tube, add 5 drops of methyl red indicator. A red colour indicates positive MR test. *E. coli* ferments glucose to produce acid, which brings down pH of the medium to less than 4.4 indicated by the red colour of methyl red indicator. If the colour is orange, pH is 5.0 - 5.8 and if yellow, pH is more than 6.0

VP Test: Using 1 ml of the culture from the second tube, do the VP test by adding 0.6 ml of α-naphthol solution and 0.2 ml

of 40 % KOH. Add few crystals of creatine. Shake and let it stand for 2 h. Eosine pink colour indicates positive VP test.

3. **Simmon's Citrate agar:** A change in the colour of the medium from green to blue indicates a positive test for citrate utilization by the bacterial culture. *E. coli* do not utilize citrate and hence give a negative reaction.

A culture giving the following results is confirmed as *E. coli*

Indole	**Positive**
Methyl Red	**Positive**
VP	**Negative**
Citrate	**Negative**

IMViC: + + - -

2. Total Enterobacteriaceae Count

Enterobacteriaceae, that includes *Klebsiella, Salmonella, Shigella* and *E. coli* are important non-indigenous contaminants, occurring on products as the result of contamination from animals, birds and humans. They may survive in products and processing areas for long times even for months. Major contamination routes are from unhygienic handling of fish, utensils and equipments or from water or water environments, such as ice. Good hygienic practice and maintenance of equipment, with hygiene awareness to food handlers are essential in the control of *Enterobacteriaceae* contamination.

Method

Media used: Violet Red Bile Glucose Agar (VRBGA)

- 25g of fish/shrimp is aseptically cut from the sample lot and macerated with 225ml sterile diluent (Butterfields phosphate-buffered dilution water) in stomacher or mortar and pestle. Make serial dilutions as required. Pour plate technique is followed.
- One ml each of 10^{-2} and 10^{-3} dilutions are pipetted on sterile petri plates and pour 18 – 20 ml of molten VRBGA.
- Plates are allowed to set, invert and incubate at 37ºC for 18-24 h.
- Count red, small (2 - 4 mm diameter) colonies as *Enterobacteriaceae* colonies. Take average count of duplicate plates.

Total Enterobacteriaceae count/g = Average count x dilution factor.

3. **Faecal *Streptococci***

Faecal *Streptococci* is also considered as indicator of Faecal contamination

Method

Media used: Kenner Faecal Streptococci agar (KF)

- 25 g of the fish/shrimp is aseptically cut from the sample lot and macerated with 225 ml sterile diluent (butterfields phosphate-buffered dilution water) in stomacher or mortar and pestle. Make serial dilutions as required. Pour plate technique is followed.
- One ml each of 10^{-2} and 10^{-3} dilutions are pipetted on sterile petri plates and pour 18 - 20 ml of molten KF. (Note: After cooling the molten KF to 45 ºC, add 1 ml of 0.1% Triphenyl Tetrazolium Chloride (TTC) per 100 ml medium before plating.
- Plates are allowed to set and incubate at 37 ºC for 36 - 48 h.
- Count all surface and sub-surface red to pink colonies (some will be with a thin white margin) as faecal Streptococci.

  Faecal *Streptococci* count/g = Average count x dilution factor.

If Total Plate Count, *E. coli,* Total Enterobacteriaceae count and Faecal *Streptococci* has to be done with the same sample then dilutions has to be made only once.

References

1. Feng, P., Weagant, S.D., Grant, M. A. and Burkhardt, W. (2002)**.** Bacteriological Analytical Manual, Chapter 4, USFDA.
2. Surendran, P.K., Thampuran, N., Nambiar, V.N., Lalitha, K.V and Joseph, T.C., 2013, *Laboratory techniques for microbiological examination of seafood*. 4th edition, Central Institute of Fisheries Technology, Cochin-682029, India.48p

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MPN (Most Probable Number) Method for Determination of Coliforms in Water

Anupama T.K

The Most Probable Number (MPN) method is a statistical, multi-step assay consisting of presumptive, confirmed and completed phases. In the assay, serial dilutions of a sample are inoculated into broth media. The numbers of gas positive (fermentation of lactose) tubes are counted, from which the other 2 phases of the assay are performed, and then uses the combinations of positive results to consult a statistical table to estimate the number of organisms present. Typically, only the first 2 phases are performed in coliform and faecal coliform analysis, while all 3 phases are done for *E. coli*. The 3 - tube MPN test is used for testing most foods. The 5-tube MPN is used for water, shellfish and shellfish harvest water testing.

5 Tube MPN method

Step I: Presumptive test for Total Coliforms

Media required:

a) Double strength MacConkey broth -1 flask - 50 ml;
   Place inverted Durham's tube in the flask
b) Double strength MacConkey broth - 5 tubes - 10 ml;
   Place inverted Durham's tube in each tube

c) Single strength MacConkey broth -10 tubes -10 ml;
Place inverted Durham's tube in each tube

Method:

- Collect water samples aseptically for analysis.
- Transfer 50 ml of water sample to the flask containing 50 ml double strength MacConkey broth; 10ml each of water sample to 5 tubes containing double strength MacConkey broth; 1 ml each into 5 tubes of single strength MacConkey broth. Make a serial dilution of the water sample by adding 1 ml of the water to 9 ml of sterile diluent and add 1 ml each of the first dilution (equivalent to 0.1 ml of the original sample) to the next five tubes of single strength MacConkey broth
- Label appropriately. Incubate the flask and tubes at 35 ± 2 ºC.
- Examine the tubes after 24 ± 2 h for growth and gas formation. If the tubes are negative at 24 h, reincubate tubes for an additional 24 h and examine again for gas. If acid (yellow colour) and gas production (bubbles in Durham's tube) are noticed, the reaction is noted as positive.
- Note the results as number of positives in each set of 50 ml, 10 ml and 1 ml and 0.1 ml tubes.

- Compare the results with standard 5 tube MPN table and perform confirmation test for all the positive tubes.

Step II: **Confirmed test for coliforms**

Media required: Brilliant Green Lactose Bile Broth (BGLB 2%) 5 ml each in test tubes; place inverted Durham's tube in each tube

- Transfer a loopful of suspension from the positive tubes of MacConkey broth to a tube of BGLB broth, avoiding pellicle if present. (a sterile wooden applicator stick may also be used for these transfers).
- Incubate BGLB tubes at 35 °C and examine for gas production at 48 ± 3 h.
- Note the results as number of positives in each set of 50 ml, 10 ml, 1 ml and 0.1 ml tubes.
- Calculate most probable number (MPN) of coliforms from the 5 tube MPN table. Give the result as Coliforms MPN per 100 ml.

Step III: Confirmed test for Faecal coliforms

Media required: EC Broth, 5 ml each in test tubes; place inverted Durham's tube in each tube.

- Transfer a loopful of suspension from the positive BGLB tubes to EC broth. Label appropriately.

- Incubate EC tubes 24 ± 2 h at 44.5 °C and examine for gas production.
- Note the results as number of positives in each set of 50 ml, 10 ml, 1 ml and 0.1 ml tubes.
- Use results of this test to calculate faecal coliform MPN and express the result as Faecal Coliforms MPN per 100 ml.

Note: Faecal coliform analyses are done at 45.5 °C for food testing, except for water, shellfish and shellfish harvest water analyses, which use 44.5 °C.

Step IV: **Completed test for** *E. coli*

Media Required:

a) Eosin-methylene blue (EMB) agar
b) Tryptone (tryptophan) broth
c) MR-VP broth
d) Koser's citrate broth
e) Simmons citrate agar
f) Plate count agar (PCA)

Method:

- Melt 100 ml of EMB agar and cool to 45-50 ºC, pour into petri dishes and allow to set, the set plates are dried at 56 ºC for 45 min and cool to room temperature.

- Streak loopful of culture from positive tubes of EC broth on EMB agar plate and incubate for 18 - 24 h at 35 °C ± 0.5 °C.
- Examine plates for suspected *E. coli* colonies, i.e., dark centered and flat, with metallic sheen. Transfer up to 5 suspected colonies from each EMB plate to PCA slants; incubate them for 18 - 24 h at 35 °C ± 0.5 °C and use for further testing.

StepV: IMViC tests

(a) Indole production: Inoculate suspected *E. coli* into tryptone broth and incubate 24 ± 2 h at 35 °C ± 0.5 °C. Test for indole by adding 0.2 - 0.3 ml of Kovacs' reagent. Appearance of distinct red color in upper layer is positive test.

(b) Voges-Proskauer (VP) test: Inoculate suspected *E. coli* into MR-VP broth and incubate 48 ± 2 h at 35 °C ± 0.5 °C. Transfer 1 ml of the incubated culture to 13×100 mm tube. Add 0.6 ml α-naphthol solution and 0.2 ml 40 % KOH, and shake. Add a few crystals of creatine. Shake and let stand for 2 h. Test is positive if eosin pink color develops.

(c) Methyl red test: Inoculate suspected *E. coli* into MR-VP broth and incubate 48 ± 2 h at 35 °C ± 0.5 °C. Add 5

drops of methyl red solution to each tube. Distinct red color is positive test. Yellow is negative reaction.

(d) Simmon's Citrate agar: Streak a little of the culture to citrate agar slants and incubate at 37 ºC for 48 - 96 h. Growth is indicated by a change in the colour of the medium from green to blue indicates a positive. *E. coli* do not utilize citrate and hence give a negative reaction.

Interpretation: IMViC tests of ++-- are considered to be *E. coli*.

Calculate MPN of *E. coli* from the 5 tube MPN table and express the result as *E. coli* MPN per 100 ml.

Table: 5 Tube MPN Table for water samples

Number of tubes giving a positive reaction			MPN per 100 ml of water
1 of 50 ml	**5 of 10 ml**	**5 of 1 ml**	
0	**0**	**0**	**<1**
0	**0**	**1**	**1**
0	**0**	**2**	**2**
0	**1**	**0**	**1**
0	**1**	**1**	**2**
0	**1**	**2**	**3**
0	**2**	**0**	**2**
0	**2**	**1**	**3**
0	**2**	**2**	**4**
0	**3**	**0**	**3**
0	**3**	**1**	**5**
0	**4**	**0**	**5**
1	**0**	**0**	**1**

1	**0**	**1**	**3**
1	**0**	**2**	**4**
1	**0**	**3**	**6**
1	**1**	**0**	**3**
1	**1**	**1**	**5**
1	**1**	**2**	**7**
1	**1**	**3**	**9**
1	**2**	**0**	**5**
1	**2**	**1**	**7**
1	**2**	**2**	**10**
1	**2**	**3**	**12**
1	**3**	**0**	**8**
1	**3**	**1**	**11**
1	**3**	**2**	**14**
1	**3**	**3**	**18**
1	**3**	**4**	**21**
1	**4**	**0**	**13**
1	**4**	**1**	**17**
1	**4**	**2**	**22**
1	**4**	**3**	**28**
1	**4**	**4**	**35**
1	**4**	**5**	**43**
1	**5**	**0**	**24**
1	**5**	**1**	**35**
1	**5**	**2**	**54**
1	**5**	**3**	**92**
1	**5**	**4**	**161**
1	**5**	**5**	**>180**

Table: 5 Tube MPN Table for water samples

Number of tubes giving a positive reaction			MPN per100 ml of water
5 of 10 ml	5 of 1 ml	5 of 0.1 ml	
0	0	0	0
0	0	1	2
0	0	2	4
0	1	0	2
0	1	1	4
0	1	2	6
0	2	0	4
0	2	1	6
0	3	0	6
1	0	0	2
1	0	1	4
1	0	2	6
1	0	3	8
1	1	0	4
1	1	1	6
1	1	2	8
1	2	0	6
1	2	1	8
1	2	2	10
1	3	0	8
1	3	1	10
1	4	0	11
2	0	0	5
2	0	1	7
2	0	2	9
2	0	3	12
2	1	0	7
2	1	1	9
2	1	2	12

2	**2**	**0**	**9**
2	**2**	**1**	**12**
2	**2**	**2**	**14**
2	**3**	**0**	**12**
2	**3**	**1**	**14**
2	**4**	**0**	**15**
3	**0**	**0**	**8**
3	**0**	**1**	**11**
3	**0**	**2**	**13**
3	**1**	**0**	**11**
3	**1**	**1**	**14**
3	**1**	**2**	**17**
3	**1**	**3**	**20**
3	**2**	**0**	**14**
3	**2**	**1**	**17**
3	**2**	**2**	**20**
3	**3**	**0**	**17**
3	**3**	**1**	**20**
3	**4**	**0**	**20**
3	**4**	**1**	**25**
3	**5**	**0**	**25**
4	**0**	**0**	**13**
4	**0**	**1**	**17**
4	**0**	**2**	**20**
4	**0**	**3**	**25**
4	**1**	**0**	**17**
4	**1**	**1**	**20**
4	**1**	**2**	**25**
4	**2**	**0**	**20**
4	**2**	**1**	**25**
4	**2**	**2**	**30**
4	**3**	**0**	**25**
4	**3**	**1**	**35**

4	**3**	**2**	**40**
4	**4**	**0**	**35**
4	**4**	**1**	**40**
4	**4**	**2**	**45**
4	**5**	**0**	**40**
4	**5**	**1**	**50**
4	**5**	**2**	**55**
5	**0**	**0**	**25**
5	**0**	**1**	**30**
5	**0**	**2**	**45**
5	**0**	**3**	**60**
5	**0**	**4**	**75**
5	**1**	**0**	**35**
5	**1**	**1**	**45**
5	**1**	**2**	**65**
5	**1**	**3**	**85**
5	**1**	**4**	**115**
5	**2**	**0**	**50**
5	**2**	**1**	**70**
5	**2**	**2**	**95**
5	**2**	**3**	**120**
5	**2**	**4**	**150**
5	**2**	**5**	**175**
5	**3**	**0**	**80**
5	**3**	**1**	**110**
5	**3**	**2**	**140**
5	**3**	**3**	**175**
5	**3**	**4**	**200**
5	**3**	**5**	**250**
5	**4**	**0**	**130**
5	**4**	**1**	**170**
5	**4**	**2**	**225**
5	**4**	**3**	**275**

5	**4**	**4**	**350**
5	**4**	**5**	**425**
5	**5**	**0**	**250**
5	**5**	**1**	**350**
5	**5**	**2**	**550**
5	**5**	**3**	**900**
5	**5**	**4**	**1600**
5	**5**	**5**	**>1800**

References

1. Feng, P., Weagant, S.D., Grant, M. A. and Burkhardt, W. (2002)**.** Bacteriological Analytical Manual, Chapter 4, USFDA.
2. Surendran, P.K., Thampuran, N., Nambiar, V.N., Lalitha, K.V and Joseph, T.C., 2013, *Laboratory techniques for microbiological examination of seafood*. 4th edition, Central Institute of Fisheries Technology, Cochin-682029, India.55p.

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Isolation and Identification of *Staphylococcus aureus* from Seafood

Remya. S

Introduction

- Gram-positive round shaped (coccal) bacterium
- Appear as "**bunches of grapes**"
- The genus name *Staphylococcus* is derived from the Greek word "stapyle" (grape-cluster berry/bunch of grapes) and species name "*aureus*" (golden) is Latin.
- Facultative anaerobe, which can grow without the need for oxygen
- Member of the natural flora of the human body
- Frequently found in the nose, respiratory tract and on the skin
- It is highly vulnerable to destruction by heat treatment and nearly all sanitizing agents.
- Hence, the presence of this bacterium or its enterotoxins in processed foods or on food processing equipment generally ***indicates poor sanitation***.
- It is an important human pathogen that causes skin and respiratory infections.
- It can cause severe ***food poisoning*** through its production of ***enterotoxins*** and other superantigens.

- The emergence of **antibiotic-resistant strains** of *S. aureus* such as methicillin-resistant *S. aureus* (**MRSA**) is a worldwide problem in clinical medicine.

Direct plate count method (Source: Bacteriological Analytical Manual, Chapter 12, 2016, USFDA)

- This method is appropriate for the analysis of foods in which more than 100 *S. aureus* cells/g are expected.

Media and reagents

1. Baird-Parker (BP) medium
2. Trypticase (tryptic) soy agar (TSA)
3. Brain heart infusion (BHI) broth
4. Coagulase plasma (rabbit) with EDTA
5. Toluidine blue-DNA agar
6. Lysostaphin
7. Tryptone yeast extract agar
8. Paraffin oil, sterile
9. 0.02 M phosphate-saline buffer containing 1% NaCl
10. Catalase test

Preparation of pre-set Baird-Parker (BP) plates

Basal medium

Tryptone	10 g
Beef extract	5 g
Yeast extract	1 g
Sodium pyruvate	10 g
Glycine	12 g
Lithium chloride·$6H_2O$	5 g
Agar	20 g

- Autoclave the medium for 15 min at 121 °C.
- Final pH, 7.0 ± 0.2.
- If intended for immediate use, maintain melted medium at 48-50 °C before adding enrichment.
- If not, store solidified medium at 4 ± 1 °C up to 1 month. Melt one 100 ml flask of stored BP medium on water bath.
- Cool to about 50 ^oC

Enrichment & Complete medium

- Aseptically add 5 ml pre-warmed (45-50 °C) ***Bacto EY (egg yolk) tellurite enrichment*** to 95 ml melted base.

*Alternatively, add 1 ml of sterile 1 % *potassium tellurite* solution followed by 5 ml of 50 % egg yolk emulsion.

- Mix well (avoiding bubbles) and pour into sterile petri dishes. The medium must be densely opaque.
- Allow to set and dry at 56 ˚C for 45 min
- Cool to room temperature
- Store prepared plates at 20-25 °C for up to 5 days.

Preparation of sterile egg yolk (50 %) for BP medium

- Take 5 numbers of hen's eggs and wash it free of dirt.
- After wiping and drying, keep the eggs immersed in alcohol (rectified spirit) in a 1 L beaker for 2 h.
- Drain off the alcohol and take out the eggs one by one.
- Make a small opening at one end of the egg using a sterile scalpel and pour out all the egg white carefully.
- Break the shell a little more carefully and transfer the egg yolk (yellow) into a sterile conical flask.
- Each egg will provide about 15 ml of egg yolk.
- Add an equal volume of sterile normal saline, agitate well and allow to stand.
- Pipette 5 ml each of the egg yolk saline into sterile test tubes, plug with sterile cotton and keep in a refrigerator.

Preparation of diluent (Butterfield's Phosphate-Buffered Dilution Water)

- Potassium dihydrogen phosphate (KH_2PO_4): 34 g
- Distilled water: 500 ml

- Adjust pH to 7.2 with 1 N NaOH.
- Bring volume to 1 L with distilled water.
- Sterilize for 15 min at 121 °C.
- Store in refrigerator.
- Take 1.25 ml of above stock solution and bring volume to 1 liter with distilled water.
- Dispense into bottles to 90 or ± 1 ml.
- Sterilize 15 min at 121 °C.

Isolation and enumeration of *S. aureus*

- Take 50 g fish sample/analytical unit
- Add 450 ml of sterile Butterfield's phosphate-buffered dilution water
- Blend for 2 min in a blender jar/stomacher. This results in a dilution of 10^{-1}

* Prepare all decimal dilutions with 90 ml of sterile diluent plus 10 ml of previous dilution

- 10 ml from above + 90 ml of sterile diluent (10^{-2})
- 10 ml from above + 90 ml of sterile diluent (10^{-3})
- From each dilution, aseptically transfer 0.3, 0.3 and 0.4 ml sample suspension in to 3 BP plates (Distributing 1 ml of inoculum equitably to 3 BP plates).

*Spread inoculum over surface of agar plate, using sterile bent glass streaking rod.

*Retain plates in upright position until inoculum is absorbed by agar (about 10 min on properly dried plates).

*If inoculum is not readily adsorbed, place plates upright in incubator for about 1 h.

- Invert plates and incubate 45 - 48 h at 35 - 37 °C.
- Select plates containing 20-200 colonies, unless only plates at lower dilutions (>200 colonies) have colonies with typical appearance of *S. aureus*.
- Count black colonies with off-white margin surrounded by opaque zone

*Colonies of *S. aureus* are circular, smooth, convex, moist, 2-3 mm in diameter on uncrowded plates, grey to jet-black, frequently with light-coloured (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone; colonies have buttery to gummy consistency when touched with inoculating needle.

Number of *S. aureus*/ g = Number of positive colonies x Dilution factor

*Add number of colonies on triplicate plates represented by colonies giving positive coagulase test and multiply by the sample dilution factor. Report this number as number of *S. aureus*/g of food tested.

Coagulase test

- This test is used to identify *S. aureus*, which produces the enzyme coagulase.
- Transfer suspect *S. aureus* colonies into small tubes containing 0.2 - 0.3 ml BHI (Brain heart infusion) broth and emulsify thoroughly.
- Inoculate agar slant of suitable maintenance medium, e.g., TSA (Trypticase soy agar), with loopful of BHI suspension.
- Incubate BHI culture suspension and agar slants for 18 - 24 h at 35 - 37 °C.
- Add 0.5 ml reconstituted **coagulase plasma with EDTA** (Coagulase plasma (rabbit) with EDTA) to the BHI culture and mix thoroughly.
- Incubate at 35 - 37°C and check periodically for over 6 h for **clot formation/coagulation/gel formation.**
- Only firm and complete clot that stays in place when tube is tilted or inverted is considered positive for *S. aureus.*

Principle of Coagulase test

- Coagulase causes plasma to clot by converting fibrinogen to fibrin.
- Two types of coagulase are produced by most strains of *S. aureus*

- **Free coagulase,** which converts fibronogen to fibrin by activating a coagulase-reacting factor present in plasma. Free coagulase is detected **by clotting in the test tube**.
- **Bound coagulase** (clumping factor), which converts fibrinogen directly to fibrin without requiring a coagulase-reacting factor. It can be detected by the **clumping of bacterial cells** in the rapid slide test.

Ancillary tests (Optional)

1. Catalase test

- Used to differentiate bacteria, which produce the enzyme catalase, such as staphylococci, from non-catalase producing bacteria such as streptococci.
- Use growth from TSA slant for catalase test on glass slide or spot plate, and illuminate properly to observe **production of gas bubbles**.

Principle

- Catalase acts as a catalyst in the breakdown of **hydrogen peroxide to oxygen and water**. An organism is tested for catalase production by bringing it into contact with hydrogen peroxide. If the organism is a catalase producer, bubbles of oxygen are released. The culture should not be more than 24 h old.

2. Anaerobic utilization of glucose

3. Anaerobic utilization of mannitol
4. Lysostaphin sensitivity
5. Thermostable nuclease production

Typical characteristics of *S. aureus*

Characteristic	***S. aureus***
Coagulase production	+
Catalase activity	+
Anaerobic utilization of glucose	+
Anaerobic utilization of Mannitol	+
Lysostaphin sensitivity	+
Thermostable nuclease production	+

*+, Most (90 % or more) strains are positive

Coagulase-positive *staphylococci* – *Staphylococcus aureus* and other species (Source: ISO 6888-1:1999/Amd. 1:2003) Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) - Part 1: Technique using Baird-Parker agar medium

Sample preparation:

Transfer 50 g of the analytical unit in a blender jar, add buffered dilution water and blend for 2 min.

- Prepare all decimal dilutions.
- If possible, dilutions should be selected that will give colony counts of between 10 and 300 colonies per plate.

Inoculation

- From decimal dilutions in the appropriate diluent, 0.1 ml aliquots of each appropriate dilution are spread on the surface of each of two Baird-Parker agar plates.
- If low numbers of Coagulase-Positive *Staphylococci* are required to be counted, 1 ml of the test sample, if liquid, or the initial suspension, can be spread on to the surface of a large (140 mm) agar plate or three 90 mm agar plates, in duplicate.
- Plates are allowed to dry for some 15 min with their lids on at laboratory temperature.

Incubation

- The plates are incubated inverted at 35 °C or 37 °C for 24 h (± 2 h).
- After this time, the bottoms of plates are marked to show the positions of any typical colonies.
- The plates are re-incubated at 35 °C or 37 °C for a further 24 h (±2 h) and the positions of any new colonies are marked.
- Atypical colonies should also be marked at this point.

Selection of plates and interpretation

- Typical colonies are black or grey, shiny and convex, and are surrounded by a clear zone that may be partially opaque.
- After incubation for at least 24 h, an opalescent ring immediately in contact with the colony may appear in this clear zone.

- The colonies are 1 - 1.5 mm in diameter after 24 h, and 1.5 - 2.5 mm in diameter after 48 h incubation.
- Atypical colonies are similar in size to typical colonies, but may be shiny black colonies with or without a narrow white edge, the clear zone is absent or barely visible and the opalescent ring is absent or hardly visible, or grey colonies without a clear zone.
- Colonies should only be counted on plates containing a maximum of 300 colonies with 150 typical and/or atypical colonies at two successive dilutions.
- One plate should contain at least 15 colonies.
- Select five typical colonies for confirmation if there are only typical colonies, five atypical colonies if there are only atypical colonies, or five of each if both types are present, from each plate.

Confirmation (Coagulase test)

- Using a sterile wire loop, remove some of each selected colony from the surface and inoculate into a separate tube of brain heart infusion.
- Incubate this at 35 °C or 37 °C for 24 h (±2 h).
- After incubation, add 0.1 ml of each culture to 0.3 ml rabbit plasma in sterile haemolysis tubes and incubate at 35 °C or 37 °C.
- Examine the plasma for clotting after 4–6 h incubation by tilting the tube.

- If there is no positive reaction, re-examine the tube after 24 h incubation.
- Suppliers of rabbit plasma may provide their own instructions that should be followed.
- The test is positive if the volume of the clot occupies more than half the original volume of liquid.
- A negative control should be set up by adding sterile brain heart infusion to the recommended quantity of rabbit plasma.

References

1. Reginald W. Bennett and Gayle A. Lancette. 2016. Chapter 12. *Staphylococcus aureus*. Bacteriological Analytical Manual (BAM). USFDA (Food and Drug Administration).
2. Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) - Part 1: Technique using Baird-Parker agar medium. ISO 6888-1:1999/Amd. 1:2003.
3. AOAC International. 1995. Official Methods of Analysis, 16th edition.

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Isolation and Identification of *Salmonella* from Seafood

Remya. S

Introduction

- *Salmonella* is named after the American Veterinary surgeon **D. E. Salmon.**
- *Salmonella* is a rod-shaped bacterium belonging to the family *Enterobacteriaceae*.
- Gram-negative organism.
- Facultative anaerobic bacteria.
- With a few exceptions, motile due to peritrichous flagella.
- Produce H_2S and colonies have a "cat-eye" appearance. Show blackening of the colonies due to H_2S production.
- *Salmonella* lives in the intestinal tracts of warm and cold-blooded animals.
- The two species of the genus *Salmonella* are ***Salmonella enterica*** and ***Salmonella bongori***.
- *S. enterica* is the type species and is further divided into six subspecies that include over 2,600 serotypes/serovars, defined on the basis of the somatic O and flagellar H antigens.

- The six subspecies of *S. enterica* are *S. e.* ***enterica***, *S. e.* ***salamae,*** *S. e.* ***arizonae***, *S. e.* ***diarizonae***, *S. e.* ***houtenae***, and *S. e.* ***indica***.
- The full name of a serotype is given as, for example, *Salmonella enterica* subsp. *enterica* serotype Typhimurium, but can be abbreviated to *Salmonella Typhimurium*.
- In humans, Salmonella causes two diseases. **Salmonellosis**: ***Enteric fever*** (typhoid), resulting from bacterial invasion of the bloodstream and ***acute gastroenteritis***, resulting from a foodborne infection/intoxication.
- The most frequent sources of *Salmonella* food poisoning are poultry, meat, milk, cream and egg.

Detection of *Salmonella* (Source: Bacteriological Analytical Manual, Chapter 5, December, 2019, USFDA)

1. *Non-Selective Enrichment (Pre enrichment)*: ***Lactose broth***
2. *Selective Enrichment*: ***Tertathionate (TT) broth & Rappaport –Vassiliadis (RV) medium***
3. *Selective Plating*: ***XLD, HEA & BSA***
4. *Purification*: ***MacConkey agar***
5. *Biochemical Screening*
6. *Serological Identification*: ***Polyvalent somatic (O) antisera***

Sample preparation for isolation of *Salmonella*

- Aseptically weigh 25 g sample into sterile blending container.
- Add 225 ml sterile lactose broth and blend for 2 min.
- Aseptically transfer homogenized mixture to sterile, wide-mouth, screw-cap jar/flask (500 ml) or other appropriate container and let stand 60 ± 5 min at room temperature.
- Mix well by swirling and determine pH with test paper.
- Adjust pH, if necessary, to 6.8 ± 0.2.
- Mix well and loosen jar cap about 1/4 turn.
- Incubate 24 ± 2 h at 35 °C.

Lactose Broth: Pre-enrichment

- A pre-enrichment medium provides a higher ratio of *Salmonella* to non-*Salmonella* bacteria after incubation.
- Lactose broth provides an environment favourable for the recovery of *Salmonella*.
- The media allows damaged cells to recover, dilutes toxic substances that may be present, and favours growth of *Salmonella* over other species.
- Most non-*Salmonella* bacteria ferment lactose, while *Salmonella* does not.

- When lactose-fermenting bacteria metabolize lactose in the medium, the pH decreases, creating a bacteriostatic effect on competing microorganisms.

Isolation of *Salmonella*

Selective Enrichment

- Gently shake incubated sample.
- Transfer 0.1 ml mixture to 10 ml Rappaport-Vassiliadis (RV) medium and another 1 ml mixture to 10 ml tetrathionate (TT) broth.
- Vortex mixer.
- Incubate RV medium at 42 ± 0.2 ℃ (circulating, thermostatically controlled, water bath) for 24 ± 2 h.
- Incubate TT broth at 35 ± 2 ℃ for 24 ± 2 h.

Selective Plating

- Mix (vortex, if tube) and streak 3 mm loopful (10 µl) incubated TT broth on bismuth sulfite (BS) agar, xylose lysine desoxycholate (XLD) agar, and Hektoen enteric (HE) agar.
- Prepare BS plates the day before streaking and store in dark at room temperature until streaked.
- Repeat with 3 mm loopful (10 µl) of RV medium.
- Incubate plates 24 ± 2 h at 35 ℃.

- Examine plates for presence of colonies that may be *Salmonella*.

Typical *Salmonella* colony morphology

- Pick 2 or more colonies of *Salmonella* from each selective agar after 24 ± 2 h incubation.
- Typical *Salmonella* colonies will have following characteristics:

  Bismuth sulfite (BS) agar: Brown, gray, or black colonies; sometimes they have a metallic sheen. Surrounding medium is usually brown at first, but may turn black in time with increased incubation, producing the so-called halo effect.

  Hektoen enteric (HE) agar: Blue-green to blue colonies with or without black centers. Many cultures of Salmonella may produce colonies with large, glossy black centers or may appear as almost completely black colonies.

  Xylose lysine desoxycholate (XLD) agar: Pink colonies with or without black centers. Many cultures of Salmonella may produce colonies with large, glossy black centers or may appear as almost completely black colonies.

Triple sugar iron agar (TSI) & lysine iron agar (LIA)

- Lightly touch the centre of the colony to be picked with sterile inoculating needle and inoculate TSI slant by streaking slant and stabbing butt.
- Without flaming, inoculate LIA slant by stabbing butt twice and then streaking slant.
- Since lysine decarboxylation reaction is strictly anaerobic, the LIA slants must have deep butt (4 cm).
- Store picked selective agar plates at 5-8°C.
- Incubate TSI and LIA slants at 35 °C for 24 ± 2 h.
- Cap tubes loosely to maintain aerobic conditions while incubating slants to prevent excessive H_2S production.
- Salmonella in culture typically produces alkaline (red) slant and acid (yellow) butt, with or without production of H_2S (blackening of agar) in TSI.
- In LIA, Salmonella typically produces alkaline (purple) reaction in butt of tube. Consider only distinct yellow in butt of tube as acidic (negative) reaction.
- Most Salmonella cultures produce H_2S in LIA.
- All cultures that give an alkaline butt in LIA, regardless of TSI reaction, should be retained as potential *Salmonella* isolates and submitted for biochemical and serological tests.

- Cultures that give an acid butt in LIA and an alkaline slant and acid butt in TSI should also be considered potential *Salmonella* isolates and should be submitted for biochemical and serological tests.
- Cultures that give an acid butt in LIA and an acid slant and acid butt in TSI may be discarded as not being *Salmonella*.

Apply biochemical and serological identification tests to:

a.) Three presumptive TSI cultures recovered from set of plates streaked from RV medium, if present, and 3 presumptive TSI agar cultures recovered from plates streaked from TT broth, if present.

b.) If 3 presumptive-positive TSI cultures are not isolated from one set of agar plates, test other presumptive-positive TSI agar cultures, if isolated, by biochemical and serological tests. Examine a minimum of 6 TSI cultures for each 25 g analytical unit or each 375 g composite.

Identification of Salmonella

1. **Mixed cultures**
   - Streak TSI agar cultures that appear to be mixed on MacConkey agar, HE agar, or XLD agar.
   - Incubate plates 24 ± 2 h at 35 °C.
   - Examine plates for presence of colonies suspected to be Salmonella.

- **MacConkey agar**: Typical colonies appear transparent and colourless, sometimes with dark centre.

2. **Pure cultures**

Urease test (conventional)

- With sterile needle, inoculate growth from each presumed-positive TSI slant culture into tubes of urea broth.
- Since occasional, uninoculated tubes of urea broth turn purple-red (positive test) on standing, include uninoculated tube of this broth as control.
- Incubate 24 ± 2 h at 35 °C.
- Urease test is used to identify bacteria capable of hydrolyzing urea using the enzyme urease.
- The hydrolysis of urea forms the weak base, ammonia, as one of its products.
- This weak base raises the pH of the media above 8.4 and the pH indicator, phenol red, turns from yellow to pink.
- *Salmonella* is urease negative

Serological polyvalent flagellar (H) test

a.) Inoculate growth from each urease-negative TSI agar slant into either

1) BHI broth and incubate 4-6 h at 35 °C until visible growth occurs (to test on same day);

2) or to trypticase soy-tryptose broth and incubate 24 ± 2 h at 35 °C (to test on following day).

- Add 2.5 ml formalinized physiological saline solution to 5 ml of either broth culture.

b.) Select 2 formalinized broth cultures and test with Salmonella polyvalent flagellar (H) antisera.

- Place 0.5 ml of appropriately diluted Salmonella polyvalent flagellar (H) antiserum in 10 x 75 mm or 13 x 100 mm serological test tube.
- Add 0.5 ml antigen to be tested.
- Prepare saline control by mixing 0.5 ml formalinized physiological saline solution with 0.5 ml formalinized antigen.
- Incubate mixtures in 48-50 °C water bath.
- Observe at 15 min intervals and read final results in 1 h.

Positive--agglutination in test mixture and no agglutination in control.

Negative--no agglutination in test mixture and no agglutination in control.

- Cultures, which show agglutination in test mixture and no agglutination in control are taken as positive and continued for further biochemical tests.

Additional biochemical tests

(Testing of urease-negative cultures)

a.) Lysine decarboxylase broth:

- If LIA test was satisfactory, it need not be repeated.
- Use lysine decarboxylase broth for final determination of lysine decarboxylase if culture gives doubtful LIA reaction.
- Inoculate broth with small amount of growth from TSI slant suspicious for *Salmonella*.
- Replace cap tightly and incubate for 48 ± 2 h at 35 °C but examine at 24 h intervals.
- *Salmonella* species causes alkaline reaction indicated by purple colour throughout medium.
- Negative test is indicated by yellow colour throughout medium.
- If medium appears discoloured (neither purple nor yellow) add a few drops of 0.2 % bromocresol purple dye and re-read tube reactions.

b.) Phenol red dulcitol broth or purple broth base with 0.5% dulcitol:

- Inoculate broth with small amount of growth from TSI culture.

- Replace cap loosely and incubate for 48 ± 2 h at 35 °C, but examine after 24 h.
- Most Salmonella species give positive test, indicated by gas formation in inner fermentation vial and acid pH (yellow) of medium.
- Production of acid should be interpreted as a positive reaction.
- Negative test is indicated by no gas formation in inner fermentation vial and red (with phenol red as indicator) or purple (with bromocresol purple as indicator) colour throughout medium.

c.) Tryptone (or tryptophane) broth:

- Inoculate broth with small growth from TSI agar culture.
- Incubate for 24 ± 2 h at 35 °C and proceed as follows:

1.) Potassium cyanide (KCN) broth:

- Transfer 3 mm loopful of 24 h tryptophane broth culture to KCN broth.
- Heat rim of tube so that good seal is formed when tube is stoppered with wax-coated cork.
- Incubate for 48 ± 2 h at 35 °C but examine after 24 h.
- Interpret growth (indicated by turbidity) as positive.
- Most Salmonella species do not grow in this medium, as indicated by lack of turbidity.

2.) Malonate broth:

- Transfer 3 mm loopful of 24 h tryptone broth culture to malonate broth.
- Since occasional uninoculated tubes of malonate broth turn blue (positive test) on standing, include uninoculated tube of this broth as control.
- Incubate for 48 ± 2 h at 35 °C, but examine after 24 h.
- Most Salmonella species cultures give negative test (green or unchanged colour) in this broth.

3.) Indole test:

- Transfer 5 ml of 24 h tryptophane broth culture to empty test tube.
- Add 0.2-0.3 ml Kovacs' reagent.
- Most *Salmonella* cultures give negative test (lack of deep red colour at surface of broth).
- Record intermediate shades of orange and pink as ±.

4.) Serological flagellar (H) tests for Salmonella:

If either polyvalent flagellar (H) test has not already been performed, it may be performed here.

5.) Discard as not Salmonella any culture that shows either positive indole test and negative serological flagellar (H) test, or positive KCN test and negative lysine decarboxylase test.

Serological somatic (O) tests for *Salmonella*

Polyvalent somatic (O) test:

- Using wax pencil, mark off 2 sections about 1 x 2 cm each on inside of glass or plastic petri dish (15 x 100 mm).
- Commercially available sectioned slides may be used.
- Emulsify 3 mm loopful of culture from 24-48 h TSI slant or, preferably, tryptose blood agar base (without blood) with 2 ml 0.85 % saline.
- Add 1 drop of culture suspension to upper portion of each rectangular crayon-marked section.
- Add 1 drop of saline solution to lower part of one section only.
- Add 1 drop of Salmonella polyvalent somatic (O) antiserum to other section only.
- With clean sterile transfer loop or needle, mix culture suspension with saline solution for one section and repeat for other section containing antiserum.
- Tilt mixtures in back and forth motion for 1 min and observe against dark background in good illumination.
- Consider any degree of agglutination a positive reaction.
- Classify polyvalent somatic (O) test results as follows:

Positive - agglutination in test mixture; no agglutination in saline control.

Negative - no agglutination in test mixture; no agglutination in saline control.

Additional biochemical tests: Classify as *Salmonella*, those cultures, which exhibit typical *Salmonella* reactions for tests 1-11, shown in the below given Table.

Biochemical and serological reactions of *Salmonella*

Test or substrate	Result		Salmonella species reaction(a)
	Positive	Negative	
Glucose (TSI)	Yellow butt	Red butt	+
Lysine decarboxylase (LIA)	Purple butt	Yellow butt	+
H_2S (TSI and LIA)	Blackening	No blackening	+
Urease	Purple-red colour	No colour change	−
Lysine decarboxylase broth	Purple colour	Yellow colour	+
Phenol red dulcitol broth	Yellow colour and/or gas	No gas; no colour change	+ (b)
KCN broth	Growth	No growth	−
Malonate broth	Blue colour	No colour change	− (c)
Indole test	Violet colour at surface	Yellow colour at surface	−

Polyvalent flagellar test	Agglutination	No agglutination	+
Polyvalent somatic Test	Agglutination	No agglutination	+
Phenol red lactose Broth	Yellow colour and/or Gas	No gas; no colour change	– [(c)]
Phenol red sucrose Broth	Yellow colour and/or Gas	No gas; no colour Change	–
Voges-Proskauer Test	Pink-to-red colour	No colour change	–
Methyl red test	Diffuse red colour	Diffuse yellow colour	+
Simmons citrate	Growth; blue colour	No growth; no colour change	v

[a] +, 90% or more positive in 1 or 2 days; -, 90% or more negative in 1 or 2 days; v, variable.
[b] Majority of *S. arizonae* cultures are negative and [c] Majority of *S. arizonae* cultures are positive.

a. Phenol red lactose broth or purple lactose broth

- Inoculate broth with small amount of growth from unclassified 24-48 h TSI slant.
- Incubate for 48 ± 2 h at 35 °C, but examine after 24 h.

- **Positive**-acid production (yellow) and gas production in inner fermentation vial. Consider production of acid only as positive reaction.
- Most cultures of Salmonella give negative test result, indicated by no gas formation in inner fermentation vial and red (with phenol red as indicator) or purple (with bromocresol purple as indicator) throughout medium.
- Discard as not *Salmonella*, cultures that give positive lactose tests, except cultures that give acid slants in TSI and positive reactions in LIA, or cultures that give positive malonate broth reactions.
- Perform further tests on these cultures to determine if they are *S. arizonae*.

b. Phenol red sucrose broth or purple sucrose broth

- Inoculate broth with small amount of growth from unclassified 24-48 h TSI slant.
- Incubate for 48 ± 2 h at 35 °C, but examine after 24 h.
- Discard as not *Salmonella*, cultures that give positive sucrose tests, except those that give acid slants in TSI and positive reactions in LIA.

c. MR-VP broth

- Inoculate medium with small amount of growth from each unclassified TSI slant suspected to contain *Salmonella*.

- Incubate for 48 ± 2 h at 35 °C.

1) Perform **Voges-Proskauer (VP) test** at room temperature as follows:
   - Transfer 1 ml 48 h culture to test tube and incubate remainder of MR-VP broth an additional 48 h at 35 °C.
   - Add 0.6 ml α-naphthol and shake well.
   - Add 0.2 ml 40 % KOH solution and shake.
   - To intensify and speed reaction, add a few crystals of creatine.
   - Read results after 4 h; development of pink-to-ruby red colour throughout medium is positive test.
   - Most cultures of *Salmonella* are VP-negative, indicated by absence of development of pink-to-red colour throughout broth.

2. Perform **methyl red test** as follows:
   - To 5 ml of 96 h MR-VP broth, add 5-6 drops of methyl red indicator.
   - Read results immediately.
   - Most *Salmonella* cultures give positive test, indicated by diffuse red colour in medium.
   - A distinct yellow colour is negative test.
   - Discard, as not *Salmonella*, cultures that give positive KCN and VP tests and negative methyl red test.

3. **Simmons citrate agar**

- Inoculate this agar, using needle containing growth from unclassified TSI agar slant.
- Inoculate by streaking slant and stabbing butt.
- Incubate for 96 ± 2 h at 35 °C.
- Read results as follows: Positive-presence of growth, usually accompanied by colour change from green to blue.
- Most cultures of *Salmonella* are citrate-positive.

Detection of *Salmonella* (Source: ISO 6579-1: 2017 (E))

As per this method, the detection of Salmonella requires four successive stages.

1. **Pre-enrichment in non-selective liquid medium**
    - Sample + Buffered peptone water (BPW)
    - Tenfold dilution (25 g test portion+225 ml of BPW)
    - Incubation for 18 ± 2 h at 34 °C to 38 °C.
2. **Enrichment in/on selective media**
    - 0.1 ml culture + 10 ml RVS broth or on MSRV agar
    - RVS: Rappaport-Vassiliadis medium with Soya
    - MSRV: Modified Semi-solid Rappaport-Vassiliadis
    - Incubate for 24 ± 3 h at 41.5 °C ± 1 °C
    - 1 ml culture + 10 ml MKTTn broth
    - MKTTn: Muller-Kauffmann tetrathionate-novobiocin

- Incubate for 24 ± 3 h at 37 °C ± 1 °C.

3. Plating out on selective solid media

- From the cultures obtained, two selective solid media are inoculated.

  - Xylose Lysine Deoxycholate agar (XLD agar)

  - Any other solid selective medium complimentary to XLD agar.

  - The XLD agar is incubated for 24 ± 3 h at 37 °C ± 1 °C.

  - Typical colonies of *Salmonella* on XLD agar have a black centre and a lightly transparent zone of reddish colour due to the colour change of the indicator.

4. Confirmation

- Colonies of presumptive *Salmonella* are sub-cultured and their identity is confirmed by means of appropriate biochemical and serological tests.
- For characterization of *Salmonella* strains, full serotyping is needed.

Selection of colonies for confirmation

- Mark suspect colonies on each plate
- Select at least 1 typical or suspect colony for subculture and confirmation

- If this is negative, select up to 4 more suspect colonies ensuring that these colonies are sub-cultured from different selective enrichment/isolation medium combinations showing suspect growth.

Non-selective agar medium

- Streak the selected colonies onto the surface of a pre-dried non-selective agar medium.
- Incubate the inoculated plates between 34 °C and 38 °C for 24 ± 3 h.
- Alternatively, if well-isolated colonies (of a pure culture) are available on the selective plating media, the biochemical confirmation can be performed directly on a suspect, well-isolated colony from the selective plating medium.
- The culture step on the non-selective agar medium can then be performed in parallel with the biochemical tests for purity check of the colony taken from the selective agar medium.
- Use pure cultures for biochemical and serological confirmation.

Biochemical testing

- Inoculate the biochemical confirmation media with each of the cultures obtained from the colonies selected.

- For confirmation of *Salmonella spp.*, at least the following tests should be performed.

1. TSI Agar

- Streak the agar slant surface and stab the butt
- Incubate at 37 °C for 24 ± 3 h
- Interpret the changes in the media as follows:

a. Butt

- Yellow: Glucose positive (Glucose formation)
- Red or unchanged: Glucose negative (No glucose fermentation)
- Black: Formation of hydrogen sulphide
- Bubbles or cracks: Gas formation from glucose

b. Slant surface

- Yellow: Lactose and/or sucrose positive (Lactose and/or sucrose fermentation)
- Red or unchanged: Lactose and sucrose negative

- The majority of the typical *Salmonella* cultures show alkaline (red) slants and acid (yellow) butts with gas

formation (bubbles) and (in about 90 % of the cases) formation of hydrogen sulfide (Blackening of the agar).

2. **Urea agar**
    - Streak the agar slant surface
    - Incubate at 37 °C for upto 24 h
    - If the reaction is positive, urea is hydrolyzed, liberating ammonia.
    - This changes the colour of phenol-red to rose-pink and later to deep cerise.
    - This reaction is often apparent after 2 h to 4 h.
    - Typical *Salmonella* cultures don't hydrolyze urea so that the colour of the urea agar will remain unchanged.
3. **L-Lysine decarboxylation medium (LDC)**
    - Inoculate just below the surface of the liquid medium
    - Incubate at 37 °C for 24 ± 3 h
    - Turbidity and purple colour after incubation indicate a positive reaction.
    - A yellow colour indicates a negative reaction.
    - The majority of the typical Salmonella cultures show a positive reaction in LDC.

Optional tests

1. β-galactosidase test
2. Indole test

Serological testing

- The pure colonies showing typical biochemical reactions for *Salmonella* are also tested for the presence of *Salmonella* O- and H- antigens (and, in areas where *Salmonella* typhi is expected in the food supply, also for Vi antigen) by slide agglutination using polyvalent antisera.
- The pure colonies are cultured on a non-selective agar medium and tested for auto-agglutination.
- Strains that are auto-agglutinable cannot be tested for the presence of *Salmonella* antigens.

Elimination of auto-agglutinable strains

- Place one drop of saline solution on a cleans glass slide
- Using a loop, disperse part of the colony to be tested in the saline to obtain a homogeneous and turbid suspension.
- Rock the slide gently for 5 s to 60 s.
- Observe the suspension, preferably against a dark background.
- If the bacteria have formed granules in the suspension, this indicates auto-agglutination and serological confirmation will be complicated.

Examination for O-antigens

- Using one non-auto-agglutinating pure colony, proceed as above described in the portion of elimination of auto-agglutinable strains.
- Use one drop of polyvalent anti-O sera, in place of saline solution.
- If agglutination occurs, this is considered a positive reaction.

Examination for Vi antigens (Optional)

- Using one non-auto-agglutinating pure colony, proceed as above described in the portion of elimination of auto-agglutinable strains.
- Use one drop of polyvalent anti-Vi sera, in place of saline solution.
- If agglutination occurs, this is considered a positive reaction.

Examination for H-antigens

- Using one non-auto-agglutinating pure colony, proceed as above described in the portion of elimination of auto-agglutinable strains.
- Use one drop of polyvalent anti-H sera, in place of saline solution.

- If agglutination occurs, this is considered a positive reaction.

Interpretation of biochemical and serological reactions

Biochemical reactions	Auto-agglutination	Serological reactions	Interpretation
Typical	No	O- and H-antigens positive (and Vi positive, if tested)	Strains considered to be *Salmonella*
Typical	No	O-and/or H-antigens negative	Presumptive
Typical	Yes	Not tested because of auto-agglutination	*Salmonella*
No typical reactions	-	-	Not considered to be *Salmonella*

Serotyping

- Strains that are confirmed as *Salmonella* spp. can be further typed to serovar level.

Expression of results

- In accordance with the interpretation of the results, indicate *Salmonella* detected or not detected in a test

portion of x g or x ml of product, or on the surface area or in an object.

References

1. Wallace H. Andrews, Hua Wang, Andrew Jacobson, and Thomas Hammack. 2019. Chapter 5. *Salmonella*. Bacteriological Analytical Manual (BAM). USFDA (Food and Drug Administration).
2. Microbiology of the food chain- Horizontal method for the detection, enumeration and serotyping of *Salmonella*. ISO 6579-1: 2017 (E)
3. AOAC INTERNATIONAL. 2000. Official Methods of Analysis, 17th ed., Methods 967.25-967.28, 978.24, 989.12, 991.13, 994.04 and 995.20. AOAC INTERNATIONAL, Gaithersburg, MD.

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Isolation and Identification of *Vibrio cholerae* from Seafood

Anupama T. K

V. cholerae, the type species of the genus *Vibrio*, is the causative agent of cholera outbreaks and epidemics. Cholera enterotoxin (CT) is the primary virulence factor of the disease cholera. The *V. cholerae* strains recovered from epidemic cholera cases contain a common somatic antigen and include serogroup O1. Over 150 known somatic antigenic types have been identified. Strains that are agglutinable in Inaba or Ogawa serotypes of O1 antiserum are well-documented human pathogens. Until recently, only the O1 serogroup was associated with cholera epidemics. *V. cholerae* strains that are identical to, or closely resemble, clinical strains in biochemical characteristics, but fail to agglutinate in either anti-O1 or O139 sera are now referred to as *V. cholerae* non-O1/O139. These serologically diverse strains are abundant in estuarine environments. The non-O1/O139 strains cause cholera-like diarrheal disease but rarely in outbreaks. Some non-O1/O139 strains also produce heat stable toxin and can cause septic infections in individuals. Most strains do not produce Cholera toxin, the key difference between these and epidemic *V. cholerae* O1/O139.

The primarily source of infection of *V. cholerae* O1 is the feaces of cholera patients. The disease is transmitted through contaminated water and food. Direct person-to-person spread is not common. The non-O1/O139 strains are commonly isolated from estuarine water and shellfish. Studies have found that *V. cholerae* O1 is a component of the autochthonous flora of brackish water, estuaries, and salt marshes of coastal areas, which is serious hazard to public health.

Media and Reagents

1. Alkaline peptone water (APW)
2. Arginine glucose slants (AGS)
3. Motility test medium-1% NaCl
4. Oxidase reagent (1% N,N,N,N'-tetramethyl-p-phenylenediamine.2HCl in dH_2O)
5. Phosphate buffered saline (PBS)
6. Trypticase soya agar (TSA)
7. Normal saline solution - 0.85% in dH_2O
8. Sodium desoxycholate - 0.5% in sterile dH_2O
9. Thiosulfate citrate bile salts sucrose (TCBS) agar
10. T_1N_0, T_1N_1 and T_1N_3 broths (Tryptone 1% and NaCl 0g for T_1N_0 ; Tryptone 1% and NaCl 1% for T_1N_1 and Tryptone 1% and NaCl 3% for T_1N_3)
11. *V. cholerae* polyvalent O1 and O139 antiserum

Procedure

A. Enrichment and plating

- Weigh 25 g of sample into a stomacher bag.
- Add 225 ml APW and blend 2 min at high speed in stomacher blender and transfer to 500ml sterile conical flask.
- Incubate APW at 35 ±2 °C for 6 to 8 h.
- Prepare dried plates of TCBS agar.
- Transfer a 3-mm loopful from the surface pellicle of APW culture to the surface of a dried TCBS plate and streak in a manner that will yield isolated colonies.
- Incubate TCBS overnight (18 to 24 h) at 35±2°C.
- Typical colonies of *V. cholerae* on TCBS agar are large (2 to 3 mm), smooth, yellow and slightly flattened with opaque centres and translucent peripheries.
- For biochemical identification, colonies from crowded plates must be streaked to a non-selective agar (T_1N_1, T_1N_3, or TSA-2% NaCl agar) for purity. Incubate overnight at 35 ±2° C and proceed with identification using a single isolated colony.

B. Screening and Confirmation

- Arginine glucose slant (AGS). Inoculate each suspect T_1N_1 culture to AGS by streaking the slant and stabbing the butt. Incubate AGS with loose cap overnight at 35° ± 2 °C. *V.*

cholerae will have an alkaline (purple) slant and an acid (yellow) butt, as arginine is not hydrolyzed. No gas or H_2S is produced.

- Salt tolerance. From T_1N_1 culture, lightly inoculate one tube each of T_1N_0 and T_1N_3 broths. Incubate tubes overnight at 35° ± 2 °C. *V. cholerae* will grow without NaCl.
- String test. The string test is a useful presumptive test for suspected *V. cholerae* as all strains are positive. Emulsify a large colony from a T_1N_1 agar culture in a small drop of 0.5% sodium desoxycholate in sterile distilled water. Within 60 sec the cells lyse (loss of turbidity) and DNA strings when a loopful is lifted (up to 2 to 3 cm) from the slide.
- Oxidase reaction. Transfer the overnight T_1N_1 growth using a platinum wire (nichrome wire should not be used) or wood applicator stick to a filter paper saturated with oxidase reagent (1% N,N,N,N'-tetramethyl-p-phenylenediamine. 2HCl). A dark purple color developing within 10 sec indicates a positive test growth. *V. cholerae* is oxidase positive.
- Serologic agglutination test. Serotyping of suspect *V. cholerae* cultures passing the string test using somatic or O antigens gives important epidemiological evidence. Two major serotypes of serogroup O1, Ogawa and Inaba, and

serogroup O139 are recognized as human pathogens. The two serotypes of O1 are seen in both the classical *V. cholerae* and the El Tor biotypes. The O139 serogroup resembles only the El Tor biotype.

a. For each culture, mark off three sections (with wax pencil) about 1 × 2 cm on the inside of a glass petri dish or on a 2 × 3-inch glass slide and add one drop of 0.85% saline solution to the lower part of each marked section. With a sterile transfer loop or needle, emulsify the T_1N_1 culture in the saline solution for one section, and repeat for the other section. Check for agglutination.
b. Add a drop of polyvalent *V. cholerae* O1 antiserum to one section of emulsified culture and mix with a sterile loop or needle. Add a drop of anti-O139 to a separate section. (Third section)
c. Tilt the mixture back and forth for one min and observe against a dark background. A positive reaction is indicated by a rapid, strong agglutination in a clear background.
d. If positive, test separately with Ogawa and Inaba antisera. The Hikojima serotype reacts with both antisera.

C. Biochemical tests

The following table presents the minimal number of characters needed to identify *V. cholerae* strains. The ability of *V. cholerae* to grow in 1% tryptone without added NaCl differentiates it from other sucrose-positive vibrios.

Table: Biochemical tests for the confirmation of *V. cholerae*

	Biochemical tests		*V. cholerae*
1.	TCBS agar		Smooth yellow, 2-3 mm diameter colonies; slightly flattened with translucent periphery and opaque centre
2.	AGS		KA
3.	Oxidase		+
4.	Arginine dihydrolase		-
5.	Ornithine decarboxylase		+
6.	Lysine decarboxylase		+
7.	**Growth in (w/v):**	0% NaCl	+
8.		3% NaCl	+
9.	Growth at 42°C		+
10	**Acid from:**	Sucrose	+
11		D-Cellobiose	-

12	Lactose	-
13	Arabinose	-
14	D-Mannose	+
15	D-Mannitol	+
16	ONPG	+
17	Voges-Proskauer	V

Abbreviations: TCBS, thiosulfate-citrate-bile salts-sucrose; AGS, arginine-glucose slant; Y=yellow, S=susceptible, V=variable among strains, KA=slant alkaline/Butt slightly acidic

Reference

Kaysner, C.A and DePaola, Jr. A (2004) Bacteriological Analytical Manual, Chapter 9, USFDA.

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Isolation and Identification of *V. parahaemolyticus* from Fish and Fishery Products

Toms C. Joseph

Vibrio parahaemolyticus is a curved, rod-shaped, Gram-negative bacterium found in brackish and saltwater environments. When the bacteria are ingested, it causes gastrointestinal illness in humans. *V. parahaemolyticus* is oxidase positive, facultatively aerobic, and does not form spores. This bacteria is motile with a single polar flagellum, like other members of the genus *Vibrio*. Most clinical isolates of *V. parahaemolyticus* can be differentiated from environmental strains by their ability to produce a thermostable direct hemolysin (TDH), (Kanagawa phenomenon).

Methods of Isolation

Vibrio species are facultatively anaerobic and grow best under alkaline conditions. They grow in the presence of relatively high levels of bile salts. Isolation of *Vibrio* species from foods is facilitated by the use of media with an alkaline pH. Alkaline peptone water (APW) is used commonly used as enriching media for isolating Vibrios. Media used for isolation and

biochemical reactions of *V. parahaemolyticus* should contain 2 % or 3 % NaCl. TCBS agar is a medium, which is commonly used for isolation of *V. cholerae, V. parahaemolyticus*, and other species from seafood. This medium supports good growth of most species of Vibrio while inhibiting most non-vibrios.

Equipments and materials

1. Biosafety cabinet/laminar flow
2. Petridishes
3. Micropipettes of 1 ml and 10 ml capacity and sterile pipette tips or glass pipettes of similar capacity
4. Mortar and pestle or Stomacher blender and bags
5. Incubator set at 35 ± 1^{o}C
6. Sterile forceps, scissors
7. Electronic balance (sensitivity of 0.1 g)

Media and Reagents

1. Alkaline peptone water (APW) with 3%NaCl
2. Oxidase reagent (1% N,N,N,N'-tetramethyl-p phenylenediamine dihydrochloride in distilled water)
3. Phosphate buffered saline (PBS)
4. Thiosulfate citrate bile salts sucrose (TCBS) agar (3 %)
5. T_1N_1 and T_1N_3 agars (1% tryptone and either 1 % or 3 % NaCl)

6. T_1N_0, T_1N_3, T_1N_6, T_1N_8, T_1N_{10} broths
7. Urea broth or Christensen's urea agar with added NaCl (2 %)

A. Seafood samples: Enrichment, isolation, and enumeration.

1. Weigh 50 g of seafood sample into a blender or mortar and pestle. Surface tissues, gills, and gut of fish may be obtained. For shellfish samples meat and liquor may be included. Normally 12 animals may be pooled, blended at high speed for 90 sec in blender or mortar and pestle. Fifty gram of sample is used for analysis. For crustaceans such as shrimp, use the entire animal if possible; if it is too large, use the central portion including gill and gut.
2. Macerate 25 g of sample with 225 ml of alkaline peptone water with 3 % salt in a sterile blender or stomacher blender to a sterile 500 ml flask and incubate overnight at 35 ± 2 °C.
3. Streak a 3-mm loopful from the top 1 cm of APW tubes containing the three highest dilutions of sample showing growth onto TCBS (3 %).
4. Incubate TCBS plates at 35 ± 2 °C overnight. *V. parahaemolyticus* appear as round, opaque, green or

bluish colonies, 2 to 5 mm in diameter on TCBS agar. Interfering, competitive *V. alginolyticus* colonies are, large, opaque, and yellow.

B. Screening and Confirmation

1. Biochemical identification of isolates. Unless otherwise specified, all media in this section are prepared to contain 2 % or 3 % NaCl. The API 20E diagnostic strip can be alternatively used here. Prepare a cell suspension of the suspect cultures in 2 % NaCl for the API 20E.

Triple sugar Iron agar with 3% salt (TSI+N_3)

Streak the slant, stab the butt, and incubate overnight at 35 ± 2 °C. *V. parahaemolyticus* produce an alkaline (Red) slant and an acid (yellow) butt, but no H_2S production.

Screen suspect cultures of *V. parahaemolyticus*, using TSI+N3, and T_1N_0 and T_1N_3. Incubate tubes at 35 ± 2 °C for 18 - 24 h.

a. Transfer two or more suspicious colonies from TCBS agar with a needle to arginine glucose slant (AGS). Streak the slant, stab the butt, and incubate overnight at 35 ± 2°C. *V. parahaemolyticus* produce an alkaline (purple) slant and

an acid (yellow) butt (arginine dihydrolase negative), but no gas or H_2S in AGS.

b. For TSB and TSA slants (supplemented with 2 %NaCl),inoculate both media and incubate overnight at 35±2° C.These cultures provide inocula for other tests as well as material for the Gram stain and for microscopic examination. *V. parahaemolyticus* are oxidase positive, Gram-negative, pleomorphic organisms exhibiting curved or straight rods with polar flagella.

c. Inoculate a tube of motility test medium by stabbing the column of the medium to a depth of approximately 5 cm. Incubate overnight at 35 ± 2 °C. A circular outgrowth from the line of stab constitutes a positive test. *V. parahaemolyticus* are motile.

d. *V. parahaemolyticus* will only grow in T_1N_3 but not in T_1N_0. Only the salt-requiring cultures need to be tested further.

Only motile, Gram-negative rods that produce an acid butt and an alkaline slant on AGS, do not form H_2S or gas, and are salt-requiring require further examination.

e. The identifying characteristics of *V. parahaemolyticus* are presented in Table. Biochemically, *V. parahaemolyticus*

and *V. vulnificus* are phenotypically similar, but can be differentiated by differences of the ONPG, salt-tolerance, cellobiose and lactose reactions (Table). By using selected biochemical traits, *V. parahaemolyticus* and *V. vulnificus* can be distinguished from most interfering marine vibrios and other marine microorganisms.

All *V. parahaemolyticus* isolates should be tested for the presence of urease, by either using urea broth supplemented with 2 % NaCl or on Christensen's urea agar supplemented with NaCl, 2 % final concentration or using the API 20E. Urease production is correlated with the presence of the *tdh* and/or *trh* genes. The urease reaction is a valuable screening test for potentially pathogenic strains.

Inoculate urea broth - 3 % NaCl with a heavy inoculum of culture or spot the culture to surface of Christensen's-urea-NaCl agar plate or slant. Incubate 35 ±2°C 18-24 h.

Production of urease is determined by a pink (alkaline) color to the medium.

Negative cultures should be incubated an additional 24 h for the rare, slow urease producing strains.

Biochemical identification of *Vibrio parahaemolyticus*

	Test/reaction		*Vibrio parahaemolyticus*
1.	TCBS agar		Green/bluish green/3-5 mm colonies with green blue centre
2.	TSIA & KIA		Alkaline (red) slant and acid (yellow) butt. No H_2S
3.	AGS		Alkaline (red) slant and acid (yellow) butt. No H_2S
4.	Gram stain and motility		Gram negative short or curved rods, motile
5.	Catalase		+
6.	Oxidase		+
7.	Arginine dihydrolase		−
8.	Ornithine decarboxylase		+
9.	Lysine decarboxylase		+
10.	Growth in (w/v):	0 % NaCl	−
11.		3 % NaCl	+
12.		6 % NaCl	+
13.		8 % NaCl	+
14.		10 % NaCl	−
15.	Growth at 42°C		+
16.	Acid from:	Sucrose	−

17.		D-Cellobiose	V
18.		Lactose	−
19.		Arabinose	+
20.		D-Mannose	+
21.		D-Mannitol	+
22.		ONPG	−
23.		Voges-Proskauer	−
24.	Sensitivity to:	10 µg O/129	R
25.		150 µg O/129	S
26.		Gelatinase	+
27.		Urease	V

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Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

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Isolation and Identification of *Listeria monocytogenes* from Seafood

Renuka. V

Listeria monocytogenes is a gram-positive, catalase positive, motile, facultative anaerobic bacteria with psychrotrophic characteristics. It is the causative agent of listeriosis, a severe disease with high hospitalization and fatality rates. It has ability to form a biofilm in seafood contact surfaces of seafood establishments. The genus Listeria contains 6 species as follows

- *Listeria monocytogenes*
- *Listeria ivanovii*
- *Listeria innocua*
- *Listeria welshimeri*
- *Listeria seeligeri*
- *Listeria grayi*

Sample treatment:

Fresh seafood samples should be stored at 4 ^{o}C. However, frozen samples should not be thawed until analysis.

Pre-enrichment & enrichment

25 gram of seafood sample is blended or stomached in 225 ml of BLEB broth (Buffered Listeria enrichment broth) and incubated at 30 ^{o}C for 4 h. Aseptically, 10 mg/L acriflavin, 40

mg/L cycloheximide and 50 mg/L sodium nalidixic acid are added in the BLEB as selective agent and continue incubating for a total time of 48 h.

Isolation procedure

BLEB enrichments are streaked into by 2 different base agars

- Esculin based *Listeria* selective agar
- Chromogenic based agar

Esculin based selective agar

1. *Oxford agar (OXA)/Modified Oxford Agar (MOX)*

BLEB broth samples are streaked in OXA agar/MOX agar and incubated for 24 h at 35 ^{o}C. Colonies are approximately 1mm diameter gray to black colonies surrounded by a black halo. After 48 h of incubation, the colonies are approximately 2 – 3 mm diameter, black with a black halo and sunken center.

2. *LPM fortified with Esculin and $Fe3^{+}$*

Appearance of Listeria species colonies are the same as for Oxford agar except that the incubation temperature is 30^{o}C for 24 h.

3. PALCAM agar

Incubation conditions and appearance of Listeria species colonies are the same as for Oxford agar except that the background plate colour is red.

Chromogenic *based* agars

1. **R&F *Listeria monocytogenes* Chromogenic Plating Medium (R&F LMCPM)**

After 24 h of incubation at 35 ºC, *L. monocytogenes* and *L. ivanovii* produce a 1 - 3 mm diameter, smooth, convex, blue/green colony and small blue/green halo. All other *Listeria* species produce a 1 - 2 mm, smooth, convex white colony with no halo.

2. RAPID *L. mono* agar

After 24 h of Incubation at 37 ºC, *L. monocytogenes* and *L. ivanovii* produce a 1 - 3 mm diameter, smooth, convex, blue/green colony. Typical colonies appear dark blue/green in the red background of RAPID *L. mono* agar.

3. Agar *Listeria* according to Ottaviani and Agosti (ALOA) or Oxoid Chromogenic *Listeria* agar (OCLA)

Incubate plates at 37 °C, after 24 h all *Listeria* species appear as 1 - 3 mm diameter blue/green colonies. Additionally, *L. monocytogenes* and *L. ivanovii* have an opaque white halo surrounding the colony.

4. CHROMagar *Listeria*

Incubation conditions and appearance of *Listeri*a colonies are the same as for ALOA except that the background plate color is light blue.

In **R&F LMCPM and RAPID *L. monocytogenes* agars,** the chromogen agar based methods, *L. monocytogenes* and *L. ivanovii,* will appear blue-green due to the phosphatidylinositol-specific phospholipase C (PI-PLC) activity and all other Listeria species will not develop the blue-green color and remain white in appearance.

In case of **ALOA and CHROM agar,** all the species will appear as blue-green colonies. *L. monocytogenes* and *L. ivanovii* is determined by blue-green colonies with the additional opaque white halo surrounding the colony.

Identification procedure

1. 5 typical colonies from each esculin based agar may be selected and streak to Trypticase soy agar with 0.6% yeast extract (TSAYE) and incubate the plates at 30 ºC for 24 - 48 h. Typical colonies are 1 - 3 mm diameter smooth convex white colonies.
2. The isolated colonies from esculin based agar or Chromogenic based agars are stabbed to 5 % sheep blood agar plate and Incubated at 35 °C for 24 - 48 h.

Haemolysis test

L. monocytogenes is beta haemolytic. Inoculate the isolated colonies on the surface of 5 % sheep blood agar by stabbing

plates. Draw grid of 20 - 25 spaces on plate bottom. Stab one culture per grid space. The stabbing should be as near as to the bottom of the agar layer without touching bottom of agar layer and fracturing of agar. Incubate the plates for 24 – 48 h at 35 ^{o}C. *L. monocytogenes* and *L. seeligeri* produce a slightly cleared zone around the stab. *L. innocua* shows no zone of hemolysis, whereas *L. ivanovii* produces a well-defined clear zone around the stab. If mixed culture was observed on the TSAYE plate repeat the hemolysis test with an isolated colony.

Christie-Atkins-Munch-Peterson (CAMP) test

CAMP test is performed by using 5 % sheep blood agar. The strain of *Staphylococcus aureus* (FDA strain ATCC 49444 or ATCC 25923) and *Rhodococcus equi* (ATCC 6939) are streaked vertically on the sheep blood agar

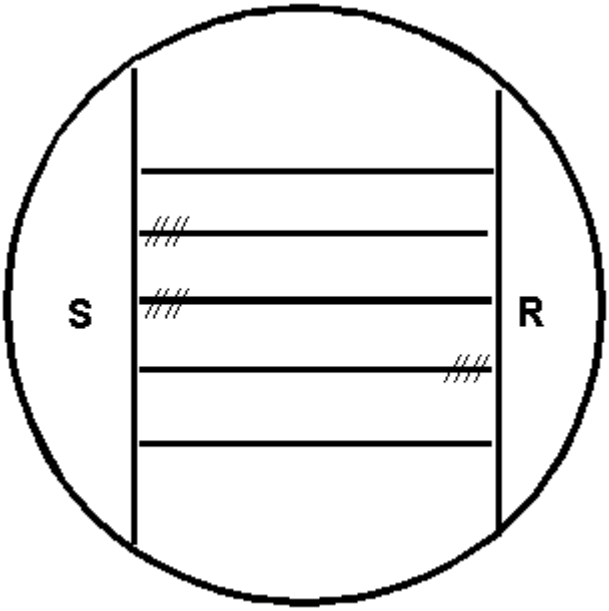

plate. The test strains of *Listeria* sp are streaked horizontally between the *S. aureus* and *R. equi* streaks without quite touching them. (Figure shows the arrangement of the culture streaks on a CAMP plate). Incubate the CAMP plate 24 to 48 h at 35 °C. Hemolysis of *L. monocytogenes* and *L. seeligeri* is enhanced near the *S. aureus* streak; *L. ivanovii* hemolysis is enhanced near the *R. equi* streak.

Biochemical tests for *Listeria*

Gram staining	Gram positive short rods
Motility	Motile
Methyl Red test	Positive
Voges Proskauer test	Positive
Urease test	Negative
Catalase test	Positive (O_2 gas evolution is slow, so observe under microscope)
Cytochrome oxidase test	Negative
Esculin hydrolysis	Positive
Acid from glucose	Positive (Acid only. No gas produced)
Acid from salicin	Positive

Motility Test

Inoculate into BHI broth and incubate at 28 ± 2 °C. Take a drop of culture on a microscopic slide and observe under high power (40X) in dark field of a microscope. Listeria show tumbling motility.

Alternatively, stab the presumptive Listeria culture into the umbrella motility medium and incubate at 25-30 ˚C for 36 - 48 h. Listeria shows an umbrella like growth pattern.

Tests for confirmation of *Listeria monocytogenes*

Nitrate reduction	Negative
Acid from D-mannitol	Negative
Acid from L-Rhamnose	Positive
Acid from D-xylose	Negative
Acid from alpha-methyl-D-manno-pyranoside	Positive
Beta-haemolysis on blood agar	Positive

Differentiation of *Listeria* species

Species	**Mannitol**	**Rhamnose**	**Xylose**	**Virulence**	**β-Hemolysis**	**Hemolysis enhancement with *Staphylococcus aureus*(S)**	**Hemolysis enhancement with *Rhodococcus equi* (R)**
L. monocytogenes	-	+	-	+	+	+	-
L. ivanovii	-	-	+	+	+	-	+
L. innocua	-	V	-	-	-	-	-
L. welshimeri	-	V	+	-	-	-	-
L. seeligeri	-	-	+	-	+	+	-
L. grayi	+	V	-	-	-		

V - Variable reaction

Microbiological media

Buffered Listeria Enrichment Broth (BLEB)

Trypticase soy broth	30 g
Yeast extract	6 g
Monopotassium phosphate (anhydrous)	1.35 g/liter
Disodium phosphate (anhydrous)	9.6 g/liter
Sodium Pyruvate (Sodium salt)	1.11 g/liter
Distilled water	1 liter
Final pH	7.3 ± 0.1

[**Note**: Optionally a filter-sterilized 10% (w/v) sodium pyruvate solution may be added after autoclaving (11.1 ml/L)]

Oxford agar (OXA)

Columbia blood agar base	39.0 g
Esculin	1.0 g
Ferric ammonium citrate	0.5 g
Lithium chloride	15.0 g
Cycloheximide	0.4 g
Colistin sulphate	0.02 g
Acriflavin	0.005 g
Cefotetan	0.002 g
Fosfomycin	0.010 g
Distilled water	1 liter

Modified Oxford Agar (MOX agar)

Columbia Blood Agar Base (brand dependent)	39.0-44.0 g
Agar	2.0 g
Esculin	1.0 g
Ferric ammonium citrate	0.5 g
Lithium chloride (Sigma L0505 quality or equivalent)	15.0 g
Buffered colistin methane sulfonate (1 % w/v) solution	1.0 ml
Distilled water	1.0 L
pH	7.2±0.1

LPM fortified with Esculin and $Fe3^{+}$

Phenylethanol agar (Difco)	35.5 g
Glycine anhydride (NOTE: not glycine)	10 g
Lithium chloride	5 g
Moxalactam stock solution,1% in phosphate buffer, pH 6.0	2 ml
Distilled water	1 liter
Esculin	1.0g
Ferric ammonium citrate	0.5g

PALCAM agar

Peptone	23 g
Starch	1 g

NaCl	5 g
Columbia agar	13 g
Mannitol	10 g
Ferric ammonium citrate	0.5 g
Esculin (aesculin)	0.8 g
Dextrose (glucose)	0.5 g
Lithium chloride	15.0 g
Phenol red	0.08 g
Distilled water	1000 ml
pH	7.2 ± 0.1

Selective agents

Polymyxin B sulphate	10 mg
Acriflavin	5 mg
Ceftazidine	20 mg
Distilled water	2 ml

R&F *Listeria monocytogenes* Chromogenic Plating Medium (R&F LMCPM) – available commercially.

RAPID L. mono agar

Peptones	30 g
Meat extract	5 g
Yeast extract	1 g
Lithium chloride	9 g

Selective supplements	20 ml
D-Xylose	10 g
Phenol red	0.12 g
Agar Base	13 g
Chromogenic substrate	1 ml
Water, distilled	1000 ml
pH	7.3 ± 0.1

Store at 2 - 8 °C in dark in original packaging. Shelf life is 4 months under these conditions.

Agar *Listeria* according to Ottaviani and Agosti (ALOA) or Oxoid Chromogenic *Listeria* agar (OCLA)

Meat peptone	18 g
Tryptone	6 g
Yeast extract	10 g
Sodium pyruvate	2 g
Glucose	2 g
Magnesium glycerophosphate	1 g
Magnesium sulphate	0.5 g
Sodium chloride	5 g
Lithium chloride	10 g
Disodium hydrogen phosphate anhydrous	2.5 g

5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside	0.05 g
Agar, according to gelation-strength	12 g to 18 g
Water, according to volume of fungistat supplement	925-930 ml
pH	7.2 ± 0.2

Trypticase Soy Agar with 0.6% Yeast Extract (TSAYE)

Trypticase soy agar	40 g
Yeast extract	6 g
Distilled water	1 liter
pH	7.3 ± 0.2

Reference

Hitchins, A.D., Jinneman, K and Chen, Y. (2017). Detection of *Listeria monocytogenes* in Foods and Environmental Samples, and Enumeration of *Listeria monocytogenes* in Foods Bacteriological Analytical Manual. Chapter 10.

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Enumeration of Yeasts and Molds

Toms C. Joseph

The fungus that can grow as multicellular filaments (hyphae) are called molds while yeasts are fungi that are single-celled. Majority of molds and yeasts are aerobic and they can grow in pH between 2 and 9. Molds can grow in foods that has low moisture contents like dry fish (water activity (a_w) of 0.85 or less), while yeasts require higher water activity for growth. The growth of yeasts and molds can result in reduction in quality and can result in severe economic loss to the processors and consumers. The growth of mold on fish will be manifested as discoloration of various sizes and colors, white cotton like or colored mycelium. Fish or fishery product may appear to be free of fungi, but can be established only after mycological examination.

Many of the foodborne molds and yeasts, may also be harmful to human or animal health because many of them have the capability to produce toxic metabolites known as mycotoxins. Even though the organisms that produce the toxin may be killed by food processing or during cooking, most of the mycotoxins are stable compounds that are not destroyed by heat. Some of the foodborne molds and yeasts may cause allergic reactions. Most of the fungi are non-infectious, but some of the

species can cause infection in aged population, HIV-infected persons and persons receiving chemotherapy.

Antibiotics are usually added to mycological media (media used for the growth of molds and yeasts) to inhibit bacterial growth. Chloramphenicol is the most commonly used antibiotic since it is stable even under autoclave conditions. The recommended concentration of this antibiotic is 100 mg/liter medium. Prepare stock solution by dissolving 0.1 g chloramphenicol in 40 ml distilled water. Add this solution to 960 ml medium mixture before autoclaving.

Sampling and preparation of sample homogenate

- Aseptically weigh 50 g of fish into a sterile sample dish.
- Transfer 50 g of sample to a stomacher bag and add 450 ml of sterile diluent (0.1 % peptone water) and blend for 2 min in stomacher. This results in a dilution of 10^{-1}. Alternatively, mortar and pestle can be used for homogenising the sample.
- Using separate sterile pipette, transfer 10 ml from the above 10^{-1} dilution to 90 ml of sterile diluent and mix well. This gives 10^{-2} dilution.
- Then transfer 10 ml from the above 10^{-2} dilution to 90 ml of sterile diluent and mix well. This gives 10^{-3} dilution.
- Similarly prepare further dilutions (10^{-4}, 10^{-5}, 10^{-5} etc.) depending on the microbial load of the sample.

Plating and incubation of sample

Spread-plate method

Pipet 0.1 ml of each dilution in triplicate aseptically on DRBC agar plates and the inoculum may be spread with a sterile, bent glass rod. When the water activity of the analyzed sample is less than 0.95, DG18 media is preferred.

Pour-plate method

One ml of each dilution is pipetted on sterile petri plates and pour 20 - 25 ml of boiled and cooled DG18 agar at 45 $^{\circ}$C. Mix contents by gently swirling plates clockwise, then counterclockwise, taking care to avoid spillage on dish lid. Each dilution may be plated in triplicate.

For enumeration of yeast and mold, spread plating is considered to be better than the pour plate method. The fungal colonies on the surface grow faster than the colonies beneath the surface in pour plating and thus the colonies below the surface may not be clearly visible. DRBC agar should be used for spread plates only.

Incubate plates in the dark at 25 $^{\circ}$C. Do not invert the plates and do not disturb the plates till counting.

Counting of plates

The colonies on plates may be counted after 5 days of incubation. Re-incubate for another 48 h, if there is no growth at 5 days. Counting of colonies before the incubation period may result in secondary growth from spores that are dislodged and thus the final count may become invalid. Plates with 10 -150 colonies may be counted. Average count of the triplicate plates may be taken and the results may be reported in colony forming units CFU/g. The Mold and yeast counts may be reported as less than 1 times the lowest dilution, when plates from all dilutions have no colonies.

Media

Dichloran 18% glycerol (DG18) agar

Reagent	**Quantity**
Glucose	10.0 g
Peptone	5.0 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Dichloran (2,6-dichloro-4-nitroaniline) solution (0.2% (w/v) in ethanol)	1.0 ml
Chloramphenicol	0.1 g
Agar	15.0 g
Distilled water	800 ml

All the above items are mixed and heated in a boiling water bath to dissolve agar. Then make up volume to 1000 ml with distilled water. Sterilize by autoclaving at 121°C for 15 min after adding 220 g glycerol (analytical reagent grade). Cool medium to 45 °C and pour plates under aseptic conditions. The final a_w of this medium is 0.955.

DG18 agar is used for enumeration of mold and is preferred when the a_w of the analyzed food is less than 0.95. The low water activity of the medium will reduce the interference by bacteria and fast-growing fungi. When both yeasts and molds have to be enumerated, DRBC agar should be used.

Dichloran rose bengal chloramphenicol (DRBC) agar

Reagent	**Quantity**
Glucose	10.0 g
Bacteriological peptone	5.0 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Rose bengal (5% aqueous soln., w/v)	0.5 ml
Dichloran (0.2% in ethanol, w/v)	1.0 ml
Chloramphenicol	0.1 g
Agar	15.0 g
Distilled water	1.0 liter
pH= 5.6	

Mix ingredients, heat to dissolve agar and sterilize by autoclaving at 121 °C for 15 min. Cool to 45 °C in a water bath and pour plates.

The presence of dichloran and rose bengal in DRBC agar slow down the growth of fast-growing fungi and hence it is especially useful for analyzing samples containing "spreader" molds (e.g. *Mucor*, *Rhizopus*, etc.), since the added effectively, thus readily allowing detection of other yeast and mold propagules, which have lower growth rates.

Media containing rose bengal are **light-sensitive**; relatively short exposure to light will result in the formation of inhibitory compounds. Keep these media in a dark, cool place until used. DRBC agar should be used for spread plates only.

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Biochemical Tests for Bacterial Identification

Anupama T.K

Biochemical tests are most important methods for identification of bacteria. The commonly used biochemical tests are mentioned below.

1. Carbohydrate fermentation test

Carbohydrate fermentation test is used to determine whether bacteria can utilise a specific carbohydrate. The patterns of carbohydrate fermentation are useful for differentiating bacterial groups or species

- Inoculate one tube each of carbohydrate media (containing specific sugars such as glucose, fructose, maltose, lactose, sucrose etc.) with wire loop.
- Make sure inoculum reaches bottom of tube.
- Incubate 24-48 h at 35-37 °C.
- Acid production is indicated by change in colour and gas production can be detected by the formation of gas bubble in inverted Durham's tube.
- Run controls simultaneously (positive and negative cultures).

2. **Catalase test**

Catalase test is done to test the ability of microorganism to produce catalase enzyme.

- Place a loop of young culture on clean glass slide or spot plate using a wire loop.
- Add 2-3 drops of 30% hydrogen peroxide on the culture. Evolution of gas from the culture indicates positive reaction.

3. **Citrate test**

Citrate utlilization test is used to determine the ability of bacteria to utilize sodium citrate as the only carbon source.

- Inoculate Simmons citrate agar lightly on the slant by touching the tip of a needle to a colony that is 18 to 24 hours old.
- Incubate at 35 °C for 18 to 24 h. Some organisms may require up to 7 days of incubation due to their limited rate of growth on citrate medium.
- Observe the development of blue color, denoting alkalinization.

4. **Coagulase test**

Coagulase test is used to determine the production of coagulase enzyme by bacteria. *Staphylococcus aureus* produces coagulase while Coagulase Negative

Staphylococcus (CONS) does not produce. Coagulase enzyme produced by *S. aureus* converts soluble fibrinogen in plasma to insoluble fibrin.

- Transfer suspect *S. aureus* colonies into small tubes containing 0.2-0.3 ml BHI (brain heart infusion) broth and emulsify thoroughly.
- Inoculate agar slant of suitable maintenance medium, e.g., TSA (trypticase soy agar), with loopful of BHI suspension.
- Incubate BHI culture suspension and slants 18-24 h at 35-37 °C.
- Add 0.5 ml reconstituted coagulase plasma with EDTA to the BHI culture and mix thoroughly.
- Incubate at 35-37 °C and examine periodically over 6 h period for clot formation.
- Only firm and complete clot that stays in place when tube is tilted or inverted is considered positive for *S. aureus*.
- Test known positive and negative cultures simultaneously with suspect cultures of unknown coagulase activity.

5. **Decarboxylase tests**

Decarboxylase test is used to measure the ability of decarboxylase enzyme in an organism to decarboxylate (or hydrolyze) an amino acid to form an amine. Decarboxylation or hydrolysis, of the amino acid results in an alkaline pH. The increased pH of the medium is detected by color change of the pH indicators bromcresol purple and cresol red present which results in a color change from orange to purple.

- Inoculate decarboxylase medium (containing lysine, arginine or ornithine) with young culture from TSA slants.
- Add sterile liquid paraffin (approx.1cm height) and incubate at 35 ± 2 ºC.
- Examine daily for four days.
- The medium first becomes yellow because of acid production. Later if decarboxylation occurs, the medium changes to alkaline (purple).
- The control tubes remain acid (yellow) throughout the period.

6. **Hydrogen Sulphide production**

Hydrogen sulphide (H_2S) production test is used to detect the production of hydrogen sulphide (H_2S) gas by an organism and is mainly used in the identification of members of family

Enterobacteriaceae. H_2S is produced by certain bacteria through reduction of sulphur containing amino acids like cystine, methionine or through the reduction of inorganic sulphur compounds such as thiosulfates, sulfates or sulfites. The hydrogen sulphide production is detected by incorporating a heavy metal salt containing iron or lead as H_2S indicator to a nutrient culture medium containing cystine and sodium thiosulfates as the sulfur substrates. The Hydrogen sulphide produced by the microorganism reacts with the metal salt forming visible insoluble black precipitate of ferrous sulphide.

- Inoculate young culture on TSI (Triple Sugar Iron) agar by stabbing the butt and streaking the slope.
- Incubate for 24 - 48 h at 35 ± 2 ºC.
- Observe the tubes for blackening along the line of stab inoculation.

7. Indole production

Indole test is used to determine the ability of an organism to split amino acid tryptophan to form indole. Tryptophan is hydrolysed by **tryptophanase** produced by the bacteria to produce indole. Indole production is detected by Kovac's or Ehrlich's reagent which contains ***p* -**

Dimethylaminobenzaldehyde, which reacts with indole to produce a red coloured compound.

- Inoculate suspected culture into tryptone broth and incubate 24 ± 2 h at 35 ± 0.5 °C.
- Test for indole by adding 0.2 - 0.3 ml of Kovacs' reagent.
- Appearance of distinct red color in upper layer is positive test.

8. Voges-Proskauer (VP) test

The Voges-Proskauer (VP) test is used to determine the production of **acetylmethyl carbinol** by bacteria from glucose fermentation. The a**cetylmethyl carbinol, if present,** is converted to **diacetyl** in the presence of α-**naphthol,** strong alkali (**40 % KOH**), and atmospheric oxygen.

- Inoculate suspected culture into MR-VP broth and incubate 48 ± 2 h at 35 ± 0.5 °C.
- Transfer 1 ml of the incubated culture to 13 × 100 mm tube.
- Add 0.6 ml α-naphthol solution and 0.2 ml 40 % KOH, and shake. Add a few crystals of creatine.

- Shake and let stand 2 h. Test is positive if eosin pink color develops.

9. Methyl red test

Methyl Red (MR) test determines the ability of bacteria to produce stable acid end products (lactate, acetate, succinate and formate) from glucose. The acid causes the medium to acquire acidic pH. Methyl Red is used as the pH indicator which is red in color at a pH of 4.4 or less.

- After VP test, incubate MR-VP tube additional 48 ± 2 h at 35 ± 0.5 °C.
- Add 5 drops of methyl red solution to each tube.
- Distinct red color is positive test.
- Yellow is negative reaction.

10. Oxidase (Cytochrome oxidase) test (Kovac's method)

Kovac's cytochrome oxidase reagent:

Oxidase test is used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. C**ytochrome c oxidase when present in bacteria** oxidizes the reagent **(Tetramethyl-p-phenylenediamine)** to **indophenols** which is purple. When the enzyme is not present, the reagent remains reduced and is colorless.

N,N,N',N'-Tetra-methyl-*p*-phenylenediamine
dihydrochloride - 0.25 g
Distilled water - 25 ml
Store at refrigerator and fresh solution has to made before use.
Scrape some of the young culture with a glass rod or platinum loop and rub on the test paper (a piece of filter paper already impregnated with Kovac's cytochrome oxidase reagent).
Development of a blue colour within 30-60 s indicates a positive test.

12. Urease test

The urease test is used to determine the ability of an organism to produce urease. The urease produced by bacteria will result in hydrolysis of **urea** and produces **ammonia** and **carbon dioxide**. The **ammonia produced** alkalinizes the medium, and the pH of the media changes from **light orange** at pH 6.8 to **magenta (pink)** at pH 8.1.

- With sterile needle, inoculate growth from TSI (triple sugar iron) slant culture into tubes of urea broth.
- Incubate 24 ± 2 h at 35 °C.

- If urease is present, the urea is spilt to ammonia, which changes the colour of the indicator from yellow to pink.

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Microbiology of Seafood Spoilage

Remya. S

Introduction

- Fish is a highly perishable food, which starts spoiling at the moment they are taken out of water.
- As soon as fish dies, a series of changes starts to take place, which is collectively known as spoilage.
- The degradation of the tissue is brought about both by indigenous fish enzymes and by microorganisms, which are present on the surface of the skin, on the gills and in the intestine.
- Spoilage reduces the shelf life/keeping quality of fish

How is 'freshness' of fish judged?

Characteristic	Description
Eyes	Bright and bulging, not sunken or cloudy
Gills	Bright red or pink
Skin	Bright
Slime	May be present on skin or gills. Should be clear or colourless
Odour	Gill odour should be sharp and seaweedy

Spoilage of fish/How fish goes bad?

- Microbial spoilage

- Enzymatic spoilage/Self-digestion by enzymes (Autolytic changes)
- Chemical spoilage (Oxidation & hydrolysis)

Microbial spoilage of fish

- Not all bacteria growing on fish will lead to the production of objectionable characteristics. A minority of the bacteria species are often associated with majority of the spoilage, often termed the ***Specific spoilage organisms (SSOs).***
- Microbial spoilage of fish is usually described as a proteolytic process, with the exception of Molluscans, therefore pH increases.

Where do bacteria come from?

- Bacteria are found practically everywhere in nature, including the skin, the gills and in the gut of the fish.
- The newly caught healthy fish have sterile tissues and bacteria can only be found on the skin, gills and in the intestines.
- When the fish is alive, its natural defence mechanisms prevent the invasion of these bacteria into the fish tissues. So, they feed, grow and multiply on the surface of the fish without causing any damage. In fact, many of these are

useful to the fish. For example, bacteria in the gut help the fish to breakdown its food.

- The fish and bacteria exist in a state of equilibrium, whilst still alive and it is only after death that the bacteria can invade the tissues and spoil the fish.
- Invasion of the muscle from the gut is made easier by the autolysis brought about by the gut enzymes.
- The number of bacteria in the gut is highest, when the fish has recently been feeding.
- Fish caught from polluted waters will be more contaminated than fish from clean waters.
- Once the fish is caught, it will be contaminated to some extent by all the materials with which it comes into contact like ice, fish boxes, the boat itself and even the crew.

What do bacteria do?

- The bacteria grow using the fish as a food source and produce various waste products, which accumulate and produce off-odours and bad flavours.
- In a quest for nutrients, bacteria make use of the simplest compounds first and intact proteins may only be used, when they have been broken down by autolytic enzymes.

- As they increase in numbers, they produce a thick slime on the skin and gills of the fish.
- Unpleasant odours are also produced, often with a strong smell of ammonia.
- The flesh becomes softened and in un-gutted fish, the gut wall eventually bursts.
- This process of breakdown of dead tissues by bacteria is known as putrefaction.

Action of bacteria on the chemical components of fish:

1. **Degradation of amino acids**: Decarboxylation and Deamination
   - Amino acids contain carboxyl and amino group

   Decarboxylation: Removal of carboxyl group from amino acids using the enzyme amino acid decarboxylase producing a primary amine and carbon dioxide.

   - Results in the accumulation of primary amines in the putrefying fish flesh.
   - Example: Conversion of amino acid Histidine into histamine, lysine into cadaverine.
   - **Scombroid fish poisoning/Histamine poisoning**: Scombroid fishes like tuna, mackerel etc. have high contents of histidine in their muscle. Histidine decarboxylase enzyme

acts on histidine and produces histamine, which causes food poisoning.

Deamination: Removal of amino group from amino acids. Ammonia is the product of deamination of amino acids. This causes strong ammoniacal odour in spoiled fish.

2. **Reduction of Trimethylamine Oxide (TMAO)**
    - Fresh bony marine fish contains 0.1 % - 0.5 % TMAO in their muscles
    - Bacteria reduces Trimethylamine Oxide (TMAO) to Trimethylamine (TMA)
    - TMA contributes to the typical smell of marine fish
3. **Action on Urea**
    - Urea in the flesh of elasmobranchs is converted to ammonia by bacteria
4. **Microbial rancidification of fat**
    - Microbial enzyme lipoxidase acts on lipid/fat and results in formation of aldehydes and ketones

Methods of prevention/control of fish spoilage

1. **Lowering temperature of the food**
    - The control of spoilage by reduction of temperature offers the most common and practical way of keeping fish fresh.
    - Example: Icing (Chilling) and Freezing

- The lower the temperature, the longer the fish will take to spoil.

Chilling/Icing (temperature near 0 degree Celsius (0-2 ^{o}C), but not below 0):

- Fishes are held at a temperature as close to 0 °C as possible (but not below).
- At low temperature, the enzymes in the cell cannot function at their optima and since the metabolism of the whole cell relies on enzymes, the cells are slow to grow and divide.
- At chilling temperature, many bacteria become inactive, but psychrophiles/psychrotrophs may grow.
- The effectiveness of a particular temperature in preserving a food will depend on a number of factors, including;
    1. What proportion of the flora is psychrotrophic
    2. The growth rate of the organisms at the given temperature
    3. The previous treatment given to the fish
- Shelf life of chilled stored fish is 5 to 15 days
- Chilling is regarded as a short-term storage method. However, it can increase the storage life of some fish by between 14-21 days.

Freezing (temperature below 0 degree Celsius (0 to -20 ^{o}C):

- Long term method of preserving fish
- Freezing is achieved by

  Quick freezing: where temperature is lowered to -20 ^{o}C within 30 min.

  Slow freezing: where temperature is lowered to -20 ^{o}C within 3~72 hours.
- Freezing converts water into ice crystals and making water not available for bacterial action
- In most cases, growth is completely stopped and the change in state of the water may well kill a large proportion of the cells.
- Death can be attributed to many factors including mechanical damage, dehydration, concentration in cellular solution, cold shock and metabolic injury.
- Shelf life of frozen stored fish is 6 to 8 months

2. Lowering water activity/moisture content of the food

- **Drying**: Removing a considerable portion of water from fish by evaporation
- **Salting**: Reduces the availability of water for biological functions of the bacteria
- All the reactions which take place in the cell require an aqueous environment for their proper functioning. Thus,

reducing the amount of available water in the foodstuff will bring about a slowing, or complete cessation, of bacterial growth.

- Water content is usually recorded as percentage moisture but, in bacterial terms, it is the free water which is important.
- Microbiologists measure water content as **water activity** (a_w), which is derived from the following formula:

  Equilibrium relative humidity = 100 a_w
- Here is a table showing the minimum aw at which different groups of microorganisms can grow:

Organisms	**a_w**
Most spoilage bacteria	0.91
Most spoilage yeasts	0.88
Most spoilage moulds	0.80
Halophilic bacteria	0.75
Xerophilic moulds	0.65
Osmophilic moulds	0.60

- The water activity of a food can be lowered by removal of water or the addition of a solute, which makes the water no longer available to the cells.
- Sodium chloride is such a solute; the a_w obtained for different concentrations of salt are given in the table below:

Per cent salt w/v	**a_w**
0.9	0.995
1.7	0.99
3.5	0.98
7.0	0.96
10.0	0.94
13.0	0.92
16.0	0.90
19.0	0.88
22.0	0.86

- It is obvious that although a 22 per cent salt solution is too salty for the average palate, it still does not give complete control of spoilage organisms, especially moulds and halophilic bacteria.
- In order to provide the best protection to the food, it is usual to remove some of the water and add salt.
- The removal of water can be by the direct application of heat but a more interesting technique is the **smoking of foods**.

3. Heat processing/Thermal Processing

Canning:

- Bacteria is destroyed or the number of bacteria is reduced in food by heat treatment.
- This process kills all viable pathogenic and spoilage organisms.
- Reduction in the number of bacteria slows down the development of spoilage flora and delays spoilage.
- Commercially sterile or commercial sterility is often used for canned foods to indicate the absence of viable microorganisms detectable by culture methods or the number of survivors is so low that they are of no significance under condition of canning and storage.
- Shelf life of canned fish is 2 years.

Pasteurisation:

- Partial destruction of bacteria by application of heat.
- Pasteurization refers to use of heat at the range of 60~80 ^{o}C for a few minutes for the elimination/ destruction of all disease-causing microorganisms and reduction of potential spoilage organisms.
- Pasteurization process which is commonly employed in milk preservation can be achieved by heating the

milk at 63 ^{o}C for 30 min, called low temperature long time (LTLT) process; or 72 ^{o}C for 15 sec, called high temperature short time (HTST) process

4. Shifting the pH of the environment

- The growth of bacteria can also be arrested by shifting the pH of the environment so that the cell's enzymes can't function at their optima.
- Examples: Pickles and Marinades
- Many spoilage organisms find the low pH so hostile that they die during storage.

  The following table shows the minimum and maximum pH that a few common organisms can survive.

Organism	**Minimum pH**	**Maximum pH**
Escherichia coli	4.4	9.0
Salmonella typhi	4.5	8.0
Streptococcus lactis	4.3-4.8	-
Lactobacillus spp.	3.8-4.4	7.2
Moulds	1.5-2.0	11.0
Yeasts	2.5	8.0-8.5

- It is obvious that since the pH of fish tissue is 5.6 or more, almost any microorganism can grow on and spoil it.
- Some bacteria, particularly *Lactobacillus* spp., have the ability to reduce the pH to a level where the normal spoilage

flora is inhibited, the usual mechanism being the production of lactic acid from the carbohydrate in the substrate or food. Many of the **traditional fermented foods** of South East Asia owe their long shelf life to such a mechanism as this.

5. Use of radiation

- Radiations have potential application in food preservation, because of their destructive effect on microorganisms.
- Generally, radiations of shorter wavelength are more damaging to microorganisms than long wavelengths.
- In food preservation ionizing radiations of wavelength of 2000 A^o or less are important.
- These include beta rays, gamma rays and X-rays.
- Ionizing radiations ionize molecules on their path and thus destroy microorganisms without raising temperature.
- Killing of microorganisms in foods using electromagnetic radiations without raising temperature is termed as **cold sterilization**.
- **Radiation process**: The radiation process given to food is of 3 types.
    - Radappertization
    - Radicidation
    - Radurization

6. **Use of chemicals**

- A large number of chemicals have potential as food preservatives because of their ability to prevent/ delay spoilage of foods caused by microorganisms and thus extend shelf life of food.
- Among several chemical substances only a few are permitted to be used in food products because of strict rules of safety by enforcement agencies and change in the antimicrobial property when incorporated to certain foods.
- Some of the chemicals having preservative effect are permitted to be used in foods and are generally recognized as safe (GRAS) are.
    - Benzoic acid and parabens
    - Sorbic acid and sorbates
    - Propionates
    - Sulphur dioxide and sulphites
    - Nitrites and nitrates

Good practices

- **Separate fish caught at different times** Fish caught at different times will be at a different state of spoilage. The older fish would therefore contaminate the freshest, if they were not separate.

- **Separate small fish from large fish** Small fish spoil faster than large fish.
- **Separate fish with 'soft bellies'** 'Soft belly' is a sign of spoilage. So, these fish will be spoiled more than the others. Also, there is a risk of contamination with gut, if the belly were to burst.
- **Separate gut and fish.** Of course, guts contain large quantities of the bacteria and enzymes which cause spoilage. So, they must be kept separate from the fish.
- Use clean, plastic boxes for distributing fish.
- Do not re-use surplus ice, left in fish boxes after distribution.
- Do not put fish directly on the ground.
- Do not mix different species of fish during distribution or storage.

Good Handling Guide for fish merchants and processors

- Do buy the best quality fish.
- Don't leave boxes of fish lying around on the market or on your premises. Remove them quickly and keep them cool.
- Do ensure all surfaces and equipment are kept clean and hygienic.
- Don't throw or tread on fish. Handle them gently.
- Do separate different types of fishes to avoid tainting.

- Don't use chill stores to cool down fish. They are not designed for this and ice does it better anyway.
- Do rotate stock properly, operating first in first out policy to ensure fish is moved quickly to the next distribution stage.
- Don't store waste and offal near fish working areas. This will encourage flies which can carry disease and introduce bugs onto the fish.
- Do ensure fish is adequately iced before despatch.
- Don't fill boxes so full that fish get crushed.

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Public Health Microbiology

Renuka. V

Bacteria that may cause illness in humans are considered as pathogenic bacteria. In fish higher microbial load is mainly found in gills, guts and skin. Most bacteria associated with fish are not pathogenic. Pathogens may be present at low levels when fish or shellfish are harvested, and others may be introduced during handling and processing or by unsanitary practices.

The food borne pathogens may arise from the aquatic environment, general environment or from animal/human reservoir (Table 1). During fish harvesting, the indigenous aquatic bacteria and the general environment bacteria may cause the food borne illness to the consumers. While, during handling and processing, bacteria from the general environment or from the animal/human reservoir may cause the food borne illness. Food borne illness are categorised into 2 different groups.

1. **Infection** (ingestion of live pathogenic organism)
2. **Intoxication** (ingestion of toxin produced by microorganism in food)

The infections may be due to bacteria, virus or parasite.

Table 1: Food borne pathogens and their environment

Aquatic environment	General environment	Animal/human reservoir
Clostridium botulinum Non-proteolytic types B, E, F	*Clostridium botulinum* proteolytic type A, B	*Salmonella enterica*
Vibrio cholerae serovar O1 and O139	*Clostridium perfringens Type A*	*Shigella spp.*
Vibrio parahaemolyticus	*Listeria monocytogenes*	*Pathogenic Escherichia coli (EPEC, ETEC, EAEC, EIEC)*
Vibrio vulnificus	*Bacillus cereus*	*Campylobacter spp.*
Aeromonas spp.		*Staphylococcus aureus*
Plesiomonas shigelloides		

1. Bacterial infections

a) *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive, motile bacteria. It causes a rare but life-threatening food borne disease called listeriosis.

b) *Vibrio* spp.

Vibrio spp. are gram-negative bacteria. Among the 80 species of *Vibrio* spp. 12 were reported as human pathogens.

Vibrio parahaemolyticus, V. *vulnificus* and V. *cholerae* are the major seafood borne illness species. Of these, *V. parahaemolyticus* and V. *cholerae* cause gastrointestinal disease, while V. *vulnificus* causes septicaemia.

c) *Salmonella*

Salmonella is a gram-negative bacteria. Human infections with Salmonella could lead to several clinical conditions such as typhoid fever (enteric fever), acute gastroenteritis or systemic non-typhoid infections. Typhoid fever is caused by S. *typhi* and S. *paratyphi.*

d) *Clostridium* spp.

Clostridium spp. is a Gram-positive, spore forming bacteria. It produces a neurotoxin. 3 groups of *C. botulinum* are recognized:

- Group I - proteolytic botulinum toxin types A, B and F
- Group II - non-proteolytic botulinum toxin types B, E and F
- Group III - botulinum toxin types C and D

e) *E. coli*

E. coli is a gram-negative bacteria. All *E. coli* are not pathogenic and only few are pathogenic *E. coli* is mainly associated with the clinical syndrome of gastrointestinal illness. Based on clinical syndromes and virulence properties,

diarrheagenic *E. coli* have been categorized as: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffuse-adhering *E. coli* (DAEC).

f) *Staphylococcus aureus*

Staphylococcus aureus is a gram-positive bacteria. It produces enterotoxin and causes food borne intoxication. Disease-causing levels of toxin occur only when extensive growth of S. *aureus* has occurred, typically at levels $\geq 10^6$ cfu/g in food.

g) *Aeromonas and Plesiomonas*

Aeromonads are Gram-negative facultative anaerobic bacteria. *Plesiomonas shigelloides* is associated with traveller's diarrhoea. Currently, there are 24 validly published species under the genus *Aeromonas* in which 11 are responsible for food borne illness like gastrointestinal disease.

2. Viral infections

Food-borne viruses are derived from the human gastrointestinal tract, and their presence in water and food is a result of contamination with sewage, poor hygiene or contamination by food handlers. The food borne illness viruses are mentioned in Table 2.

Table 2: Food borne viruses

Name of virus	Disease/symptoms
Norovirus	Epidemic gastroenteritis
Astrovirus	Gastroenteritis
Hepatitis A virus	Inflammation of liver; hepatitis
Enteroviruses (eg. poliovirus, coxsackie A, B)	Poliomyelitis, meningitis, Encephalitis
Rotavirus	Gastroenteritis
Adenovirus	Respiratory, eye and gastrointestinal infections

3. Parasite infections

Fish-borne zoonotic parasites are prevalent in many regions of the world and are among the most important of all zoonotic parasites infecting humans. Fish-borne parasites are primarily helminths, and include species of nematodes (round worms), cestodes (tapeworms) and trematodes (flukes).

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Impact of Handling, Processing, Storage and Logistics of Seafood on its quality

Renuka. V

Introduction

Spoilage of fish that can be detected organoleptically in fish is due to breakdown of protein, and also a result of formation of metabolites from the growth of microorganisms. The fish starts to spoil immediately after it is captured and seafood while raw is highly perishable. The freshness of fish should be maintained to get maximum value. The spoilage bacteria that is present on the skin of fish starts invading the muscle tissue. Over time, the colour, taste and smell of fish changes and the quality of fish deteriorates if not properly preserved and sooner or later it will become unsuitable for consumption.

Bacteria and enzymes are the major reasons for fish spoilage. Higher microbial load is mainly found in gills, guts and skin. Enzymes are present in the stomach of the fish. Keeping the fish at low temperature (0 $^{\circ}$C) can slow down the growth of spoilage bacteria and hygienic handling are the basic requirements of reducing seafood spoilage.

Harvesting practices to reduce the microbial quality deterioration

Icing

Icing is a simple, economic and effective method for preservation of fish. Different types of ice like block ice, flake

ice, tube ice and dry ice are used in India. Block ice is the commonly used ice used for preservation of fish in India. The block ice is crushed into small pieces with ice crushers for better contact with the fish surface. The following points are to be remembered to reduce the microbial quality deterioration of fish

- One kg of fish requires l kg of ice to keep the fish at low temperature (0 $^{\circ}$C) and for efficient preservation. However, the ratio may be of 1:3 to 1:4 if the the insulated containers were used for transportation
- Clean potable water should be used to produce ice
- Care should be taken to prevent physical damage to fish due to icing
- The boxes used for storage of fish should not be overfilled
- Ice and fish should be placed in alternative layers to increase the surface contact

On board handling practices

Size grading and species segregation and washing the catch plays a major role to reduce the microbial deterioration. Most of the fishing vessels in India are trawlers, gillnetters and long liners and fish hold is present in all the large fishing vessels. Freshly caught fishes are usually stored in plastic insulated containers in fish hold. It is very important to use ice in required quantities for proper storage of fish.

Poor personal hygiene is one of the common causes of microbial contamination. Fish is handled during catching,

handling, processing, transportation and selling. Adaptation of good hygienic practices reduces the risk of microbial contamination. Fish handlers should be aware about the quality regulation procedure and food safety issues. Fish handlers should understand the link between poor personal hygiene and contamination of fish with disease causing bacteria. They should also know the personal hygiene steps that can be taken to prevent fish from becoming contaminated.

Table 1: Process carried out in the fresh seafood chain

Adopted from Frederiksen (2002)

Step	**Process carried**
Fishing vessels for fresh fish catch	Wash, sort in species, size grade, weigh, icepack, store and unload
Collectors	Species grade, size grade, icing, store and bring to auction
Auction	Store and auction (sell)
Wholesalers/processors	Size grade, process, weigh, icepack, store and sell. There can be one or several steps of wholesalers/ processors in a fish supply chain.
Transport companies	Load, store and unload
Retailers/markets	Process, weigh, icepack, store and sell

Transportation and storage practices

The cold chain in seafood industry determines the eating quality of final product. The cold chain process begins once the fish is caught. As per the international, national and food safety

regulations the seafood should be stored at 0 ^{o}C from the sea to the consumer. The fluctuations in the cold chain process affects the quality, which cannot be reversed by any means after the event. Traditionally, Indian seafood process follow a lengthy marketing channel to reach the consumers. There are many avoidable and unavoidable occurrences that cause fluctuation in the cold chain.

The best practice to maintain the seafood quality and minimise the microbial contamination is by good hygienic handling and maintaining low product temperature in each step of seafood processing.

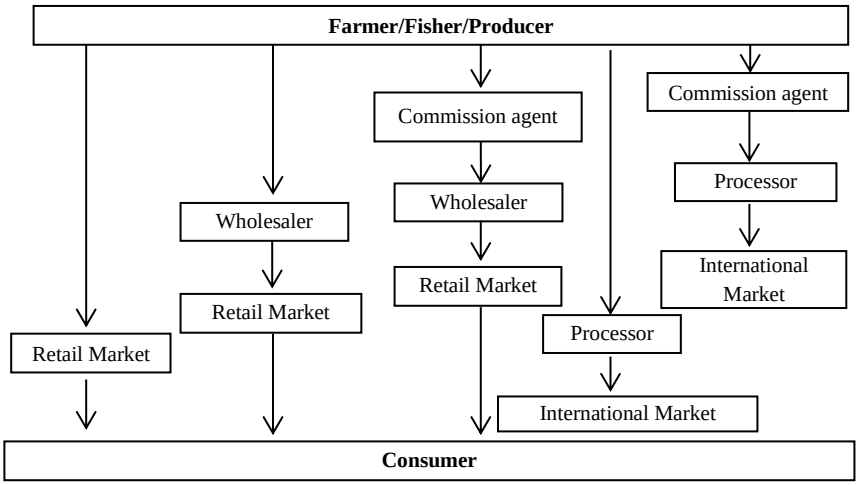

Figure 1: Seafood chain for fresh and frozen products in the fish industry of India

**The number of commission agent and wholesalers depends on the market price and place*

Processing factories should have:

- Proper infrastructure to achieve sanitation
- Proper lighting and ventilation
- Good water supply
- Chlorinated water with residual level of less than 2 ppm for processing
- Utensils for fish handling
- Washing of utensils and food contact surfaces with chlorinated level of 100 ppm
- Workers hygiene and health condition
- Rodent control measures
- Fly control measures
- Proper waste disposal process
- Proper toilet facilities

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Implementation of HACCP and Microbial Quality of Seafood

Renuka. V

HACCP is implemented in seafood industry to prevent hazards and ensure food safety and consumer protection. HACCP is the integral part of food safety management system. HACCP is a preventive system of hazard control rather than a reactive one. Food processors can use it to ensure safer food products for consumers. HACCP is not a stand-alone programme but is part of a larger system of control procedures.

HACCP system is designed to prevent and control food safety hazards associated with food from the time a seafood establishment receives raw material through production to distribution to the consumer. HACCP systems must be built upon a firm foundation of current Good Manufacturing Practices (GMPs) and Sanitation Control Procedures (SCPs). GMPs and sanitation standard operating procedures (SSOPs) affect the processing environment and should be considered pre-requisite programmes to HACCP.

The primary thing in developing a HACCP system is a commitment from the management. It is extremely important to have the support of top company officials such as the proprietor

or Director without which HACCP will not become a company priority. Availability of adequately trained work force is also to be ensured for effective implementation of HACCP. HACCP is often thought of in terms of its 7 basic principles. Before the preparation of HACCP Plans with the application of 7 principles of HACCP, 5 preliminary steps are also to be essential addressed. The steps involved in the effective implementation of HACCP for the seafood establishment is as follows

Preliminary steps in developing HACCP

1. Assemble the HACCP team
2. Describe the product
3. Describe the intended consumer use
4. Diagram of process flow from raw material receiving to shipping
5. Verification of process flow

HACCP Principles

1. Conduct a hazard analysis
2. Determine the Critical Control Points (CCP)
3. Determine the Critical Limit (CL)
4. Establish the monitoring procedure
5. Establish the corrective actions procedures
6. Verification and validation

7. Establish good Record keeping

1. Assemble the HACCP team

The team develops the HACCP Plan, writes SSOP, verifies and implements HACCP.

2. Describe the product

The team should list out all the materials, ingredients, packaging and storage condition of the products made.

3. Describe the intended consumer use

It includes the type of targeted consumer and how the product is to be prepared and used.

4. Diagram of process flow

The flow diagram should be simple and clear. The flow diagram should indicate all specific steps in the manufacturing process from the time raw materials are received until the finished product is shipped.

5. Verification of process flow

The flowchart must be physically verified by visiting the facility during operating hours for effective implementation of HACCP.

6. Conduct a hazard analysis

The HACCP team should list all the physical, chemical and biological hazards that may be reasonably expected to occur at

each step from primary production, processing, manufacture, and distribution until the point of consumption.

Biological hazard

Biological hazards include pathogenic bacteria, fungi, viruses and parasites that can cause food borne infection or intoxication to the consumers.

Bacteria of public health significance in fish and fish products include

1. *Escherichia coli* (*E. coli*)
2. *Salmonella* spp.
3. *Vibrio cholera*
4. *V. parahaemolyticus*
5. *Listeria monocytogenes*
6. *Campylobacter jejuni*
7. *Bacillus cereus*
8. *Clostridium botulinum*
9. *Aeromonas* spp.

Determine the control measures

The HACCP team must now consider what control measures, if any, exist which can be applied for each hazard.

Examples of Control measures:-

Biological Hazards: Time / temperature control and hygienic practices for reducing the microbial contamination.

The sources of contamination for microbial hazards are

- Fishing from polluted water.
- Use of contaminated water for processing.
- The primary habitat of the organism in intestinal tract of man and animals.
- Improper personal hygiene.
- Handling food material by workers who are carriers of Salmonella.
- Contaminated food, person to person contact, cross contamination of food by dirty food contact surface etc.

Preventive measures

- Adequate control over the health and hygiene of fish handlers.
- Refrigeration below $4^{o}C$ of the material during handling and processing.
- Minimise time/temperature abuse of seafood especially after cooking.
- Avoid fishing from polluted water.

- Avoid washing with costal/harbour water.
- Avoid the workers who are suffering from diarrhoea/vomiting in seafood handling.
- *Chemical Hazards*: raw materials testing, application of food additives

Physical Hazards: use of metal detectors

7. Determine the Critical Control Points (CCP)

CCP is a process step at which control can be applied to prevent or eliminate a food safety hazard or reduce it to an acceptable level.

It is the best and suitable point at which a significant hazard can be effectively controlled. CCPs are product and process specific. Eg: raw material receiving step, cooking step and metal detection step.

Effect of freezing on microbial hazards

- *E. coli* is very sensitive to freezing and frozen storage. 95% of the organism can be eliminated by freezing at -40^{0}C. The complete destruction of *E. coli* is possible on frozen storage (-18±2^{0}C) for about 3 months.
- All serotypes of Salmonella can survive during freezing at -40^{o}C and also survive for months together at frozen

condition at -18^{o}C even up to 9 months depending upon the serotype and initial load.

8. Establish Critical Limit (CL)

CL is a maximum and/or minimum value to which a biological, chemical or physical parameter must be controlled at a CCP to prevent, eliminate or reduce to an acceptable level the occurrence of a Food Safety Hazard.

CL is a criterion which separate acceptability from unacceptability.

9. Establish the monitoring procedure

To conduct a planned sequence of observations or measurements to assess whether a CCP is under control and to product an accurate record for future use in verification.

The purpose of the monitoring procedure is to assess whether the CCP is under control or not.

10. Establish corrective action procedures

Corrective action must be taken when critical limits are violated. A corrective action plan must be prepared and implemented in advance, so that there will not be any delay in taking corrective action.

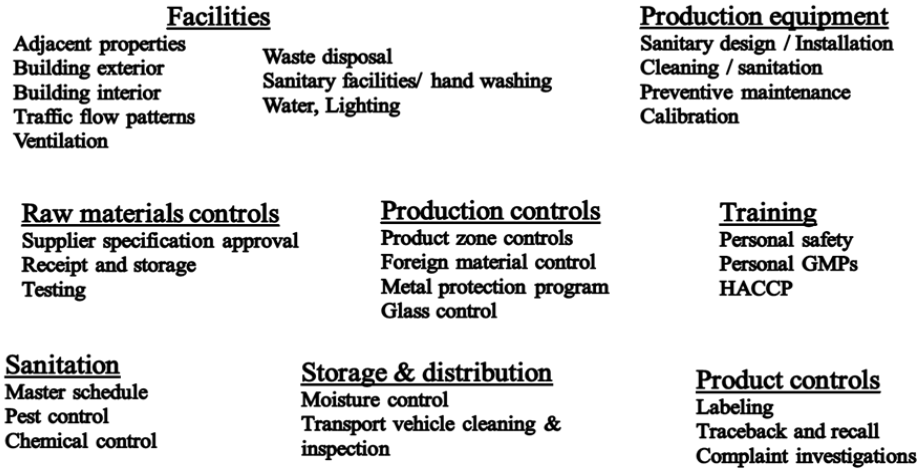

Figure 1: Pre-requisite programs that aid HACCP

11. Verification and validation

The purpose of verification is to provide a level of confidence that the HACCP plan is based on solid scientific principles is adequate to control the hazards associated with the product and process, and is being followed. As the countries requirement, the verification may be done internally or externally with the collaboration with government agencies or external agencies.

12. Establish good record keeping procedures

Records are important tools that make it possible to operate an effective HACCP system.

HACCP AUDIT **ANNEXTURE 1**

Sr. No.	Particulars	Observations				Remarks
I Safety of Water and ice used in the facility						
			Sufficient	Adequate	Efficient	
1	Source of water	Borewell water/water tankers				
2	Source control	Protection from outside contamination and testing of raw water from source by the QC department				
3	Cleanness & conditions of pipelines / hoses	Pipes and hoses provided for distribution of water				
4	Water purification system	Filtration plant consists sand cum carbon filter, softener, auto chlorine dozer				
5	Back washing system	Automated backwashing system at daily frequency				
6	Water storage tank	Ground and overhead water tanks and hygienic condition of the same				

Sr. No.	Particulars	Observations				Remarks
7	Water tank cleaning	As per the cleaning schedule, once in a month for underground tank and overhead tank				
8	Ease of cleaning water tanks	The design of water tanks are in such a way to facilitate easy cleaning. Man holes and ladders provided for entry of workers inside.				
9	Chlorination	Process water -2ppm (by automatic chlorine dosing) Water for Hand dip- 20 ppm Foot dip 50 -100 ppm Utensil washing and floor washing- 50-100 ppm (by manual chlorination)				
10	UV Treatment	UV irradiation after purification and prior to distribution.				
11	Controls to prevent cross contamination	Only potable water line. Nowhere the chances to cross with drainage line. The pipe lines are fully leak proof				
12	Color coding of pipelines	Only Potable water line				

Sr. No.	Particulars	Observations				Remarks
13	Prevention of back suction	Hose holders and non-returned valves are provided.				
14	Chances of contact with non potable water / sewage	The design of plumbing line is in such a way to avoid the chances for crossing with sewage line, nowhere.				
15	Numbering of water taps	Serially numbered at each sections				
16	Sources of water for making ice	Flake ice manufacturing unit is integrated				
17	Source control	As specified in the SSOP part of the own check system manual				
18	Prevention of cross contamination from machineries	All the machineries were installed away from the ice making sections (Separate area provided)				
19	Cross contamination from workers	By following good hygiene practices and daily audit of the personnel hygiene and employees practices				
20	Contamination during handling	Prevented by adhering to good handling practices				

Sr. No.	Particulars	Observations				Remarks
21	Contamination during storing	Provisions /precautions made for not keeping the flake ice directly on the floor				
22	Test reports of water & ice	Satisfactory as per EC Directive 98/83/EC				
23	Monitoring results	Satisfactory				
II. Condition and cleanliness of food contact surfaces, including utensils, gloves and outer garments						
1	Design, workmanship, materials and maintenance of FCS	The design workmanship and material used for all the equipments or utensils coming in contact with food or food contact surfaces in respect of maintaining the cleanliness				
2	Condition of FCS					
	A) Gloves	Hygienic condition and usage of the gloves used by the workers for handling final product				
	B) Outer garments	Condition and cleanliness of outer garments worn by the workers are found satisfactory				

Sr. No.	Particulars	Observations				Remarks
	C) Equipment for ice production	Hygienic conditions of equipments used for collection of ice				
	D) Storage and packing materials	Hygienic conditions of the storage premises and of packing materials while on storage				
3	Cleanliness/ and sanitation of FCS	Condition of FCS in the sections of raw material receiving, pre-processing, processing and while on transportation				
4	Type and concentration of sanitizer used	Liquid chlorine (Sodium hypochlorite solution) concentration of 6.00%				
5	Training and education	Training specified in the GMP part of HACCP manual				
III. Prevention of cross contamination from insanitary objects to food packaging materials and other food contact surfaces, including utensils, gloves and other outer garments and from raw product to cooked product.						
1	Cross contamination through					
	A) Workers hand, gloves, outer garments	Hand washing and sanitizing facilities, Use of single use				

Sr. No.	Particulars	Observations				Remarks
		disposable gloves for handling final product. Washing and ironing of uniforms after each operation.				
	B) FCS comes in contact with waste	Mode of separation of waste, handling and frequency of waste disposal				
	C) Temperature of the waste	Temperature of the waste is reduced by addition of ice to prevent bacterial multiplication in waste				
2	Direction of flow of raw product to blanched product	Observed unidirectional flow of the product from R/M receiving to blanched product				
3	Adequate separation of raw and blanched products	High risk and low risk area Separation of raw, frozen and blanched observed				
4	Flow of personnel	Unidirectional				
5	Flow of drainage	Opposite to the flow of product				
6	Employee practices	Employees practices (Monitored by responsible person)				

Sr. No.	Particulars	Observations				Remarks
7	Cleaning schedule	As per the cleaning schedule described in the HACCP manual				
8	Personal hygiene	As per the GMP & SSOP part of their HACCP manual				
9	Employees food handling practices	Raw material/ finished product handling practices of employees				
10	Facility for washing aprons and other clothing	Out-sourced arrangements				
IV. Maintenance of hand washing and sanitizing and toilet facilities						
1	Hand washing facilities	Location design and condition				
2	Concentration of sanitizer used	Sodium hypochlorite solution - Less than 20 ppm				
3	Adequate No. of Non hand operated taps	Number and maintenance of non hand operated taps				
4	No. of Toilet facilities	Adequacy and accessibility of location				
5	Condition of toilet facilities	Location Cleanliness /maintenance/ fly proof arrangements				
6	Maintenance and repair	The practice and schedule for maintenance				

Sr. No.	Particulars	Observations				Remarks
7	Foot washing at each entry points	(Foot dips) Numbers and location				
8	Strength of sanitizer in hand dips and foot dips	20 ppm for hand dips and 50 to 100 ppm for foot dips				
9	Numbering of water taps	Satisfactory				
10	Non hand operatable water taps and faucets	Functioning and location of all the non hand operatable water taps				
11	Liquid soap dispensers	Number in relation to the strength of workers and to number of wash basins				
12	Paper napkins, towels	Numbers or quantity of sterile paper and towel				
13	Specific instructions and sign boards	Instructions for hand washing and sign boards for effective hand washing and sanitizing provided in all the area				

Sr. No.	Particulars	Observations				Remarks
V. Protection of food, food contact surfaces, food packaging materials from fuels and lubricants, chemicals, pesticides, cleaning compounds, sanitizing agents, condensate and other chemical physical and biological contaminants. Windblown dust & water and other extraneous matter						
1	Fuels and lubricants	Storage and location -away from the food production area under the supervision of a responsible person				
2	Floor splash, drips condensate	Practices or operations for prevention of floor splash and drip condensate are found satisfactory				
3	Windblown dust	Concreting of the premises and compound wall				
5	Food packaging material & FCS	Measures and practices during storage of the packaging materials to prevent contamination				
VI. Proper labeling, storage and use of toxic compounds						
1	Number of compounds used					
2	Source of the compounds					
3	Legal permission to use					

Sr. No.	Particulars	Observations				Remarks
4	Proper labeling of containers	Proper labeling of the containers for easy identification of the compounds				
	Name of the compound					
	Instruction for proper use					
	Working container label					
5	Proper storage of toxic compounds					
6	Room with limited accessibility	Restriction of persons				
7	Proper use of toxic compounds	No toxic compounds used				
VII. Control of employee health conditions that could result in the microbiological contamination of food, food packaging materials and food contact surfaces						
1	Set company policy					
	a) Health and personnel hygiene	Procedure or practices as per in the GMP & SSOP part of HACCP manual				
	b) To deal with sick employees	Procedure or practices as per in the GMP & SSOP part of HACCP manual				

Sr. No.	Particulars	Observations				Remarks
2	Health status of the employees	Procedure or practices as per in the GMP & SSOP part of HACCP manual				
3	Protocol for assessing the health status	Registration of Health card of each employees by the Medical practitioner				
4	Training of the employees	In house training of each workers by the QC in charge				
5	Monitoring of employees health	Procedure or practices as per in the SSOP part 7 of the HACCP manual				
6	Personnel responsibility	Self reporting system of illness by each workers				
7	Maintain good health	Self responsibility of the workers to maintain their health in good condition				
VIII. Exclusion of pests from the food plant						
1	Elimination of harborage and attractant areas	Removal of scraps, debris and other waste from the premises				

Sr. No.	Particulars	Observations				Remarks
2	Bait maps	Maintenance of bait maps at the required stations as per bait maps				
3	Exclusion and extermination of pests	The maintenance for preventing attraction of pests/ harborage/ and breeding areas of the same				
4	All entries					
	Air/strip curtains to doors and chute	Strip curtains or air curtains to all entries or chute doors to prevent the entry of pests and rodents				
	Passage for the pests	Sealing, screening of all holes				
	Doors and windows	Prevention of gaps and provision of self-closing of the doors				
5	Spraying of insecticides and fogging	By the outside pest control agency				
6	Birds nesting area	Preventive measures for birds nesting areas in nearby places of outside premises				
7	Application of rodenticides	Chemicals used for the same				
8	Rodent map showing bait stations /traps	Maintenance of rodent traps in the most required places				

Sr. No.	Particulars	Observations				Remarks
9	Electrocuting of fly catchers	Fixing of fly catchers near to all the entry points and time to time removal of dead flies				
10	Effective sanitation programme for surroundings	Planned schedule for effective cleaning and sanitation programmes				

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Seafood Quality Standards

Ashish Kumar Jha

Fish or fishery products intended to be used as food must not contain microorganisms or their toxins or the metabolites of the toxin in quantities that may cause an unacceptable risk to the human health. The safety of the food product is mainly ensured by a preventive approach like implementation of Hazard Analysis Critical Control Point (HACCP) system.The fish business operators must comply with the microbiological criteria pertaining to fish and fishery products. This includes testing against the values set for the criteria through the taking of samples, the conduct of analyses and the implementation of corrective actions, in accordance of the food law and the instructions given by the competent authority. Different country has laid down its own set of rules and specifications for the presence or absence of microorganisms in food/fish/fishery products.

The government of India's (GOI) Food Safety and Standards Authority of India (FSSAI) notified the Food Safety Standards (Food Product Standards and Food

Additives) Third Amendment Regulations, 2017 to the earlier existing Food Safety Standards Regulations 2011. The new regulation defines the microbiological standards for fish and fish products. These regulations came into force on 1st January 2018.

Sampling Plan:

The terms n,c,m and M used in the above table have following meanings

n = Number of units comprising a sample

c= Maximum allowable number of units having microbiological counts above m

m = Microbiological limit that may be exceeded number of units c

M =Microbiological limit that no sample unit may exceed.

Indian Standard

Microbiological requirements for Fish and Fishery products-Hygine Indicator Organisms

Sl. No.	product category	Aerobic plate count				Coagulase positive Staphylococci				Yeast & mold			
		Sampling plan		Limits (cfu/g)		Sampling plan		Limits (cfu/g)		Sampling plan		Limits (cfu/g)	
		n	c	m	M	n	c	m	M	n	c	m	M
1.	Chilled/ Frozen Fish	**5**	**3**	**$5x10^5$**	**$1x10^7$**								
2.	Chilled /Frozen Crustacean	**5**	**3**	**$1x10^6$**	**$1x10^7$**								
3.	Chilled/Frozen Cephalopods	**5**	**2**	**$1x10^5$**	**$1x10^6$**								
4.	Live Bivalve Molluscs												
5.	Chilled Frozen Bivalve	**5**	**2**	**$1x10^5$**	**$1x10^6$**								
6.	Frozen Cooked Crustaceans/Frozen Heat Shucked Molluscs	**5**	**2**	**$1x10^5$**	**$1x10^6$**	**5**	**2**	**$1x10^2$**	**$1x10^3$**				
7.	Dried/Salted and Dried fishery Products	**5**	**0**	**$1x10^5$**						**5**	**2**	**100**	**500**
8.	Thermally Processed Fishery Products	**Commercially sterile**											
9.	Fish minced/Surimi and analogues	**5**	**2**	**$1x10^5$**	**$1x10^6$**	**5**	**2**	**$1x10^2$**	**$1x10^3$**				
10.	Fish Pickle	**5**	**0**	**$1x10^3$**		**5**	**1**	**$1x10^2$**	**$1x10^3$**				
	Test Methods suggested	**IS:5402/ISO 4833**				**IS: 5887 part 8 (sec 2)/ISO 6888-2**				**IS: 5403/ISO 21527**			

Microbiological Requirements for Fish and Fishery products-Safety Indicator Organisms

Sl.no.	product category	*Escheichia coli*				*Salmonella*				*Vibrio cholerae* (O1 and O139)				*Listeria monocytogens*			
		Sampling plan		Limits (MPN/g)		Sampling plan		Limits (cfu/g)		Sampling plan	Limits (cfu/g)			Sampling plan		Limits (cfu/g)	
		n	c	m	M	n	c	m	M	n	c	m	M	n	c	m	M
1.	Chilled/ Frozen Fish	**5**	**3**	**11**	**500**	**5**	**0**	**absent in 25g**		**5**	**0**	**absent in 25g**					
2.	Chilled /Frozen Crustacean	**5**	**3**	**11**	**500**	**5**	**0**	**absent in 25g**		**5**	**0**	**absent in 25g**					
3.	Chilled/Frozen Cephalopods	**5**	**2**	**20**		**5**	**0**	**absent in 25g**		**5**	**0**	**absent in 25g**					
4.	Live Bivalve Molluscs	**5**	**1**	**230/ 100g**	**700/ 100g**												
5.	Chilled Frozen Bivalve	**5**	**2**	**46**		**10**	**0**	**absent in 25g**		**5**	**0**	**absent in 25g**					
6.	Frozen Cooked Crustaceans/Frozen Heat Shucked Molluscs	**5**	**2**	**1**	**10**	**5**	**0**	**absent in 25g**				**absent in 25g**		5	0	**absent in 25g**	
7.	Dried/Salted and Dried fishery Products	**5**	**0**	**20**		**5**	**0**	**absent in 25g**									
8.	Fermented Fishery Products	**5**	**2**	**4**	**40**	**10**	**0**	**absent in 25g**									
9.	Fish minced/Surimi and analogues	**5**	**0**	**20**		**5**	**0**	**absent in 25g**		**5**	**0**	**absent in 25g**		5	0	**absent in 25g**	
10.	Fish Pickle	**5**	**0**	**20**		**5**	**0**	**absent in 25g**									

Adopted from the gazette of India (778GI/2017)

Note: For *Clostridium botulinum* there is no limit prescribed for chilled or frozen products but in thermally processed fishery and fermented products there must be absence of viable spores or vegetative cells of Clostridium botulinum and absence of botulinum toxin.

European standard

The European Parliament and of the council laid down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matter of Food Safety through the regulation (EC) No. 178/2002 and Commission Regulation (EC) No.2073/2005 deals with the microbiological criteria for food stuffs.

Some of the important microbiological standard for fish and fishery products followed by EU countries

Sl.no.	product category	*Escheichia coli*				*Salmonella*				*Listeria monocytogens*				*Coagulase-Positive Staphylococci*			
		Sampling plan		Limits (MPN/g)		Sampling plan		Limits (cfu/g)		Sampling plan		Limits (cfu/g)		Sampling plan		Limits (cfu/g)	
		n	c	m	M	n	c	m	M	n	c	m	M	n	c	m	M
1.	Ready to eat foods unable to support the growth of *L.monocytogens*, other than those intended for infants and for special medical purpose									**5**	**0**	**absent in 25g**					

2.	Cooked crustaceans and molluscs shell-fis					**5**	**0**	**absent in 25g**							
3.	Shelled and shucked products of cooked crustaceans and molluscan shellfish	**5**	**2**	**1** cfu/g	**10** cfu/g							**5**	**2**	**100** cfu/g	1000 cfu/g
4.	Live bivalve molluscs and live echinoderms, tunicates and gastropods	**1**	**0**	230MPN/100 g of flesh and intravalvular liquid		**5**	**0**	**absent in 25g**							

Microbial standard for fish and fishery products in China

National health and Family Planning Commission (NHFPC) of Republic of China published the National Food Safety Standard for processed Aquatic Products of Animal Origin. The standard is applied to all the processed aquatic animal products which are made from fresh and frozen aquatic animal products as the raw material.

Microbiological limits

Sl.no.	product category	Aerobic plate count				Coliform colonies			
		Sampling plan		Limits (cfu/g)		Sampling plan		Limits (cfu/g)	
		n	c	m	M	n	c	m	M
1.	Aquatic Products of Animal Origin	**5**	**2**	**$5x10^4$**	**$1x10^5$**	**5**	**2**	**10**	**10^2**

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