Training Manual on
"Microbiological examination of seafood pathogens with special reference *V.mimicus* & *V.valnificus"*

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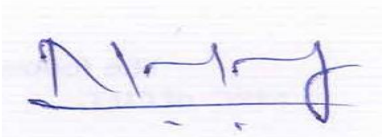
PREFACE

In India nearly 10 billion cases of food poisoning occur and most of them unnoticed for many reasons. Poisoning is going to be 1500 million by 2050.In this scenario the question is microbial safety of foods. This training manual on **'Microbiological examination of seafood pathogens with special reference to *Vibrio mimicus* and *V vulnificus*'** is intended for technologist working in fish processing industry, to keep abreast of all recent modification in the microbiological and biochemical techniques. The training manual is mainly focus on foodborne pathogens associated with sea food industry, monitoring control of the pathogens in seafood industry and Critical Control point (HACCP), microbial growth and sensory changes, seafood quality indicators, biochemical composition and post mortem changes in fish. Microbiological testing protocols are depicted as step by step manner. Most of the sections in this manual are based on Bacteriological analytical Manual (BAM) and FAO, APHA standards. Each topic is begins with theory section for the information on the specific training modules. This format allows the instructor to select sections and modules according to the levels of knowledge, experience and specific responsibilities of the Technologist in sea food industry.



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Introduction

The increased awareness among consumers for the safety of food products they consume and concomitant pressure for fresh and appropriate forms of products has led to the advance of food safety practices in the food industry. Besides, the relatively high occurrence of outbreaks of food borne diseases in many countries, including the developed ones, has resulted in increasing concern and intensive investigation of food borne pathogens. As a result, there is currently an increased demand for the microbiological testing of food products. The purpose of a microbiological testing should be to identify and restrict harmful microorganisms, which can spoilage foods, and ensure safety from food borne diseases. This means that the responsible one must establish a thorough testing procedure to identify all the possible threats, which may lead to one of the two results: pathogen not detected or detected. Before performing a microbiology test, the analyst should know the necessity, purpose, and primary expectations underlying the test, the predicted certainty of identifying an issue, and possible results that may come out from the test. Accordingly, this will help understand the sampling procedure to be performed, the type of samples to be collected, the particular test method to be used, and appropriate actions to be taken before and after the test results are attained.

Laboratory techniques in microbiology: A number of techniques are routine in microbiology laboratories that enable microorganisms to be cultured, examined and identified. An indispensable tool in any microbiology laboratory is the inoculating loop. The loop is a piece of wire that is looped at one end. By heating up the loop in an open flame, the loop can be sterilized before and after working with bacteria. Thus, contamination of the bacterial sample is minimized. The inoculating loop is part of what is known as aseptic (or sterile) technique.

A Petri plate is a sterile plastic dish with a lid that is used as a receptacle for solid growth media. In order to diagnose an infection or to conduct research using a microorganism, it is necessary to obtain the organism in a pure culture. The streak plate technique is useful in this regard. A sample of the bacterial population is added to one small region of the growth medium in a Petri plate and spread in a back and forth motion across a sector of the plate using a sterile inoculating loop. The loop is sterilized again and used to drag a small portion of the culture across another sector of the plate. This acts to dilute the culture. Several more repeat yield individual colonies. A colony can be sampled and streaked onto another plate to ensure that a

pure culture is obtained. Dilutions of bacteria can be added to a Petri plate and warm growth medium added to the aliquot of culture. When the medium hardens, the bacteria grow inside of the agar. This is known as the pour plate technique, and is often used to determine the number of bacteria in a sample.

Dilution of the original culture of bacteria is often necessary to reach a countable level. Bacterial numbers can also be determined by the number of tubes of media that support growth in a series of dilutions of the culture. The pattern of growth is used to determine what is termed the most probable number of bacteria in the original sample. As a bacterial population increases, the medium becomes cloudier and less light is able to pass through the culture. The optical density of the culture increases. A relationship between the optical density and the number of living bacteria determined by the viable count can be established. The growth sources for microorganisms such as bacteria can be in a liquid form or the solid agar form. The composition of a particular medium depends on the task at hand. Bacteria are often capable of growth on a wide variety of media, except for those bacteria whose nutrient or environmental requirements are extremely restricted. So-called nonselective media are useful to obtain a culture. For example, in water quality monitoring, a non-selective medium is used to obtain a total enumeration of the sample (called a heterotrophic plate count). When it is desirable to obtain a specific bacterial species, a selective medium can be used. Selective media support the growth of one or a few bacterial types while excluding the growth of other bacteria. For example, the growth of the bacterial genera Salmonella and Shigella are selectively encouraged by the use of Salmonella Shigella agar. Many selective media exist.

The culture is shaken to encourage the diffusion of oxygen from the overlying air into the liquid. Growth occurs until the nutrients are exhausted. Liquid cultures can be kept growing indefinitely by adding fresh medium and removed spent culture at controlled rates (a chemostat) or at rates that keep the optical density of the culture constant(a turbidostat). In a chemostat, the rate at which the bacteria grow depends on the rate at which the critical nutrient is added. Living bacteria can also be detected by direct observation using a light microscope, especially if the bacteria are capable of the directed movement that is termed motility. Also, living microorganisms are capable of being stained in certain distinctive ways by what are termed vital stains. Stains can also be used to highlight certain structures of bacteria, and even to distinguish certain bacteria from others. One example is the Gram's stain, which classifies bacteria into two camps, Gram positive and Gram negative. Another example is the Ziehl-Neelsen stain, which preferentially stains the cell wall of a type of bacteria called Mycobacteria. Techniques also help detect the presence of bacteria that have become altered in their structure or genetic composition. The technique of replica plating relies on the adhesion of microbes to the support and the transfer of the microbes to a series of growth media. The

technique is analogous to the making of photocopies of an original document. The various media can be tailored to detect a bacterium that can grow in the presence of a factor, such as an antibiotic, that the bacteria from the original growth culture cannot tolerate. Various biochemical tests are utilized in a microbiology laboratory. The ability of a microbe to utilize a particular compound and the nature of the compound that is produced are important in the classification of microorganisms, and the diagnosis of infections. For example, coliform bacteria were traditionally identified by a series of biochemical reactions that formed a presumptive-confirmed-completed triad of tests. Now, media have been devised that specifically support the growth of coliform bacteria, and Escherichia coli in particular. Various laboratory tests are conducted in animals to obtain an idea of the behavior of microorganisms in vivo. One such test is the lethal dose 50 (LD50), which measures the amount of an organism or its toxic components that will kill 50 percent of the test population. The lower the material necessary to achieve the LD50, the more potent is the disease component of organism.

Microbiology Tests for seafood are carried for the following reasons

I. To assess specifications for raw material, intermediate, and finished product,

II. Risk factor identification

III. Verification of Process

IV. Strict adherence to legal limits/ regulatory guidelines

The need for Microbiological Testing

Although microbiological testing is just one component of the food safety system and does not guarantee 100% product safety, but it is a prerequisite and integral part that must take place to ensure food safety. A microbiological testing can outline important information about a manufacturing process, processing environment, as well as a specific product batch. It also informs whether a sampling/testing procedure is correctly designed and finished following regulatory guidelines or not.

However, one must understand that a microbiological testing cannot determine 100% safety from pathogens, as tests are done using samples, which are only a portion from the food products. With microbiological testing, one can, mostly achieve that no pathogens are detected from the sample and/or, realize the levels of sensitivity and assurance provided by the testing procedures and sampling plans used. To ensure the optimum food quality, the manufacturers must also establish prerequisite programs including, Hazard Analysis Critical Control Point (HACCP), Good Manufacturing Practices (GMP), Recall Management, Traceability, and Sanitation Practices.

Culture Media

A special medium that is used in microbiological laboratories to identify and detect different types of microorganisms by culturing or growing. Usually, a culture medium is composed of different nutrients to enhance the microbial growth.

Traditionally, cultural techniques have been the tests of choice for both ready-to-eat foods and fresh produce. However, today immunoassay and PCR methods are more accepted than cultural methods, because recent developments of newer testing methods and validation studies have demonstrated that cultural methods aren't suitable for all food groups.

Important factors

- Different methods are involved in culturing techniques.
- For identification and detection of microorganisms in cultures, both liquid and solid culture media are employed.
- Microscopes are usually used to detect microbes in cultures, and biochemical and serological techniques are used to differentiate various organisms.
- Both qualitative and quantitative results of microorganisms can be obtained using cultural methods. This means a culture media technique not only detects the presence or absence of an organism but also provides information about the number of organisms present in the medium. However, quantitative analysis is only possible using solid culture media, because the individually developing colonies of organisms can be counted only on the surface.
- Time to attain results can range from twelve hours to more than a week.

2. MICROBIAL QUALITY OF SEAFOOD AND ITS SAFETY

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Introduction

Fishes are classified as cold-blooded aquatic vertebrates of the super class Pisces typically showing gills, fins and a streamline body. In addition, 'fish' also refers to the flesh of such animals used as food. There are about 22,000 species of fish that began evolving around 480 million years ago (Pal and Mahendra, 2015). Fish is an important part of a healthy diet due to its high quality protein, other essential nutrients and omega 3-fatty acids, and its low fat content as compared to other meats (Rhea, 2009; Pal, 2010). Fish and seafood products constitute an important food commodity in the international trade due to its ever increasing consumption demand. Fish contributes about 60% of the world supply of protein, and 60% of the developing world derives more than 30% of their animal protein from fish (Emikpe et al., 2011). Fish allows for protein improved nutrition in that it has a high biological value in term of high protein retention in the body, low cholesterol level and presence of essential amino acids (Emikpe et al., 2011). Fishes are generally regarded as safe, nutritious and beneficial but aquaculture products have sometimes been associated with certain food safety issues (WHO, 2007). There are more kinds of fishes than all other kinds of water and land vertebrates put together, and fish differ so greatly in shape, colour, and sizes (Adebayo-Tayo et al., 2012). The contamination often occurs from human and animal sources, and thus, fish and seafood can be involved in the transmission of pathogenic microorganisms and toxins (Pal, 2012).

Consumption of fish and shellfish may cause diseases due to infection or in toxication, some of these diseases have been specifically associated with pathogens, which are resistant to antibiotics (Adebayo-Tayo et al., 2012). Microbial contamination on environmental surfaces may be transferred to the food products directly through surface contact or by vectors such as personnel, pests, air movements or cleaning regimes (Pal, 2010). Bacteria may also infect the fish from outside during careless handling of landed fish, its stowing and cutting. Among major external sources of bacterial contamination are ice and salt, crushed ice is known to carry heavy bacterial loads. Microorganisms exist on the skin/slime, gills and the gut of live and newly caught fish. The proportion of commencially occurring microorganisms on the surface and guts of fish are 102107 colony forming units (cfu) /cm^2 and 103-109 cfu/g, respectively (Huss,1995). The microbiological flora in the intestines of seafoods such as finfish, shellfish, and cephalopods is quite different being psychotrophic in nature, and to some extent believed to be a reflection of the general contamination in the aquatic environment (Adebayo-Tayo et al., 2012). Several studies have demonstrated a number of bacterial species encountered in different fish, which are potentially pathogenic under certain conditions as reported for Pseudomonas

angulluseptica and Streptococcus spp. (Emikpe et al., 2011). It is estimated that there are more than 80 million cases per annum of seafood borne illnesses on antibiotic resistance in the USA, and that the cost of these illnesses is in many billions of dollars per year (AdebayoTayo et al., 2012). The economic losses due to spoilage are rarely quantified but a report by the US National Research Council Committee (FND/NRC) estimated that one-fourth of the world food supply is lost through microbial.

Microbial Quality of Fish and Fish Products

Humans and microbes have a long history together. The normal microbial flora consists of organisms that make their home on or in some part of the body. In a healthy person, such organisms rarely cause disease. Microorganisms of the normal flora may be in symbiotic relationship, where both microorganism and host benefit. The enteric bacteria that form the normal flora of the intestine assist in the synthesis of vitamin K and some of the vitamins of the B complex. In commensalism, microorganisms are neither beneficial nor harmful to their host as in the case of the large group of microbial flora that live on the skin, and the mucous membranes of the upper respiratory tract, intestines and vagina. Fish is very important foodstuff in developing countries due to its high protein content, and nutritional value. Fish provides more than 50% of the animal protein for the populations of 34 countries (Pal, 2010). However, it spoils easily, especially in hot climates and tropical areas where cold preservation techniques are often missing. Fish salting or brining, drying or smoking, are the traditional techniques for the improvement and storage of fish (Pal, 2010).

Fish Spoilage

Fish spoilage is a complex process, in which physical, chemical and microbiological mechanisms are implicated (Adebayo-Tayo et al., 2012; Pal, 2012).Many spoilage producing bacteria (*Aeromonas, Alcaligenes, Bacillus, Enterobacter, Enterococcus, Escherichia coli, Listeria, Pseudomonas, Shewanella*) and fungi (Aspergillus, Candida, Cryptococcus, Rhodotorula) are isolated from fresh and spoiled fish and other sea foods (Pal,2012). Reports on spoilage mechanism and quality assessment of the storage quality of frozen/chilled tilapia are still not comprehensive (Sil et al., 2008; Liu et al., 2010; AdebayoTayo et al., 2012). Degradation of lipids in fatty fish produces rancid odors. In addition, marine fish and some freshwater fish contain trimethylamine oxide that is degraded by several spoilage bacteria to trim ethylamine (TMA), the compound responsible for fishy off odors. Iron is a limiting nutrient in fish and this favors growth of bacteria such as pseudomonads that produce siderophores that bind iron. Spoilage is the result of a series of changes brought about in the dead fish mainly due to enzymatic and bacterial action (Pal, 2012). It starts as soon as a fish is caught and dies. In areas where temperature is high, fish spoils within 15-20 hours depending on the species and the method of capture (Adedeji and Adetunji, 2004). Fish is extremely perishable commodity due to its high

water content (Pal and Mahendra, 2015). Spoilage is defined as a change in fish or fish products that renders it less acceptable, unacceptable or unsafe for human consumption (Pal,2012). Fish undergoing spoilage has one or more of the following signs, discolouration, slime formation, changes in texture, off-odors, off –flavors, and gas production (Adedeji and Adetunji, 2004; Pal, 2010). Properties of spoiled fish compared to fresh fish are strong odour, dark-red gills with slime on them instead of bright red ones, soft flesh with brown traces of blood instead of firm flesh with red blood, and red, milky pupils without slime instead of clear ones (Pal,2010).

Food Processors/ Handlers

Persons serving in food processing industries may be sources of microbial inoculation, food poisoning, food intoxication and food spoilage. A number of organisms including Staphylococcus aureus have been isolated from the hands of employees working in food establishments (Pal, 2012; Pal and Mahendra, 2015).Hence, it is important to mention that any person with purulent skin lesions or having respiratory infections should not be allowed to work in food industry (Pal and Mahendra, 2015).

Fish Related Food Borne Illness and Diseases

The subsurface flesh of live, healthy fish is considered sterile, and should not present any bacteria or other microorganisms. On the contrary, as with other vertebrates, microorganisms colonize the skin, gills, and the gastrointestinal tract of fish. The number and diversity of microbes associated with fish depend on the geographical location, the season and the method of harvest. In general, the natural fish microflora tends to reflect the microbial communities of the surrounding waters (Rhea, 2009). The autochthonous bacterial flora of fish is dominated by Gramnegative genera including: Acinetobacter, Flavobacterium, Moraxella, Shewanella and Pseudomonas. Members of the families Vibrionaceae (Vibrio and Photobacterium) and the Aeromonadaceae (Aeromonas spp.) are also common aquatic bacteria, and typical of the fish flora. Gram-positive organisms such as Bacillus, Micrococcus, Clostridium, Lactobacillus and coryneforms can also be found in varying proportions (Huss, 1995) Human pathogenic bacteria can be part of the initial microflora of fish, posing a concern for sea food borne illnesses (Davies, et al., 2001). These pathogens can be divided into two groups: organisms naturally present on fish such as Clostridium botulinum , pathogenic Vibrio spp. , Aeromonas spp., and Plesiomonas shigelloides; and those not autochthonous to the aquatic environment, are present there, as result of contamination or are introduced to fish during harvest, processing or storage (Listeria monocytogenes, Staphylococcus aureus, Salmonella spp., Shigella spp., Escherichia coli, and Yersinia enterocolitica) (Huss, 1997).

Annual burden of foodborne diseases in the WHO South- East Asia Region includes more than:

• 150 million illness

• 175 000 deaths

• 12 million DALYs Source: FERG Report 2010

The disability-adjusted life year (DALY) is a measure of overall disease burden, expressed as the number of years lost due to ill-health, disability or early death. It was developed in the 1990s as a way of comparing the overall health and life expectancy of different countries. The DALY is becoming increasingly common in the field of public health and health impact assessment (HIA). It "extends the concept of potential years of life lost due to premature death...to include equivalent years of 'healthy' life lost by virtue of being in states of poor health or disability." In so doing, mortality and morbidity are combined into a single, common metric.

Despite significant success at improving the safety of the food supply, current science on which safety is based does not sufficiently protect consumers from emerging issues inherent to a complex food supply. The evolving characteristics of food, technology, pathogens and consumers make it unlikely the marketplace will be entirely free of dangerous organisms at all times for all consumers. This is the conclusion made in the report, Emerging Microbiological Food Safety Issues: Implications for Control in the 21st Century was released today at IFT's International Food Safety and Quality Conference and Expo in Atlanta one and half decades back.

The report, drew upon experts specializing in food borne pathogens and microbial evolution, food borne illness, food production and processing, testing methods and regulatory measures, reveals that diligent adherence to current methods that create and monitor the food supply cannot eliminate the risk of food borne illness. The report also offered the recommendations for providing the greatest possible reduction in food safety risks.

Among its seven important issues addressed were:

- Procedures from farm to table to significantly reduce illness due to mishandling
- Processes to recognize and respond to outbreaks and to reduce their scope.
- Poor habits that make consumers more susceptible to foodborne illness,
- Education and training recommendations necessary for reducing pathogenic influence at every step
- From production to consumption (pond to plate/farm to fork
- Recommendations to enhance monitoring, data generation, and risk assessment.
- The current state and future potential of rapidly evolving illness-causing pathogens and other key issues.

To gain the greatest measure of food safety, the report stressed on the necessity of implementing flexible food safety measures so as to utilize as quickly as possible the latest scientific information as it evolves. The report also urged manufacturers, regulatory and public health agencies and allied organizations to develop partnerships to improve risk assessment and food safety management.

Seafood safety goals must achieve more than end-product probes

The absence of pathogens in final-product testing does not ensure food free of virulent microorganisms, according to a new expert report on food safety issues, and as pathogen contamination decreases this form of testing becomes more deficient. So as today's food safety continues to improve, more emphasis should be placed on monitoring processing capabilities and conditions through the application of science-based food systems.

The microbiological testing of finished sea food products and can be misleading for the following reasons

- ✓ Due to statistical limitations based on the amount of product sampled,
- ✓ The percentage of product contaminated,
- ✓ The uniformity of the contamination distributed throughout the food.

The above mentioned negative results imply an absence of pathogens in foods, the report states, and can cause consumers to assume proper food selection and handling practices are unnecessary. Instead, the report urges everyone along the farm-to-fork seafood chain to be responsible for an important role in food safety management. According to Douglas L. Archer of the University of Florida who contributed to IFT report "Current safety evaluations focus on microbes that may or may not be harmful to humans," he added,. "For example, some subtypes of *Listeria monocytogenes* found in or on food may not be associated with food borne illness. Yet their mere detection can be grounds for legal action against the manufacturer and force recalls of food that is unlikely to cause illness in the general population." The need science-based approach called Food Safety Objectives that would place specific values on public health goals, with reassurances those values are reached at key points along the pond to plate process. Those values would be flexible as hazards and public health goals change, science progresses, and unfettered data sharing improves, allowing for the quickest implementation of new safety improvements as they evolve, and a safer food supply. The report urges intentional interaction of public health, regulatory, industrial and consumer agencies, calling the implementation of a flexible, science-based approach involving all these parties "as the best weapon against emerging microbiological food safety issues."

Steps in seafood Safety Management

Foodborne illness in India is a major and complex problem that is likely to become a greater problem as we become a more global society where every 5th person walking on this planet is going to be Indian. Nearly 10 million foodborne illnesses occur per year in India. To adequately address this complex problem, the need is to develop and implement a well conceived strategic approach that quickly and accurately identifies hazards, ranks the hazards by level of importance, and identifies approaches for microbial control that have the greatest impact on reducing hazards, including strategies to address emerging hazards that were previously unrecognized. Policy Development Scientific research has resulted in significant success in improving seafood safety, but the current science supporting the safety of our seafood supply is not sufficient to protect us from all the emerging issues associated with the complexity of the food supply. As new issues emerge, some will be best addressed through the application of control technologies during seafood production and processing, but others may be best addressed at the consumer level through modification of exposure or susceptibility. Food safety policies should be developed as part of national initiatives, with input from all stakeholders. In addition, international coordination of food safety efforts should be encouraged. Globalization of the food supply has contributed to changing patterns of food consumption and food borne illness, and global food trade has the potential to introduce pathogens to new geographic areas. To achieve the maximum benefits, our food safety efforts and policies must be carefully prioritized, both in terms of research and in application of controls. As scientific advances provide a better picture of pathogenicity, the need of the hour is whether to focus the efforts on those pathogens that cause many cases of minor illness or instead focus on those pathogens with the greatest severity, despite the relatively low number of cases. In the move toward making decisions based on risk, the food safety policies need to weigh these issues, and communicate information about risk to all stakeholders, especially the public. The body of scientific knowledge must be further developed, with the research efforts carefully prioritized to yield the greatest benefit. Food safety and regulatory policies must be based on science and must be applied in a flexible manner to incorporate new information as it becomes available and to implement new technologies quickly. The seafood industry, regulatory agencies and allied professionals should develop partnerships to improve food safety management.

3. STERILIZATION TECHNIQUE USED IN MICROBIOLOGY

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Introduction

Sterilization is the process of killing all microorganisms (bacterial, viral, and fungal) with the use of either physical or chemical agents. A disinfectant is a chemical substance that kills microorganisms on inanimate objects, such as exam tables and surgical instruments. Skin can never be completely sterile. Sterilization in the microbiological laboratory denotes sterilization process implemented in preparation of culture media, reagents and equipment where the work warrants maintaining sterile condition. Sterilization in microbiology laboratory is done by following methods Physical method i.e., use of heat, filters, radiation Chemical method i.e., by use of chemicals Heat sterilization a. Dry heat sterilization.

a. Dry heat sterilization

Inoculation loops or needle are sterilized by heating to 'red' in Bunsen burner or spirit lamp flame. Sterilization in hot air oven is performed at a temperature of 160C and maintained or holding for one hour. Spores are killed at this temperature and this is the most common method of sterilization of glassware, swab sticks, pestle and mortar, mineral oil etc. Dry heat sterilization causes protein denaturation, Oxidative damage, toxic effect of elevated electrolyte in absence of water.

b. Wet heat or moist heat sterilization

Moist heat sterilization is accomplished by

1). **Boiling** at 100°C for 30 minutes is done in a water bath. Syringes, rubber goods and surgical instruments may be sterilized by this method. Almost all bacteria and certain spores are killed in this method

2). **Steaming** at 100°C for 20 to 30 minutes under normal atmospheric pressure are more effective than dry heat at the same temperature because bacteria are more susceptible to moist heat, Steam has more penetrating power and sterilizing power as more heat is given up during condensation. Suitable for sterilizing media which may be damaged at a temperature higher than 100°C

3).**Tyndallization** (Fractional Sterilization) is the steaming process performed at 100°C is done in steam sterilizer for 20 minutes followed by incubation at 37°C overnight and this cycle is

repeated for successive 2 days. Spores, if any, germinate to vegetative bacteria during incubation and are destroyed during steaming on second and third day. Heat labile media containing sugar, milk, gelatin can be sterilized using this method.

4). **Autoclaving** is done by steam under pressure. Steaming at temperature higher than 100°C is used in autoclaving. This is achieved by employing a higher pressure. The autoclave is closed and made air-tight for pressure development and at 15 lbs per sq. inch pressure, 121°C temperatures will be reached and this temperature is given as sterilizing holding time for further 15 minutes. This process kill spores and this works like a pressure cooker and one of the most common methods of sterilization.

5). **Pasteurization** is another one method of moist heat sterilization which works below 100°C heat. This process is used in heating of milk and other liquid food. The product is held at temperature and for a period of time to kill pathogenic bacteria that may be present in the product. This process does not destroy complete organism including spores.

All these moist heat sterilization causes denaturation and coagulation of protein, breakage of DNA strands, and loss of functional integrity of cell membrane.

c). **Filtration**: This method of sterilization is used for media particularly heat labile in nature (e.g. sera an media containing proteins or labile metabolites. If the study warrants bacteria-free filtrates it can be obtained through 0.45micron sized filter membranes and if the study requires viral particle free solution, then 0.22micron sized filter membranes are use. In earlier days absorptive filters of asbestos or diatomaceous earth were replaced by unglazed porcelain or sintered glass are used. Nowadays these are replaced by nitrocellulose membrane filters of graded porosity, PVDF etc.

d). **Ultraviolet Radiation:** at wavelength between 330nm and 400nm causes sterilizing effect. This method is used in surface sterilization of laminar airflow, biosafety cabinet and in certain cases in laboratory.

In microbiology laboratory autoclaving, hot air oven sterilization, filtration and UV radiation are commonly used.

Standard operating procedure for the setting up of autoclave

- Pack your media, reagents, plastic wares, in their appropriate autoclavable resistant polypropylene or borosilicated glassware
- Screw the lid of the tube and leave one thread loose in case of closed containers or plastics

- Stick at random autoclavable indicators for each run in any of the items to be autoclaved
- Check for the water level in the autoclave machine
- Donot jam pack the items in the autoclave machine
- Switch on the machine
- Keep the lid of the machine tightly closed with one valve open until it reaches boiling
- Leave heated air to escape for few minute through valve
- Completely close the valve and wait to reach the temperature for 121^0C at 15lbs pressure.
- Hold the sterilization cycle for 15 minutes
- Once the sterilization cycle end, switch off the heating and leave the machine to reach to 65^0C
- Then open the lid and take out the items back after sterilization

Standard operating procedure for the setting up of hot air oven

- Pack all the glassware such as pipette with pipette can, glass petridishes, sample dish, test tubes, pestle and mortar, mineral oil to be sterilized by hot air oven sterilization with suitable wrapping
- Switch on the hot air oven until to reach 160^0C
- Hold on in that temperature for 1 hour
- Switch off the heating of hot air oven and open the door once come below 65^0C

Standard operating procedure for the setting up of filtration

- Once the bio safety cabinet is ready for filtration
- Switch on the blower
- Filtration unit should be inside the cabinet
- Vacuum or positive pump should be kept outside of the cabinet
- Filtration assembly should be with the suitable filters
- Pour the media or reagents to be sterilized in the top of the filtration assembly
- Connect the bottom assembly to vacuum pump or top of the assembly to the positive pump

4. DO'S AND DO NOT'S IN THE MICROBIOLOGY LABORATORY

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Introduction

There is a certain element of risk in anything you do, but the potential risks in a microbiology course are greater. Persons who work in a microbiology lab may handle infectious agents in additional to other hazards such as chemicals and radioactive materials. There have been many documented cases of lab personnel acquiring diseases due to their work. About 20% of these cases have been attributed to a specific incident, while the rest have been attributed to work practices in the lab. It is possible that you can be exposed to potentially harmful microbes when you isolate bacteria from environmental materials. Working in microbiology demands a strict personal and environmental safety. Personal safety denotes protocols avoiding laboratory accidents and environmental safety denotes maintaining clean laboratory practices to prevent contamination from exogenous sources. Integral part of microbiology is the aseptic techniques since microbiology laboratory deals with microbes of public health importance. Aseptic techniques denote free from pathogenic organism and pathogens means organism capable of causing disease. All microbes handled at the laboratory should be always considered equally as potential as pathogens.

- ✓ Before entering the microbiology laboratory for handling of microbes wear lab coats.
- ✓ Keep back laboratory coats, observation note books, pen, pencils and other accessories used during observation in the specified location and strictly avoid work benches.
- ✓ Keep the doors and windows of the microbiology laboratory closed during the handling to avoid contamination from the air currents and avoiding the possible spread to the outside environment.
- ✓ Use disinfectant solution to wipe the bench top before and after handling of microbes.
- ✓ Sterilize the inoculating loops and needles by incineration in Bunsen burner.
- ✓ Discard the handled pipette in the receptacle designated for the keeping and the tips to the biohazard waste containers designated for disposing.
- ✓ Place all the cultures back in their respective places after handling either at decontamination area for disposing or at storing racks for future use.
- ✓ Fungal cultures if at all to be handled in the bacteriology laboratory should be manipulated with the utmost care and rapid and efficient way to avoid spread of reproductive spores into the laboratory environment for the personal safety.
- ✓ While leaving the laboratory wash your hands with liquid detergents
- ✓ Women should wear paper cap to avoid exposure of hairs to flame

- ✓ Wear lab coats to protect from contamination and safety during handling of cultures and chemicals and toxic substances
- ✓ Closed shoes designated to be used inside the laboratory should be available
- ✓ Not to insert contact lenses or cosmetic inside the laboratory
- ✓ Do not smoke or eat or drink inside the laboratory
- ✓ Carry cultures always in tube racks or trays while moving from one place to other or while storing on the work benches
- ✓ Do not transport media, equipment, cultures from the laboratory to outside without proper safety measure.
- ✓ In case of any spill, cover the area with the disinfectant solution to saturate the spill and leave it for 15 minutes and put paper towel or cloth towel over to cover the spill and dispose it off with decontamination procedure.
- ✓ Mouth pipetting of cultures and toxic chemicals are strictly and completely prohibited in the laboratory. Alternatively use the mechanical pipetting aid or devices as and when required.
- ✓ Use self-sticking labels inside the laboratory
- ✓ Use disposable glove while handling of known high risk group organism and dispose off the gloves after handling for decontamination
- ✓ Wear face mask, safety goggles, laboratory coats if aerosol forming procedures are going on
- ✓ Use bleach solution at 1:10 concentration for decontamination

General steps for maintaining hygiene in the laboratory

- ➢ Keep back all the media, reagents, test tubes etc in the specified respective places
- ➢ Close all the lids of the media before use and keep it back on specified rack or location (Media are hygroscopic in nature)
- ➢ Clean the weighing balance if any spill of salts, media etc., only after switching off the weighing balance
- ➢ Switch off gas, pipe connection, lights, equipment not in use after working hours
- ➢ Keep back all the handled cultures, test tube racks, petridishes, contaminated swabs, disposable pipettes, to the biohazard receptacle prior to decontamination.
- ➢ Handle potential carcinogen in fume hood.

5. PLATING TECHNIQUES IN ISOLATION OF MICRO-ORGANISMS

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Introduction

Microorganisms are present on all inanimate surfaces creating ubiquitous sources of possible contamination in the laboratory. Experimental success relies on the ability of a scientist to sterilize work surfaces and equipment as well as prevent contact of sterile instruments and solutions with non-sterile surfaces. Study of microorganism needs accurate handling or it adversely affects the handlers. Standard operating procedures are the key step in performing the microbiology study. This not only gives the reliable result but also ensure the safety of the laboratory technicians. Plating is the common technique employed and the petriplates of different sizes can be used for different purposes. It is recommended that non-pathogenic strains be used when learning the various plating methods. By following the procedures described in this protocol:

- Perform plating procedures for enumeration of bacteria without contaminating media and self.
- Isolate single bacterial colonies by the streak-plating method.
- Use pour-plating and spread-plating method for variety of applications like desired bacterial screening

General instructions

- Sterile workspace and premises is essential for microbial works
- Sterilize all instruments, solutions, and media prior to using them for plating procedures.
- Clean work area with phenol or 70 %alcohol to minimize possible contamination.
- Keep burner with flame prior to work to create a sterile field.
- In all techniques sterilization of glass wares in hot air oven and the Medias in prescribed manner should be done prior to plating.
- Media which are autoclaved and glassware should be cooled to sufficient levels before plating
- Marking of the petriplate should be done in base of the plate.

I. Pour plate technique

This method often is used to count the number of microorganisms in a mixed sample, which is added to a molten agar medium prior to its solidification. Molten agar should be cooled to 44˚C before plating otherwise it may lead to death of the desired organism. The process results in colonies uniformly distributed throughout the solid medium when the appropriate sample dilution is plated. This technique is used to perform viable plate counts, in which the total

number of colony forming units within the agar and on surface of the agar on a single plate is enumerated. Viable plate counts provide scientists a standardized means to generate growth curves, to calculate the concentration of cells in the tube from which the sample was plated, and to investigate the effect of various environments or growth conditions on bacterial cell survival or growth rate.

This method is advantageous when our organism is environment bacteria and the prevalence is less.

Materials required

Sample, sterilized petri plates, sterilized nutrient media, flame, glass marker

Procedure of Pour plate technique

- With the help of serial dilution technique, the sample should be prepared. The good dilution is one which gives colonies in between 30 to 300.
- Label the petri dishes in the bottom of plate
- Put 1 ml prepared dilution sample in the petri plate near the flame
- Cool the media and pour it in the plate. 100 ml media can be poured to 4 plates
- Mix the plate well for uniform spreading and allow it to solidify and incubate

Limitations

- Some colonies may be hidden inside agar
- Heat labile organism will die

II. Spread plate technique

- The spread plate technique is used for enumeration, enrichment, screening and selection of microorganism. In this the culture is uniformly spread over the surface of an agar plate, resulting in the formation of isolated colonies distributed evenly across the agar surface if the appropriate concentration of cells is plated.

  Materials required

  Sample, sterilized petri plates, sterilized nutrient media, flame, glass marker, glass rod (alternatively sterile plastic rod also can be used), beaker with alcohol

  Procedure

- Sterilize the petri plate and nutrient medium. Cool it to 56◦C. pour in the plate and allow it to settle.
- Then prepare the sample. Serial dilute if necessary. Add 0.1 ml of sample in the surface of dried agar plate
- Dip the spreader in alcohol, flame and cool it
- Spread the sample uniformly near the flame
- Incubate the plate in inverted position

Advantage over other methods

- Colony morphology can be seen clearly
- Can be used for screening and selection

Limitations

- Over growth may occur
- Micro aerophilic bacteria may get affected

III. Streaking

This method is used for obtaining pure culture from the mixed culture. Quadrant streaking is done in the petri plate in such way that all four corners are used for isolating a single bacterial colony

Materials required

Sample, sterilized petri plates, sterilized nutrient media, flame, glass marker,metal loop

Procedure

1. Media should be poured in petri plate and allowed to settle. Then it is dried till the condensed water becomes dry
2. Flame the loop until it becomes red hot and allows it to cool. Then pick the colony aseptically near the flame
3. Place loop with culture in petri plate and take it to other quadrat without touching the edge of the petri plate. Then flame the loop to sterilize
4. From the previous line draw another line perpendicular to the old line with sterile loop. This line also should not touch the corner.
5. Then sterilize the loop and draw another line from the previous quadrant perpendicular to the old line with sterile loop. This line also should not touch the corner
6. Then sterilize the loop and from the old line draw another line with the loop and stop in the half quadrant. This way we can get individual colony without contamination in one plate.
7. The bacteria grown in single colony are assumed to have formed from the single bacteria and they are called as clone .

Advantage over other method

- Pure culture can be obtained. If colony morphology is known contaminated cultures can be purified

Limitations

- Expertise required for getting individual colony in streaking

IV. Agar overlay method

This technique can be used for isolation bacteriophage. Phages are viruses affecting bacterial cell and they cannot live outside the cell as like other viruses. Quantification of phage as phage forming unit also can be done using this method. First Bacterial mat called lawn formed in the plate. Then the phages mixed infect the bacterial cells. So the bacterial lawn disappears. The resultant zone of clearance is called plaque. As like bacterial colonies, single plaque also formed by single phage and it is expressed as plaque forming unit.

Procedure

1. First the agar plate is prepared as like streaking or spread plate technique
2. Bacterial culture usually10^8 bacteria and phage suspension (50-200µl) is uniformly mixed with soft agar (0.5-0.7%) of 2-3 ml.
3. Pour it on top of pre-settled agar plate and shake it vigorously for uniform spreading
4. Allow it to settle and incubate 24-48 hrs.

V. Antibiotic sensitivity testing using petri plate

Antibiotic sensitivity of desired organism also can be tested with the plating procedure. For this known amount of culture with the same OD or McFarland unit concentration should be checked every time to get uniform results. The known concentration of bacteria should be inoculated as spread plate to form uniform lawn. Then the discs should be equally placed with uniform concentration of antibiotic. With the help of the ruler the zone of inhibition should be checked

Materials required

Fresh culture of bacteria to be tested, Inoculation loop , Burner, McFarland solution, Saline solution, Muller Hinton agar plate , Antibiotic disc to be tested , Incubator , Ruler , Forceps and beaker with alcohol , glass rod

Procedure

1. Take pure culture of the organism to be tested (fresh culture of 12-24 hrs. desired)
2. This should be uniformly mixed with saline and compared with McFarland standard OD
3. Alternatively, the bacteria to be tested should be well studied and compared with the OD. So the concentration to be checked for different antibiotic will be always uniform
4. Aseptically spread the colony with the sterile glass spreader to the Muller Hinton plate
5. Allow the plate for dry 5 minutes
6. Place the antibiotic disc with the help of sterile forceps and gently press
7. Incubate the plate without inverting
8. After incubation measure the zone of inhibition.

9. Compare the measurement obtained from the individual antibiotics with the standard table to determine the sensitivity zone.

6. SAMPLING OF FISH AND FISHERY PRODUCTS

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Introduction

Sampling methods vary with the type of sample being taken and the location. BAM protocol (USFDA), 10 gram of sample has to be taken randomly from 100 gram of sample lot for normal microbiological analysis like TPC, total *enterobacteriaceae* count, fecal *streptococci, staphylococci, E.coli*, spoilage bacteria, fungi and yeast and molds. For salmonella and vibrio species, 25 g sample has to be taken for analysis in 225 ml lactose broth and APW (Alkaline Peptone Water) respectively. For Salmonella detection in ready to eat (RTE) products 225g sample has to be taken in 2.025L lactose broth.

Microbiological parameters to be tested for fresh fish

Total plate count	*Staphylococcus aureus*	*Vibrio species like V. cholera, V. mimicus, V. parahemolyticus etc.,*
Total enterobacteriaceae count	*E.coli*	*Optional: Shigella and Listeria monocytogenes presence*
Fecal streptococci	*Salmonella*	

Microbiological parameters to be tested for chilled/frozen fish

Total plate count	*Staphylococcus aureus*	*Vibrio* species like *V. cholera, V. mimicus, V. parahemolyticus etc*
Total enterobacteriaceae count	*E.coli*	Spoilage indicators and *H2S producers* like *Shewanella, Pseudomonas, Brocothrix* etc
Fecal streptococci	*Salmonella*	Optional: *Shigella and Listeria monocytogens, Yersenia species* etc presence

Sample received for microbiological examination are prime importance for getting proper result. If samples are improperly collected, mishandled or not representative of original lot leads to laboratory results will be meaningless. Because, interpretations are about large consignment quality based on a relatively small sample. Hence, established sampling procedures must be applied uniformly. The number of units that comprise a representative sample from a designated lot of a food product must be statistically significant.

Sterile spoon, forceps, spatula and scissors are required for sampling techniques. Hence, all the materials used for sampling must be sterile condition. The above said materials can be easily sterilized by dry heat method. Alcohol dipping along with flaming will not be sufficient to kill all pathogens unless otherwise specified.

Sampling scale for organoleptic checks:

Organoleptic checks of raw material, process and product samples shall be analysed by the approved technologist / qualified personnel to ascertain the freshness and other organoleptic qualities of the product. To carry out the work, a sample of one Kg subject to a minimum of 10 pieces shall be tested from every 500 kg of the raw material received, variety wise and source wise for conducting the organoleptic evaluation *as per HACCP* plan. Organoleptic checks shall also be conducted during processing and after freezing / packing. For the analysis of finished products, type wise and variety wise samples shall be drawn from the days production at random as per the sampling scale

No. of package in the lot	No. of packages to be
1 to 12	2
13 to 24	3
25 to 40	4
41 to 80	5
81 to 120	6
121 to 180	7
181 to 250	8
251 to 350	10
351 to 500	12
501 to 750	14
751 to 1000	18
1001 to 1300	22
1301 to 1600	25
1601 to 2000	30
2001 and above	40

Sampling scale for Microbiological analysis:

Product samples shall also be drawn for testing the above microbiological parameters from a particular production code selected. For this purpose, each variety of fishery products (shrimps, cuttle fish, squid etc) of the selected code shall be treated as a separate lot and variety wise composite samples of 150 gms each shall be drawn aseptically for testing at EIA lab. 5 samples of 150 gms each shall be drawn aseptically from a selected code, covering maximum grades possible.

Sampling scale for residues:

Residues such as antibiotics, pesticides and heavy metals can be taken based on the formula : $\{ (n)^{1/2} + 1\}/2$,

n: Number of container / consignment

Sampling scale for histamine estimation:

For testing the histamine 9 sample has to be drawn from the different sites. In the result the mean value of the 9 samples must not exceed 100 ppm. Two values can exceed 100 ppm; but less than 200ppm. No one vale goes beyond the 200 ppm.

7. ISOLATION AND ENUMERATION OF MICROBES FROM SEAFOOD

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Introduction

Microbiology is the study of microorganisms like microscopic or barely visible single-celled life-forms such as bacteria, archaea, protozoans. Enumeration in microbiology is an estimation or determination of number of bacterial cells in a given sample. Enumeration of sea food has gained importance due to increased attention being paid to quality aspects of final product. The International Commission on Microbiological Specifications for Foods (ICMSF) established in 1962 to the need for internationally acceptable and authoritative decisions on microbiological limits for foods appropriate with public health safety, and particularly for foods in international commerce.

Methods to enumerate microbes can be divided into two categories.

a) Total cell counts include dead and inactive cells.

b) Viable methods only count cells that are metabolically active,

Direct Microscopic count/ Total cell count

Direct microscopic counts measures number of cells in a population of a given sample under a microscope. This can be possible for liquid samples using special slides known as counting chambers, consisting of a ruled slide and a cover slip. It is constructed in such a manner that the cover slip, slide, and ruled lines delimit a known volume. The number of bacteria in a small known volume is directly counted microscopically and the number of bacteria in the larger original sample is determined by extrapolation. Bacteria can be counted easily and accurately with the petroff-Hausser counting chamber. This is a special slide accurately ruled into squares that are 1/400 mm2 in area; a glass cover slip rests 1/50 mm above the slide, so that the volume over a square is 1/20,000 mm3 i.e. 1/20, 000, 000 cm3. If for example, an average of five bacteria is present in each ruled square, there is 5 x 20,000,000 or 108, bacteria per milliliter.

Advantages:

a) It is quick way of estimating microbial cell number

b) Morphology of the bacteria can be observed as they counted.

Limitations:

a) Dead cells cannot be distinguished from living ones. Only dense suspensions can be counted

b) Difficulty in to count small cells

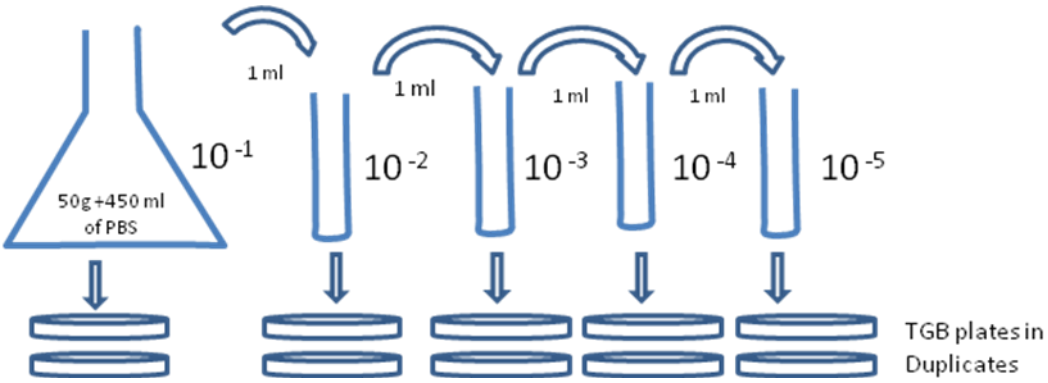
c) Precision is difficult to achieve

d) Require a phase- contrast microscope if sample is not stained.

Standard Plate Count (Viable Counts): Any cell which has a capacity to divide and form a population or colony is defined as a viable cell. Viable count is also called as plate count or colony count. A viable cell count is usually done by diluting the original sample, plating aliquots of the dilutions on to an appropriate culture medium, then incubating the plates under suitable conditions for the colonies to be grown. Colonies are counted and, from a particular dilution used, the original number of viable cells can be calculated. For accurate determination of the total number of viable cells, it is critical that each colony comes from only one cell, so chains and clumps of cells must be broken apart. However, since one is never sure that all such groups have been broken apart, the total number of viable cells is usually reported as colony-forming units (CFUs) rather than cell numbers. This method of enumeration is relatively easy to perform but major disadvantage is the time necessary for dilutions, plantings and incubation. There are two ways to perform a plate count a) pour plate technique b) spread plate technique. Plating techniques are discussed detail in chapter no.5. Enumeration protocols of significant seafood borne pathogens are given below in flow chart.

Enumeration protocols of significant seafood borne pathogens

1. Aerobic plate count (APC)



50 g of fish sample + 450 ml of sterile Butte field's phosphate buffer (BPBS)

↓

Macerate in a blender

↓

Prepare tenfold dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4},)

↓

Add 1 ml from each dilution to sterile empty and add plate count agar (PCA) and rotate firmly

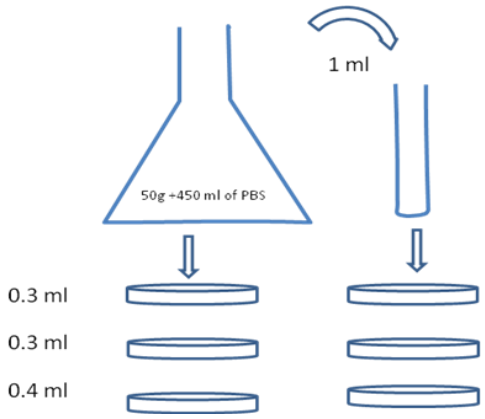
↓

Incubate at 37^{o} C for 48 hrs

↓

TPC/g= No. of colonies x dilution factor

2. Enumeration of *Staphylococcus aureus*



50 g of fish sample + 450 ml of sterile Butter field phosphate buffer

↓

Macerate in a blender

↓

Prepare tenfold dilution (10^{-1}, 10^{-2})

↓

Add 0.3, 0.3 and 0.4 ml in to three BP plates Baired Parker

↓

Incubate at 37° C for 36 – 48hrs

↓

Enumerate Black colony with white margin surrounded by opaque zone

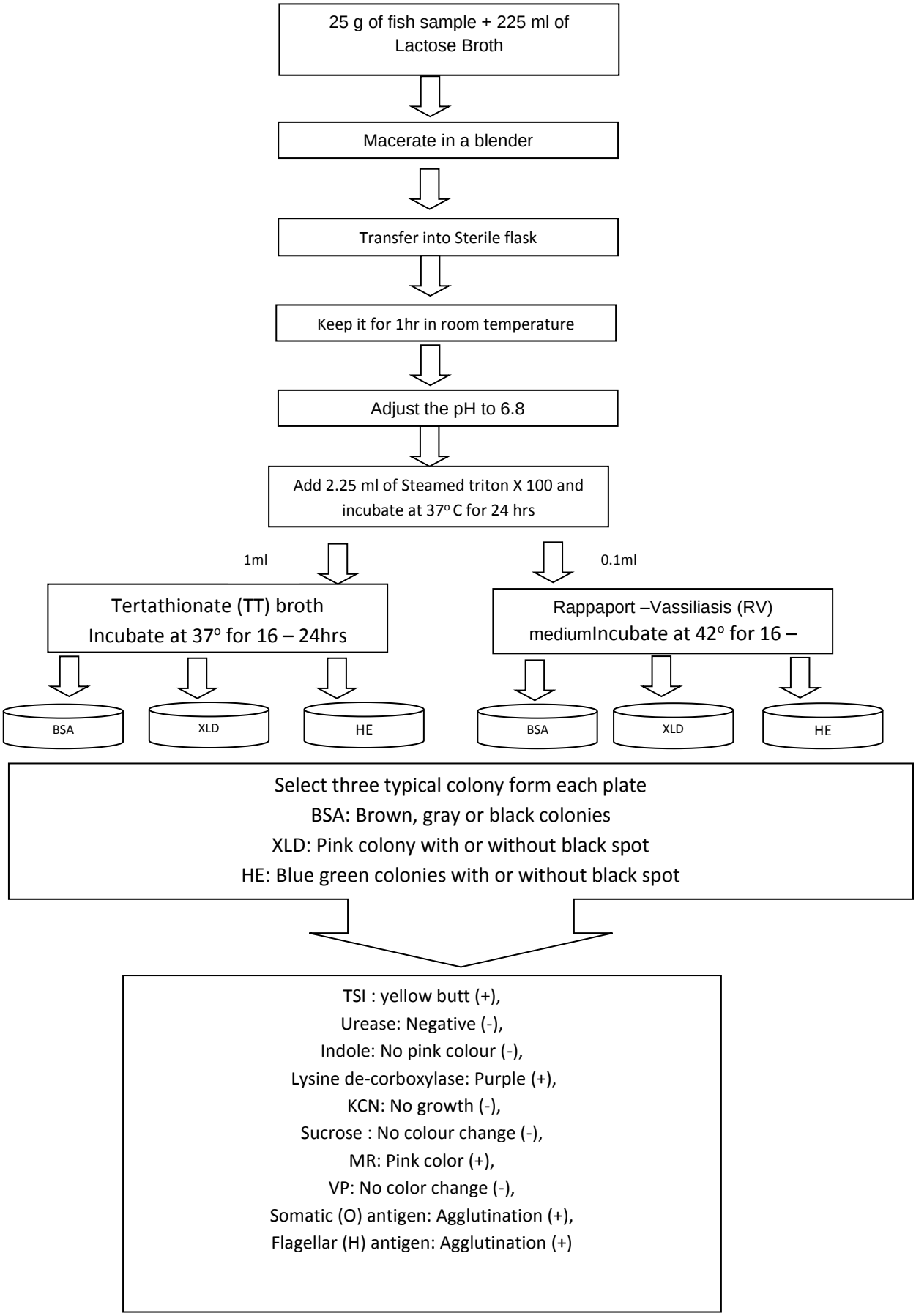
↓

Confirmatory test: catalase positive, mannitol utilization, coagulase, thermostable nuclease production

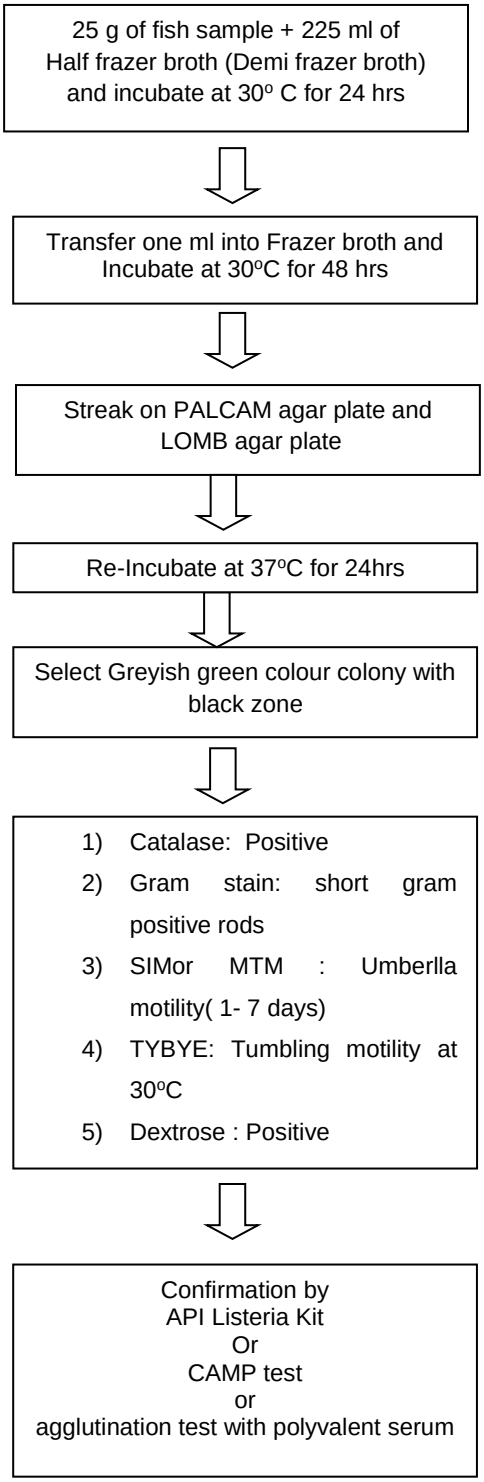
↓

No. *S. aureus*/ g= No. of positive colonies x dilution factor

3. Detection of *Salmonella*



4. Detection of *Listeria monocytogenes*



8. MPN METHOD OF ENUMERATION OF INDICATOR ORGANISM

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Most Probable Number (MPN) Test

Serial dilution tests measure the concentration of a target microbe in a sample with an estimate called the most probable number (MPN). The MPN is particularly useful for low concentrations of organisms (<100/g), especially in milk and water, and for those foods whose particulate matter may interfere with accurate colony counts. Only viable organisms are enumerated by the MPN determination. The expected result is the number of tubes and the number of tubes with growth at each dilution, will imply an estimate of the original, undiluted concentration of bacteria in the sample.

The MPN is the number which makes the observed outcome most probable. It is the solution for λ, concentration, in the following equation

$$\sum_{j=1}^{k} \frac{g_j m_j}{1-\exp(-\lambda m_j)} = \sum_{j=1}^{k} t_j m_j$$

where exp(x) means ex, and

K denotes the number of dilutions,

gj denotes the number of positive (or growth) tubes in the *j*th dilution,

mj denotes the amount of the original sample put in each tube in the *j*th dilution,

tj denotes the number of tubes in the *j*th dilution.

The 95 percent confidence intervals in the tables have the following meaning:

Before the tubes are inoculated, the chance is at least 95 percent that the confidence interval associated with the eventual result will enclose the actual concentration. MPN is used to estimate the presence of viable coliforms group of bacteria in a replicate liquid broth (ten-fold dilutions). It is commonly used in estimating microbial populations in fish, waters and ice samples. MPN is most commonly used for checking the quality of water whether it's *safe or not.* Coliform is a group of bacteria belongs to the enterobacteriaceae and assessing at three levels viz., Total coliforms, fecal coliforms and *E. coli*. The presence of fecal coliforms clearly indicates the fecal contamination and its presence in large numbers would indicate a possibility of containing the disease- producing coliforms.

Coliform bacteria are rod-shaped Gram-negative non-spore forming and motile or non-motile bacteria which fermentlactose with the production of acid and gas at 37^{o}C. The presence of

coliform group of bacteria is commonly used as an indicator of sanitary quality of foods and water. Coliforms can be found in the soil, vegetation and aquatic environment and they are normally present in large numbers in the feces of warm-blooded animals

Coliform group of bacteria belongs to the genera such as

- *Citrobacter*
- *Enterobacter* not of fecal origin
- *Klebsiella*
- *Escherichia* - originate from feces

***Escherichia coli* (*E. coli*)**, a rod-shaped member of the coliforms group, can be distinguished from most other coliforms by its ability to ferment lactose at 44°C in the fecal coliform test. Confirmed on an eosin methylene blue (EMB) plate (metallic green colonies on a dark purple media). *E. coli* are mainly of fecal sources of animal and human. Other coliform bacteria will appear as thick, slimy colonies, with non-fermenters being colorless, and weak fermenters being pink.

A **fecal coliform** is a facultatively anaerobic, rod-shaped, gram-negative, non-sporulatingbacterium and is mainly from the intestines of warm-blooded animals. Fecal coli forms are capable of growth in the presence of bile salts, oxidase negative and utilize produce lactose(produce acid and gas) at 44 ± 0.5°C within 48 hours. Because of its growth at 44 ± 0.5°C called as "thermotolerant coliform".

Principle

Sample is diluted serially and inoculated in lactose broth and the lactose is utilized by the coliforms group of bacteria and leads to produce acid and gas. The presence of acid is indicated by color change of the medium and the presence of gas is detected as gas bubbles collected in the inverted durham tube. The number of total coliforms is determined by counting the number of positive reaction *(color change and gas production) in the* tubes and checking the number of positive tubes at each dilution with standard MPN tables.

MPN test is carried out in 3 steps

1. Presumptive test – Presumptive coliforms
2. Confirmatory test- Total Fecal coliforms
3. Completed test- *E. coli*

Step 1: Presumptive test: Screening test for the coliform group of organisms.

Requirements:

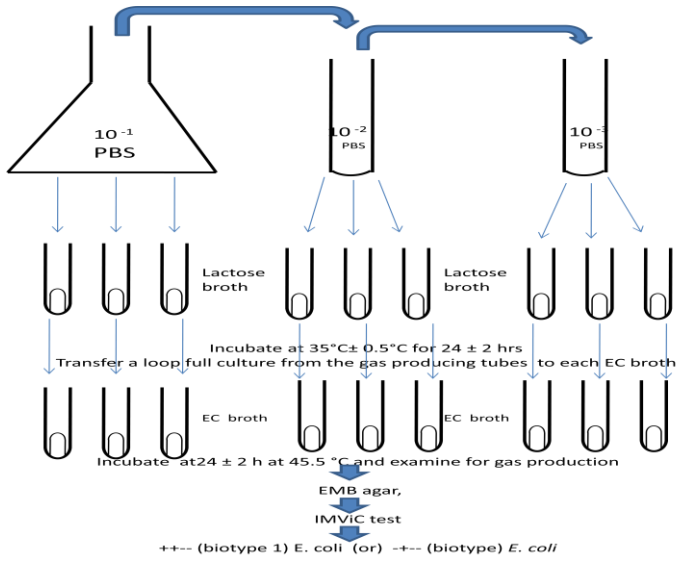
Medium: Lactose broth or Mac Conkey Broth

Glasswares: Test tubes of various capacities (20ml, 10ml, 5ml), Durham tubes

Plasticwares: Sterile pipettes and tips

Others: Discard box, Spirit .

Enumeration on E. coli by MPN method



Preparation of the Medium

Prepare the medium (Mac Conkey or Lactose broth) in single and double strength concentration. Dispense the double strength medium and single strength medium either 5 ml or 10 ml (5 tubes for solid/ semi solid samples and 10 tubes for water and ice) in each tube and put durham tube in inverted position without air bubbles. Sterilize the medium by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

MPN Testing of samples

1. Take 5 tubes of double strength and 10 tubes of single strength.
2. Add 10 ml of the samples to 5 tubes containing 10 ml double strength medium.
3. Add 1 ml of sample to 5 tubes containing 10 ml double strength strength medium and 0.1 ml water to remaining 5 tubes containing 10 ml double strength medium.
4. Incubate all the tubes at 37°C for 24 hrs.
5. Observe at 24 hrs, If no tubes shows positive for growth and gas production, re-incubate up to 48 hrs.
6. Note the number of tubes for positives from each sets and compare the number of tubes giving positive
7. reaction to the 5tubes MPN standard chart and record it.
8. The result is the total number of bacteria present in the sample as MPN values.
9. *For example: 5–4–3 (5 × 10 ml positive, 4 × 1 ml positive, 3 × 0.1 ml positive) = the MPN value is280. So sample contains an estimated 280 coliforms per 100 gram*

StepII – (For confirmed total coliforms)

Requirements :- BGLB 2% broth.

Inoculate one loopful of culture from the +ive tubes of step I to BGLB 2% broth. Incubate at 370C for 24 hrs. Note growth and gas production. Results are noted as +ives if there are growth and gas production. Compare with 3 tube MPN table.

Step III – (For faecal coliforms and *E.coli*)

From the +ive tubes of StepII, inoculate one loopful each to EC broth and Tryptone broth. (indole medium). Incubate at 44.50 °C for 24 hrs.

EC broth:- Growth and gas production.

Tryptone broth:- Test for indole produces by adding 4 drops of Kovac's indole reagent. A pink or red color at the top layer indicates a +ive test for indole.

Coliforms bacteria which products gas in EC broth and indole in tryptone broth of 44.50 °C are *E.coli*.

A loopful of sample from each tube showing positive test *(color change with gas)* is streaked onto two selective medium like Eosin Methylene Blue agar or Endo's medium. One plate each is incubated at 37°C and another at 44.5± 0.2°C for 24 hours.

High temperature incubation (44.5 ±0.2) is for detection of thermo tolerant *E.coli.*

9. BIOCHEMICAL TESTS

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Introduction

Bacteria do have the biochemical fingerprints that are properties controlled by the cellular enzymatic activity. Biochemical identification characterization of bacteria is based on the extracellular enzyme activity and intracellular enzyme activity. Extracellular enzymes are elaborated out of the bacterium and usually performs the action of hydrolysis to break down complex molecules to simpler building block units which can be further utilized by the bacteria after transporting into the cell. Whereas on the other hand the intracellular enzyme functions inside the cell for the metabolism and the metabolic products are excreted out of the bacterium. This metabolic product accumulated outside of the bacterium is detected in the biochemical test. Biochemical methods involve the identification of activity of both the types of enzymes.

- Tests used to identify the extracellular enzymes activity are starch hydrolysis, lipid hydrolysis, casein hydrolysis, chitin hydrolysis etc.,
- Tests used to identify the intracellular enzyme activity basically identifying the end product of the reaction are carbohydrate fermentation, litmus reaction, H2S production, nitrate reduction, catalase, oxidase, IMVC, TSI etc.,

For the starch, lipid and protein hydrolysis test,starch, tributyrin, skim milk powder are added in the nutrient agar or composition mentioned in appendix section and checked for their respective activity.

Starch hydrolysis

The degradation of starch molecule by amylase to shorter polysaccharides maltose and dextrin. Overnight grown cultures were streaked onto the starch agar and incubated at different temperature according to the optimum growth of the different bacteria for 24 or 48h. Pour potassium iodide solution or gram's iodine solution over the colony and observe it under the light. Observing a zone of clearance against the dark blue background is the positive and no clearance zone around the colony is negative for the starch hydrolysis test.

Lipid hydrolysis

The degradation or hydrolysis of lipid molecule by lipase to shorter fatty acid molecule and glucerol or alcohol. Overnight grown cultures were streaked onto the tributyrin agar and incubated at different temperature according to the optimum growth of the different bacteria

for 24 to 48h. observe it under the light. Observing a zone of clearance around the colony is considered as positive and no clearance zone around the colony is negative for the lipid hydrolysis test.

Protein hydrolysis

The degradation or hydrolysis of high molecular weight protein molecule by protease to shorter peptides. Overnight grown cultures were streaked onto the skim milk or casein agar and incubated at different temperature according to the optimum growth of the different bacteriafor 24 to 48h observe it under the light. Observing a zone of clearance around the colony is considered as positive and no clearance zone around the colony is negative for the lipid hydrolysis test.

Carbohydrate fermentation test

Bacteria obtain their energy through series of enzymatic reactions by majority of cases oxidation of carbohydrate substrates. Some bacteria utilize sugars either in aerobic respiration or through fermentation pathway. Whereas the facultative anaerobes use both pathways. Some of the bacteria do not use sugar also. Bacteria can be differentiated based on the carbohydrate fermentation for many types of sugars. Inoculate overnight grown fresh cultures into the carbohydrate fermentation broth incorporated individually with various sugar. Incubate at various temperature .according to the requirement of bacteria and incubate for 24h to 48h. Observe it for the characteristic colour change.

Oxidase test

During aerobic respiration, oxidase enzymes (intracellular cytochrome) catalyzes the oxidation of reduced cytochrome by molecular oxygen which results in the formation of H20 or H_2O_2 depending on the type of enzyme system they possess. Oxidase activity was found in the aerobic, facultative anaerobes and microaerophiles. Obligate anaerobes were negative for the oxidase activity. In general, Gram positive organism was oxidase negative with exception of Bacillaceae and Gram negative in exception to the Enterobacteriaceae were found in majority of the cases.

Principle

Determination of ability of bacteria to produce cytochrome oxidases. This is confirmed by the oxidization of light pink substrat (p-aminodimethyl alaniline oxalate) as electron donors and the substrate is oxidized to the blackish compound in the presence of free oxygen and oxidase enzyme.

Method

- Prepare for the young culture in TSA slant or plate
- Add directly the substrate containing solution as 1% or 0.5% on the colony or pour the solution on to the Whatman filter paper No.1 and pick a colony of the young culture and streak onto the filter paper loaded with substrate.

Observation

- Dark pink, maroon, finally black or purple colour development denotes positive for oxidase test. No colour change or light pink indicates negative for oxidase test. The result should be read within 10 to 30 seconds.

Catalase test

In aerobic respiration the bacteria produce hydrogen peroxide and toxic superoxide. Accumulation of these toxic compound result in death of cell. In order to avoid this the bacteria, produce catalase to rapidly degrade hydrogen peroxide. Superoxide dismutase is the enzyme used for the degradation of the toxic superoxide. So catalase production can be determined by the addition of 3% H2O2 and observe for the bubbles of free oxygen as gas in the slide. Keep three drops of 3% H2O2 and add a minute quantum of culture picked out from individual isolated colony or drop H2O2 on to the colony and observe for bubbling or foaming.

10. STAINING METHODS

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Introduction

Staining is technique used in microscopy to enhance contrast in the microscopic image. Stains and dyes are frequently used in biological tissues for viewing, often with the aid of different microscopes. Stains may be used to define and examine bulk tissues (highlighting, for example, muscle fibers or connective tissue), cell populations (classifying different blood cells, for instance), or organelles within individual cells. Bacteria have nearly the same refractive index as water, therefore, when they are observed under a microscope they are opaque or nearly invisible to the naked eye. Different types of staining methods are used to make the cells and their internal structures more visible under the light microscope. Microscopes are of little use unless the specimens for viewing are prepared properly. Microorganisms must be fixed & stained to increase visibility, accentuate specific morphological features, and preserve them for future use

Stain

A stain is a substance that adheres to a cell, giving the cell color. The presence of color gives the cells significant contrast so they are much more visible. Different stains have different affinities for different organisms, or different parts of organisms. They are used to differentiate different types of organisms or to view specific parts of organisms

Staining techniques

Direct staining - The organism is stained and background is left unstained Negative staining - The background is stained and the organism is left unaltered Stains are classified as

- Simple stain
- Differential stain
- Structural or special stains

Fixing Before staining it is essential to fix the bacterial sample on to the slide. Smear is prepared in the following way:

(i) With a wire loop place a small drop of the broth culture or a loop full of bacteria on a clean slide.

(ii) Place a drop of water over it.

(iii) Spread the culture so as to form a thin film.

(iv) Allow slide to dry in the air or by holding it above a bunsen flame.

(v) Avoid excess heating.

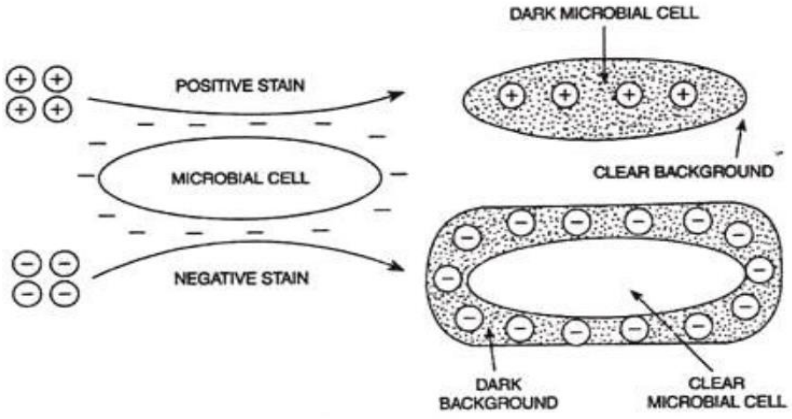
The purpose of fixation is to kill the microorganisms, coagulate the protoplasm of the cell and cause it to adhere to the slide Simple Staining The staining process involves immersing the sample (before or after fixation and mounting) in dye solution, followed by rinsing and observation. Many dyes, however, require the use of a mordant, a chemical compound that reacts with the stain to form an insoluble, coloured precipitate. When excess dye solution is washed away, the mordant stain remains. Simple staining is one step method using only one dye. Basic dyes are used in direct stain and acidic dye is used in negative stain. Simple staining techniques is used to study the morphology better, to show the nature of the cellular contents of the exudates and also to study the intracellular location of the bacteria.

Commonly used simple stains are

- Methylene blue
- Dilute carbol fuchsin
- Polychrome methylene blue

Simple Staining Procedure:

When a single staining-reagent is used and all cells and their structures stain in the same manner, the procedure is called simple staining procedure. This procedure is of two types – positive and negative. In positive staining, the stain (e.g., methylene blue) is basic (cationic) having positive charge and attaches to the surface of object that is negatively charged. In negative staining, the stain (e.g., India ink, nigrosin) is acidic (anionic) having negative charge and is repelled by the object that is negatively charged, and thus fills the spaces between the objects resulting in indirect staining of the object.



Simple staining of microbial cell.

Differential Staining

Differential Stains use two or more stains and allow the cells to be categorized into various groups or types. Both the techniques allow the observation of cell morphology, or shape, but differential staining usually provides more information about the characteristics of the cell wall (Thickness). Gram staining (or Gram's method) is an empirical method of differentiating bacterial species into two large groups (Gram-positive and Gram-negative) based on the chemical and physical properties of their cell wall. The Gram stain is almost always the first step in the identification of a bacterial organism, While Gram staining is a valuable diagnostic tool in both clinical and research settings, not all bacteria can be definitively classified by this technique, thus forming Gram variable and Gram indeterminate groups as well.

Gram staining

Gram Staining is the common, important, and most used differential staining techniques in microbiology, which was introduced by Danish Bacteriologist Hans Christian Gram in 1884. This test differentiates the bacteria into Gram Positive and Gram Negative Bacteria, which helps in the classification and differentiations of microorganisms.

Principle of Gram Staining

When the bacteria is stained with primary stain Crystal Violet and fixed by the mordant, some of the bacteria are able to retain the primary stain and some are decolorized by alcohol. The cell walls of gram positive bacteria have a thick layer of protein-sugar complexes called peptidoglycan and lipid content is low. Decolorizing the cell causes this thick cell wall to dehydrate and shrink which closes the pores in the cell wall and prevents the stain from exiting the cell. So the ethanol cannot remove the Crystal Violet-Iodine complex that is bound to the thick layer of peptidoglycan of gram positive bacteria and appears blue or purple in colour. In case of gram negative bacteria, cell wall also takes up the CV-Iodine complex but due to the thin layer of peptidoglycan and thick outer layer which is formed of lipids, CV-Iodine complex gets washed off. When they are exposed to alcohol, decolorizer dissolves the lipids in the cell walls, which allows the crystal violet-iodine complex to leach out of the cells. Then when again stained with saffranin, they take the stain and appear red in color.

Materials Required:

Clean glass slides, inoculating loop, Bunsen burner, Bibulous paper ,Microscope ,Lens paper and lens cleaner, Immersion oil, Distilled water , 18 to 24 hour cultures of organisms

Reagents:

1. Primary Stain - Crystal Violet
2. Mordant - Grams Iodine
3. Decolourizer - Ethyl Alcohol
4. Secondary Stain - Saffranin

Gram Stain Procedure

1. Place slide with heat fixed smear on staining tray.
2. Gently flood smear with crystal violet and let stand for 1 minute.
3. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
4. Gently flood the smear with Gram's iodine and let stand for 1 minute.
5. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle. The smear will appear as a purple circle on the slide.
6. Decolorize using 95% ethyl alcohol or acetone. Tilt the slide slightly and apply the alcohol drop by drop for 5 to 10 seconds until the alcohol runs almost clear. Be careful not to over-decolorize.
7. Immediately rinse with water.
8. Gently flood with saffranin to counter counter-stain and let stand for 45 seconds.
9. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
10. Blot dry the slide with bibulous paper.
11. View the smear using a light-microscope under oil-immersion.

Interpretation

Gram Positive: **Blue/Purple Color**

Gram Negative: **Red Color**

Gram Positive Bacteria:*Actinomyces, Bacillus, Clostridium, Corynebacterium, Enterococcus, Gardnerella, Lactobacillus, Listeria, Mycoplasma, Nocardia, Staphylococcus, Streptococcus, Streptomyces* ,etc.

Gram Negative Bacteria: *Escherichia coli* (*E. coli*), *Salmonella*, *Shigella*, and other Enterobacteriaceae, *Pseudomonas*,*Moraxella*, *Helicobacter*, *Stenotrophomonas*, *Bdellovibrio*, acetic acid bacteria, *Legionella* etc.

Acid-fast staining

The Ziehl–Neelsen stain, also known as the acid-fast stain, widely used differential staining procedure. The Ziehl – Neelsen stain was first described by two German doctors; Franz Ziehl (1859 to 1926), a bacteriologist and Friedrich Neelsen (1854 to 1894) a pathologist. In this type some bacteria resist decolourization by both acid and alcohol and hence they are referred as acid-fast organisms. This staining technique divides bacteria into two groups namely acid-fast and non acid-fast. This procedure is extensively used in the diagnosis of tuberculosis and leprosy. Mycobacterium tuberculosis is the most important of this group, as it is responsible for the disease called tuberculosis (TB) along with some others of this genus

Principle

Mycobacterial cell walls contain a waxy substance composed of mycolic acids. These are β-hydroxy carboxylic acids with chain lengths of up to 90 carbon atoms. The property of acid fastness is related to the carbon chain length of the mycolic acid found in any particular species.

Ziehl- Neelsen Procedure

1. Make a smear. Air Dry. Heat Fix.
2. Flood smear with Carbol Fuchsin stain
3. Carbol Fuchsin is a lipid soluble, phenolic compound, which is able to penetrate the cell wall
4. Cover flooded smear with filter paper
5. Steam for 10 minutes. Add more Carbol Fuchsin stain as needed
6. Cool slide
7. Rinse with Distilled water
8. Flood slide with acid alcohol (leave 15 seconds). The acid alcohol contains 3% HCl and 95% ethanol, or you can decolorize with 20% H_2SO_4
9. Tilt slide 45 degrees over the sink and add acid alcohol drop wise (drop by drop) until the red color stops streaming from the smear
10. Rinse with Distilled water
11. Add Loeffler's Methylene Blue stain (counter stain). This stain adds blue color to non-acid fast cells. Leave Loeffler's Blue stain on smear for 1 minute
12. Rinse slide. Blot dry.
13. Use oil immersion objective to view.

Capsule staining

The purpose of the capsule stain is to reveal the presence of the bacterial capsule, the water-soluble capsule of some bacterial cells is often difficult to see by standard simple staining procedures or after the Gram stain. The capsule staining methods were developed to visualize capsules and yield consistent and reliable results Capsule may appear as clear halo when a fresh sample is stained by Grams or Leishman stain, Negative staining- using - India ink, Nigrosin.

India ink

Commercially available India ink is used undiluted

Procedure

1. Place a loop full of India ink on the slide
2. A small portion of the culture is emulsified in the drop of ink
3. Place a clean cover slip over the preparation without bubbles. Press down gently
4. Examine under dry objective

Uses

India ink is used to demonstrate capsule which is seen as unstained halo around the organisms distributed in a black background eg. *Cryptococcus*

Endospore Staining (Bartholomew and Mittwer's Method):

Requirement

1. Cell suspension of endospore producing bacteria.
2. Malachite green stain.
3. Saffranin stain.

Procedure

1. Take a clean grease free slide and prepare a thick smear on a slide.
2. The smear is heat fixed by passing the slide from the flame for about 25 times.
3. The slide is allowed to cool.
4. Further the slide is treated with Malachite green stain and allowed it to react for about 10 minutes.
5. After 10 minutes slide is given a water wash treatment.
6. Further the slide is treated with counter stain that is saffranin for about 30 seconds.
7. After 30 seconds the slide is water washed, air dried and observed under oil immersion.

Mechanism

1. In this staining technique a longer heat treatment and prolonged staining technique.
2. Endospore gets stained due to longer heat treatment, prolonged staining and heavy concentration of stain.
3. Here we pass he slide from flame for about 25 times in addition we use concentrated stain that is 7.6 % Malachite green for about 10 minutes.
4. This technique stains the cell as well as the endospore.
5. When we give water wash treatment the water acts as a weak decolorizing agent and decolorizes cytoplasm and not endospore.

6. So here further we apply a counter stain that is Saffranin.
7. Due to application of saffranin the cytoplasm gets stain in pink colour.

Observation

The endospore appears green in colour as well as cytoplasm appears pink in colour.

Flagella Staining (Liefson's Method):

- Bacteria have two types of locomotory organs and that are Flagella and pili.
- Flagella are a thin, hair like structure made up protein called as flagellin.
- It sizes ranges from 20 µ to 200 µ in length.
- Flagella is one of the most important locomotory organ. It is mainly made up of three parts- 1) Basal body 2) Filament 3) Hook.
- Flagella are generally present in rod shape bacteria and very few cocci shape bacteria possess flagella.
- As flagella are very thin and hair like they cannot be easily observed under microscope.
- So a special technique is design to increase thickness of flagella as well as stain it.
- Due to this technique we can observe structure of flagella easily under microscope.

Requirement : Flagellated cell culture slant, Leifson's stain, 1 % Methylene blue, Distilled water.

Procedure:

- Take two hours old flagellated cell culture slant and add two to three drops of sterile distill water in the slant with the help of sterile pipette.
- The distill water is added slowly without disturbing the growth of cells.
- After addition of distill water incubated the slant for 20 minutes.
- Then take a drop of suspension from the slant and place the drop on a clean slide which is kept in slanting position.
- The drop should flow slowly from one end of slide to other end to avoid folding of flagella on cell.
- Allow smear to air dry.
- After air drying the slide is flooded with Leifson's stain till a thin film of shinny surface appear.
- After this give a gentle stream of water wash treatment to a slide.
- Treat the slide with 1 % methylene blue treatment for 1 minute.

- Give the slide water wash treatment, air dry and observe under oil immersion lens.

Mechanism

- First of all, in this procedure thickness of flagella is increase so it can be visible.
- The Leifson's stain is made up of tannic acid, basic fuschin stain prepared in alcohol base.
- When we treat Leifson's stain with cell the tannic acid get attach to the flagella and alcohol get evaporated.
- After evaporation of alcohol the thickness of flagella is increased due to deposition of tannic acid.
- Whereas Basic fuschin stain the Flagella.
- After Leifson's stain treatment cells are treated with Methylene blue stain.
- This Methylene blue stains the cell.

Result

Flagella appear red in colour and bacterial cell appears blue in colour.

11. ISOLATION AND IDENTIFICATION OF PATHOGENIC VIBRIOS FROM SEAFOOD

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Introduction

Seafood is a nutritious food that constitutes one of the desirable components of a healthy diet. Nevertheless, there is health risks associated with the consumption of seafood. One of the major risks involves the consumption of raw or undercooked seafood that may be naturally contaminated by foodborne pathogens present in the marine environment. Such risk is further increased if the food is mishandled during processing where pathogens could multiply exponentially under favorable conditions. In contrast to most other foodborne pathogens, Vibrio spp. has the aquatic habitat as their natural niche. As a result, vibrios are most commonly associated with seafood as natural contaminants. Foodborne infections with Vibrio spp. are common in Asia. Most of these foodborne infections are caused by *V. parahaemolyticus* and *V. cholerae*, and to a lesser extent by *V. vulnificus* and *V.mimicus.*

Vibrio mimicus

Vibrio mimicus is a *Vibrio* species that mimics *V. cholerae*. *V. mimicus* has been recognized as a cause of gastroenteritis transmitted by raw oysters, fish, turtle eggs, prawns, squid, and crayfish. *V. mimicus*, when carrying genes that encode cholera toxin, can cause severe watery diarrhea. Consumers and physicians should be aware that improperly handled marine and aquatic animal products can be a source of *V. mimicus* infections. Consumers should avoid cross-contamination of cooked seafood and other foods with raw seafood and juices from raw seafood and should follow FDA recommendations for selecting seafood and preparing it safely.

Vibrio cholerae

V. cholerae, a Gram-negative motile rod causes massive cholera outbreaks. Cholera is a global threat to public health and it was estimated that between 2008 and 2012 cholera caused an annual average of 2.9 million cases, and 95,000 deaths, worldwide Particular serogroups (O1 and O139) of this bacterium are responsible for cholera epidemics and pandemics. Human infection with *V. cholerae* begins with ingestion of contaminated food or water containing the bacterium.

V. cholerae colonizes the small intestine and secretes cholera enterotoxin (CT) into the host cells resulting in rapid efflux of chloride ions and water into the lumen of the intestine, leading to profuse diarrhea and severe dehydration. *V. cholerae* is commonly associated with

chitin-containing zooplankton, particularly copepods and chironomids. Recent evidence supports the hypothesis that fish and water birds may also be intermediate reservoirs and vectors of *V. cholerae*.

Vibrio parahaemolyitcus

Vibrio parahaemolyticus was first discovered by Tsunesaburo Fujino in 1950 as a causative agent of food borne disease following a large outbreak in Japan which recorded 272 illnesses with 20 deaths after consumption of *shirasu*. Virulent *V. parahaemolyticus* strains are transmitted by consumption of raw or undercooked seafood causing acute gastroenteritis. Since its discovery, *V. parahaemolyticus* has been found to be responsible for 20–30% of food poisoning cases in Japan and seafood borne diseases in many Asian countries. *V. parahaemolyticus* was also recognized as the leading cause of human gastroenteritis associated with seafood consumption in the United States. The worldwide prevalence of *V. parahaemolyticus* gastroenteritis cases stresses the need for understanding of the virulence factors involved and their effects on humans.

Vibrio vulnificus

V. vulnificus the leading cause of death in the US related to seafood consumption and nearly always associated with raw Gulf Coast oysters resembles *V. parahaemolyticus* on TCBS agar, but can be differentiated by several biochemical reactions, including β-galactosidase activity . Epidemiological and clinical investigations have shown that *V. vulnificus* causes septicemia and death following ingestion of seafood or after wound infections originating from the marine environment . Recent gene probe assays, PCR procedures , fatty acid profiles and enzyme immunoassay have been developed to detect and identify this pathogen.

Protocol for the isolation of *V. mimicus* from fish

25 g of Sample
(Surface tissue, gills, gut-pooled sample)

⇩

Mix 25 g of pooled sample with 225 ml of APW, macerate in a stomacher blender

⇩

Incubate APW at 35 ± 2 ^{0}C for 16 to 18 hours and transfer a loopful from the surface pellicle of APW culture to TCBS plate

⇩

Incubate TCBS Plates overnight at 35 ± 2 ^{0}C

⇩

V. mimicus appears as small 2-3 mm, smooth green colonies on TCBS

⇩

Pick typical colonies on to TSA slants with 2% Nacl

⇩

Proceed for biochemical tests

Biochemical confirmation:

- Oxidase positive
- Gram negative short rods
- String test postive
- Arginine decarboxylase-Negative
- Lysine, Ornithine decarboxylase- postive
- Sucrose Negative
- Growth in 0% salt, no growth in 6% salt

Protocol for the isolation of *V. cholerae* from fish

25 g of Sample

(Surface tissue, gills, gut-pooled sample)

⇩

Mix 25 g of pooled sample with 225 ml of APW, macerate in a stomacher blender

⇩

Incubate APW at 35 ± 2 ^{0}C for 6 to 8 hours and transfer a loopful from the surface pellicle of APW culture to TCBS plate

⇩

Incubate APW overnight at 35 ± 2 ^{0}C

⇩

Again transfer a transfer a loopful from the surface pellicle of APW culture to TCBS plate

⇩

Incubate overnight at 35 ± 2 ^{0}C

⇩

V. cholerae appears as large 2-3 mm, smooth yellow and slightly flattened with opaque centre and translucent peripheries on TCBS

⇩

Pick typical colonies on to TSA slants with 2% Nacl

⇩

Proceed for biochemical tests.

Biochemical confirmation:

- Oxidase positive
- String test postive
- Arginine decarboxylase-Negative
- Lysine, Ornithine Decarboxylase- postive
- Sucrose positive; Growth in 0% salt, no growth in 6% salt

Protocol for the isolation of *V. Parahaemolyticus* from seafood

All the media used for the biochemical identification of *Vibrio parahamolyticus* should contain 2 or 3% Nacl.

25 g of Sample
(Surface tissue, gills, gut-pooled sample)

⇩

Mix 25 g of pooled sample with 225 ml of APW with 3% salt and macerate in a stomacher blender

⇩

Incubate APW overnight at $35 \pm 2^{0}C$

⇩

Streak a loopful from APW onto a TCBS plate with 3% Nacl. Incubate APW overnight at $35 \pm 2^{0}C$

⇩

V. Parahaemolyticus appears as round, opaque, green or bluish colonies 2-3 mm in diameter on TCBS

⇩

Pick typical colonies on to TSA slants with 3% Nacl

⇩

Proceed for biochemical tests.

Biochemical confirmation:

- Oxidase positive
- Gram negative, straight/ curved rods
- Non H_2S producer
- Growth in 3 %, 6%, 8% Nacl, No growth in 0 % Nacl
- *V.Parahaemolyticus* can be differentiated from other Vibrios by ONPG, Salt tolerance and lactose reactions; Resistance to 10 μg of O/129, sensitive to 150 μg of O/129.

Protocol for the isolation of *V. vulnificus* from seafood

All the media used for the biochemical identification of *Vibrio vulnificus* should contain 3% Nacl

25 g of Sample
(Surface tissue, gills, gut-pooled sample)

⇩

Mix 25 g of pooled sample with 225 ml of APW with 3% salt and macerate in a stomacher blender

⇩

Incubate APW overnight at $35 \pm 2\ ^0C$

⇩

Streak a loopful from APW onto a TCBS plate with 3% Nacl. Incubate APW overnight at $35 \pm 2\ ^0C$

⇩

V. vulnificus appears as large green colonies 2-3 mm in diameter on TCBS

⇩

Pick typical colonies on to TSA slants with 3% Nacl

⇩

Proceed for biochemical tests.

Biochemical confirmation:

- Oxidase positive
- Gram negative, straight/ curved rods
- Non H_2S producer
- lactose positive
- Growth in 3 %, 6% Nacl, No growth in 0 % Nacl
- Sensitive to 10 μg of O/129, 150 μg of O/129.

12. BIOCHEMICAL QUALITY ASSESSMENT OF FISH AND FISHERY PRODUCTS

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The seafood is preferred in fresh form and being high in water content needs extra care for preservation. Therefore, fish is simply chilled to extend its shelf life or frozen or converted into different products for consumption. During various stages of post harvest handling fish is exposed to various hazards. The lack of control leads to 'quality deterioration' affecting the quality of the product. The quality of the food in general is a concern from the public health point of view as well.

The term 'quality' means "all those attributes which consciously or unconsciously the fish eater or buyer considers should be present" and which will embrace intrinsic composition, degree of spoilage, damage, deterioration during processing, storage, distribution, sale and presentation to the consumer, hazards to health, satisfaction on buying and eating, aesthetic consideration, yield and profitability to the producer and middle men. The quality of the fish and fishery products is one of the main indices in the activity of any processor. According to ISO, Quality is defined as the totality of features and characteristics of a product or service that bears its ability to satisfy stated or implied needs

Quality of a food material depends on several factors; both intrinsic and extrinsic. The intrinsic factors could be related to the fish species while extrinsic factors are environment related issue which contributes to the contamination with pathogen leading to food borne infections on consumption. Therefore preventing the onset of spoilage and preventing contamination from external sources are to be checked effectively in order to make consumer acceptable fish and shell fish and to avoid the food safety issues.

Seafood differs from other types of food because of its very nature. Fish contains more than 70% water which makes them more prone for spoilage if appropriate measures are not taken. At the time of harvesting fish contains the bacteria and other contaminants naturally present in the ecosystem. Once fish is harvested the new microbes are added from the environment and most of them are pathogenic to consumers. The post mortem changes taking place in the fish provides a suitable environment to the bacteria to multiply, if not controlled by good manufacturing practices. Being perishable, the quality of seafood deteriorates fast resulting in food borne infections on consumption of spoiled fish.

Total Volatile Base Nitrogen (TVBN)

TVBN measures the amount of volatile bases formed from solubilised nitrogen derivatives. It is a measure of decomposition of proteins. TVB-N in fish is mainly composed of ammonia and primary, secondary and tertiary amines. Bacterial catabolism of aminoacids in fish muscle results in the accumulation of ammonia and other volatile bases. Ammonia and primary amines are bound by formalin, therefore this fraction is called the formalin bound nitrogen (FBN). The trimethyl amine (TMA) represents the fraction, which is not bound by formalin. The TVB-N value is used as an index of quality for deciding the state of freshness of fish (along with TMA). A level of 35-40 mg 1VB-N /100g of fish muscle is usually regarded as the limit of acceptability, beyond which the fish can be regarded as spoiled. Generally, there is an increasing trend in TVBN values as the fish gets spoiled.

Principle:-

Volatile basic nitrogen content is mainly constituted by Ammonia. When TCA extract of the sample is treated with saturated Sodium carbonate, Ammonia will liberated which is then trapped in N/100 H2SO4 in the Conway dish. The excess acid in inner chamber is back titrated with N/100 NaOH. The calculated value gives the TVBN of the sample.

Reagents:-

1. N/100 H2SO4 & N/100
2. Saturated Sodium carbonate solution.
3. Mixed indicator.

Procedure:-

Preparation of TCA extract:-

Weigh 10 gms. of fresh muscle sample into a mortar. Add 10 ml. of 20% TCA and ground well. Filter, using Whatman's filter paper No.1 in 50 ml. standard flask. Repeat the extraction with 1% TCA & filter. Collect the washings and make the volume 50ml.

Analysis

1. TVBN is estimated by the micro diffusion Conway method. In the inner chamber of the conway unit place 1 ml. of N/100 H2SO4 and in the outer chamber, 1ml of TCA extract of the sample.
2. Cover the Conway dish with the glass cover smeared with petroleum jelly to give air-tight contact along the outer contact-ring of the unit.
3. Keep just open to draw 1ml of saturated Na_2CO_3 in the outer chamber of the unit, then closed the glass plate to air tight.

4. Mix gently by lateral circular movement.
5. Allow the unit undisturbed to stand overnight (or at 37^0C incubation for 2 hrs.).
6. The acid in the inner chamber is titrated against N/100 NaOH using two drops of mixed indicator, the indicator changing from red to green at the end point- (B).
7. Titrate a reagent blank also by taking standard acid at the central compartment- (A)
8. Perform the assay in duplicate for each sample.

Calculation:-

Value(A-B) is the vol. of N/100 acid used up by volatile base.

(1ml of N/100 acid = 0.14 mg of Nitrogen).

$$\text{TVBN mg \%} = \frac{(A-B) \times 0.14 \times 50 \times 100}{\text{Wt. of sample}}$$

Tri-methyl Amine (TMA)

Trimethylamine (TMA) is used to assess the freshness in marine fish. TMA is derived from trimethylamineoxide (TMAO) which is critical for osmo regulation in marine fish. TMAO is a tasteless non-protein nitrogen compound whose content varies with the season, size and age of fish. During spoilage, TMAO is reduced by enzymes to TMA. The concentration of amines in fish tissues is both time and temperature dependent and is related to the deterioration of fish. The determination of TMA as an indicator of freshness (actually of decay) has been a useful criterion for evaluating the quality of fish. TMA-N between 10-15 mg / 100g muscle is considered as the limit of acceptability for round, whole chilled fish. This index is not suitable for freshwater fish and heat treated fish products.

Principle:-

Tri-methyl amine is a non-protein nitrogenous volatile compound. The quantity of TMA formed is depends primarly upon the concentration of its precursor, TMA-O in the fish muscle. TMAO is reduced during spoilage to TMA. The TMA is often determined by the Conway micro-diffusion technique.

Procedure:-

The same procedure is adopted as TVBN, except that 0.5 ml Neutralized Formalin (prepared by shaking formaldehyde with magnessium carbonate and filtering through Whatman 40 no. filter paper) is added to the outer chamber and swirled to mix before adding Sat. Sod. Carbonate. Formaldehyde is added to fix all the bases except TMA.

Calculation:-

$$\text{TMA mg \%} = \frac{(A - B) \times 0.14}{\text{Wt. Of sample}} \times \frac{50}{1} \times 100$$

FREE FATTY ACIDS (FFA)

The deterioration of lipids has always been of primary concern to fishery technologists. Degradation of lipids falls into two categories: oxidation which leads to of odours and flavours and hydrolysis which splits off free fatty acids. FFA gives a measure of hydrolytic rancidity. Fish muscle contains lipase, which is able to catalyse the hydrolysis of short chain triglycerides. Free fatty acids are suspected of deriving primarily from phospholipids, as the latter disappear with time of storage which can be affected by the action of bacteria, enzymes or non-enzymic catalysis. During spoilage, the amount of free fatty acids increases, which can be measured by reacting with alkali and is expressed as %oleic acid.

Principle:-

Fat spoilage can be assessed by estimating the free fatty acids (FFA) and peroxide value (PV) on a common chloroform extract. The FFA in the sample extract is diluted with alcohol and neutralized by titration with sodium hydroxide. The FFA are expressed as % Oleic acid on the extracted fat.

Reagents:-

1. Chloroform.
2. Anhyd. Sod. Sulphate
3. Neutral Ethyl alcohol (Neutralised with NaOH)
4. Phenolphathalein indicator.
5. 0.01N NaOH

Procedure:-

1. Take about 10 gm. of fresh muscle sample in a mortar & grind well with anhyd. Na_2SO_4untill all water is removed.
2. Transfer this into 250 ml. Iodine flask. Add to this 100 ml Chloroform & keep 30min. in dark .
3. Filter the chloroform extract using filter paper and make the vol. 100ml with chloroform.
4. Weigh 2 nos. of 50 ml conical flasks. Add 20 ml of chloroform extract in each conical flask.
5. Evaporate the extract in water bath & then dry them for 3 hrs in Hot air oven at 100^0C.
6. Cool and weigh the conical flask. This will give the fat content (M) in 20 ml of chloroform extract.
7. Add 10 ml. of warm, neutral alcohol & dissolve the fat.
8. Add 1 drop of phenolphthalein indicator & titrate against 0.01 N NaOH.

Calculation:-

FFA (as oleic acid on extracted fat), % (m/m) :

$$\text{FFA \%} = \frac{V \times N \times 28.2}{M} \times \frac{100}{20} \times \frac{1}{\text{Wt. of sample}}$$

Where: M = Fat content in 20 ml.of chloroform extract
V = Vol. in ml. of NaOH
N = Normality of NaOH
28.2 = milliequivalent weight of oleic acid (include factor of 100 for %).

Peroxide Value (PV)

The highly unsaturated fatty acids found in fish lipids are very susceptible to oxidation. The primary oxidation products are the lipid hydroperoxides. These compounds can be detected by chemical methods, generally by making use of their oxidation potential to oxidize iodide to iodine or to oxidize iron(II) to iron(III). The concentration of the hydroperoxides may be determined by titrimetric or by spectrophotometric methods, giving the peroxide value (PV) as

milliequivalents (mEq) peroxide per 1 kg of fat extracted from the fish. The most common method is based on iodometric titration which measures the iodine produced from potassium iodide (KI) by the peroxide present in fat. PV is a good guide to assess the quality of fat. Fresh oil should have PV 1 mg.oxygen/kg. On storage it may increase to 10 mg/kg.

Principle:-

During oxidation of fat peroxide is formed. Peroxide value gives measure of oxidative rancidity. The peroxide value is a measure of peroxides contained in the oil.The peroxide value is usually determined volumetrically by method which depends on the reaction of potassium iodide in acid solution with the peroxide oxygen followed by titration of the liberated iodine with Sodium thiosulphate solution.

Reagents:-

1. Glacial acetic acid.
2. 1 % starch solution.
3. N/100 Sod. Thiosulphate solution
4. Pot. Iodide.

Procedure:

1. In a 250 ml. Iodine flask, take 20 ml. of chloroform extract (prepared in FFA)
2. Add about 30 ml of glacial acetic acid and 1 gm of KI &keep in dark for about 30 min. with occasionally swirling.
3. Take out and add 1 cc. 1% starch solution.
4. Titrate liberated iodine with N/100 Sod. Thiosulphate solution.

Calculation:-

$$\textbf{PV}\% = \frac{V \times N}{M} \times \frac{100}{20} \times \frac{100}{\text{Wt. of sample}}$$

V = ml of Sod. Thiosulphate solution used
N = Normality of Sod. Thiosulphate
M = Fat content in 20 ml chloroform extract

Thiobarbituric Acid (TBA)

TBA index is the most used indicator for advanced lipid oxidation. TBA measures the malonaldehyde produced during fat oxidation

Principle:-

Oxidised lipids are formed as fats become rancid. Thiobarbituricacid will react with these fatty lipids to form a red – colored complex which can be determined spectrophotometrically. Malonaldehyde is one of the end products of oxidative rancidity and is believed to be involved in the reaction with TBA. Therefore the TBA value is expressed as mg malonaldehyde per Kg sample. The TBA test is applicable to fatty foods (e.g. meat) as well as fats and oils.

Reagents:-

- TBA reagent:- 0.2883gm in 100ml of 90% glacial acetic acid.

Procedure:-

1. Weigh 10 g of prepared sample in a round bottom flask and add a glass bead and 100ml solution (3ml 2:1 HCl + 97 ml DW = 100ml) & mix.
2. Collect 50 ml distillate by steam distillation.
3. Pipette 5ml of distillate into a glass stoppered tube, add 5mlTBA reagent, stopper, shake and heat in boiling water bath for 40min.
4. Prepare a blank similarly using 5ml DW with 5ml reagent.
5. Then cool the tubes in water for 10min. and measure the absorbance (A) against the blank at 538nm.

Calculation:-

$$\text{TBA no. (as mg malonaldehyde / Kg sample)} = \frac{7.8 \times A}{\text{Wt. of sample}} \times \frac{50}{5}$$

$$= 7.8 \times A$$

7.8 is the TBA standard factor.

Free Alpha – amino Acids

In Crustaceans, the free alpha – amino acid is upto 40% of the NPN and in teleosts is only 6%. The attractive flavour invariably present in prawns and other crustaceans is attributable to their comparatively higher contents of free amino acids. The comparatively quicker rates of spoilage occurring in invertebrates than in teleosts may be attributed to the presence of large quantities of free amino – acids in their muscles.

Principle:-

The method depends on the formation of soluble copper compounds through the complex reaction between the amino acids and excess copper in the form of $CuSO_4$. The amount of copper taken into solution by amino acids or similar material is determined iodometrically.(Pope and Stevens method).

Reagents:-

1) Cupric Chloride - $CuCl_2.2H_2O$ - 27.3gms/lit.
2) Tri Sodium Phosphate - Na_3PO_4. 12 H_2O - 64.5gm.
3) Borate Buffer - $Na_2B_4O_7$.10 H_2O - 57.2gms in 1.5 lit. of water, add 100ml 1N HCl. Dilute to 2 liters with water.
4) Cupric – Phosphate Suspension:- 1 vol. of $CuCl_2$ + 2 vol. of Na_3PO_4+ 2 vol. of Borate buffer and mix.
5) Thymolphthalein indicator
6) Std. N/100 Sod. Thiosulphate solution
7) Starch solution:- 0.5% (preapare fresh)
8) 1% NaOH soln.
9) Potassium Iodide (KI)
10) Glacial acetic acid

Procedure :-

1) Pipette out 25ml of TCA extract of the sample (as prepared for TVBN) in 100ml std. Flask)
2) Add 2 drops of Thymolphthalein indicator. Neutralise this TCA acid with 1% NaOH soln. till light blue color appears.
3) Then add 35ml Cupric – Phosphate Suspention. Make the vol. upto 100ml with DW. Mix it properly and filter.
4) Pipette out 20ml of filtrate in 150ml conical flask. Add about 1gm of KI and 15ml of glacial acetic acid.

5) Titrate this rapidly with N/100 $Na_2S_2O_3$ till light yellow color.
6) Then add 1ml of starch soln. again titrate with N/100 $Na_2S_2O_3$ till blue color gets disappear. Note down the reading.

Calculation :- 1ml of 0.01 N $Na_2S_2O_3$ = 0.28 mgm of alpha amino nitrogen.

Alpha amino nitrogen (mg%)= $\frac{0.28 \times V}{\text{Wt of sample}} \times \frac{50}{25} \times \frac{100}{20} \times 100$

V = Titre value.

Non – Protein Nitrogenous Compounds (NPN)

NPN compounds generally encountered in fish muscle comprise Ammonia, TMA-bases, Guanidine and Imidazole derivatives and miscellaneous substances like Urea, Amino-acids, Purines and Pyrimidines.

NPN can be determined by the micro kjeldahl distillation method.

Procedure :-

1) Take 10ml of TCA extract in the Kjeldahldigetion flask.
2) Add a pinch of digestive mixture and 10ml of conc. H_2SO_4.
3) Digest the mixture until the contents are clear.
4) Cool and dilute to 50ml ammonia free distilled water.
5) Take 10ml for distillation.
6) Titrate the distillate with N/100 H_2SO_4.

Calculation :-

(1ml of N/100 acid = 0.14 mg of Nitrogen)

$$\% \text{ of NPN} = \frac{0.14 \times V}{\text{Wt of sample}} \times \frac{50}{10} \times \frac{50}{10} \times 100$$

Determination of Indole in Shrimp

Indole is used as an index of decomposition. Indole formation in shrimp is supposed to be due the action of bacteria such as Proteus morganii, *E. coli*. on shrimp protein. The amount of indole produced is proportional to the extent of decomposition. Shrimp can decompose in

the absence of indole-producing organisms. Therefore the presence of indole in shrimp definitely decomposition but the absence cannot ensure that the product is free from spoilage.

Indole is extracted with light petroleum from trichloroacetic acid – precipitated shrimp muscle. The extracted indole, soluble in light petroleum, is reacted and re-extracted with Ehrlich's reagent. Indole in the form of a rose indole complex can be determined spectrophotometrically.

Reagents:-

1) Trichloroacetic acid (TCA) :- 6gm of TCA dissolve in 100ml DW .
2) Petroleum ether, Boiling point 40 – 60^0C.
3) **Ehrlich's reagent**:- Dissolve 9gm para-dimethylaminobenzaldehyde in 45ml conc. HCl acid in 250ml volumetric flask and dilute to volume with ethanol.
4) Std. Indolesolutions :- Accurately prepare stock solution of 10mg indole in 100ml light petroleum. Use 1:10 dilution (with petroleum) working solution. Refrigerate indole solutions.

Procedure:-

1) Homogenise 40gm shrimp with 80ml ice-cold TCA solution in a warring blender one min. Add 80ml ice-cold light petroleum and blend for one min.
2) Transfer homogenate to 250ml centrifuge bottle and cenrifuge 10min. at 10,000 rpm. Filter supernate through whatman no. 1 paper under suction.
3) Transfer filtrate to 250ml separatory funnel. After the two layers have separated, transfer acid layer (lower) to second 250ml separatory funnel.
4) Wash TCA- denatured protein precipitate separated by centrifugation with 40ml light petroleum and filter as described above.
5) Transfer filtrate to second 250ml separatory funnel already containing TCA layer from first extraction.
6) Shake 1 min. and let 2 layers separate. Transfer lower acid layer to third separatory funnel and extract for third time with 40ml light petroleum .
7) Combine all light petroleum extracts into 1 separatory funnel.
8) Extract indole with exactly 5ml freshly prepared Ehrlich's reagent by vigorously shaking 1 min.
9) The rose indole complex formed is quantitatively transferred to Ehrlich's reagent layer.
10) When layers have separated, transfer lower layer to 1 cm path cell and read at 570nm against reagent blank solution.
11) Prepare standard curve as follows.
12) Accurately measured volumes from 0.5 to 4ml (5 to 40 microgm) stock indole solution (working solution) into 80ml TCA in separatory funnel.

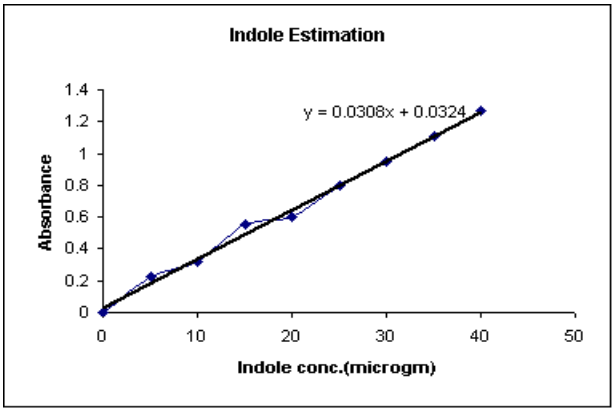
13) Extract indole by procedures described above and construct standard curve.
14) Rose indole complex from indole standard and from TCA- extracted shrimp is stable up to 4 hour.

Calculation:-

With the help of the standard curve the amount of indole present in 40gm shrimp can be determined. Indole content is usually expressed as the amount of indole in microgram per 100gm shrimp muscle. 250 microgram per kilogram is the limit.

Standard Graph

Conc.	Absorbance
0	0
5	0.23
10	0.32
15	0.55
20	0.6
25	0.8
30	0.95
35	1.11
40	1.27



Sulphur dioxide Estimation by Modified Monier – Williams Apparatus

Reagents Required:-

1) 3% Hydrogen peroxide
2) 0.25% Methyl red indicator
3) 0.1N Pot. Permangnate
4) 1:2 Hydrochloric acid
5) 0.1N Sod. Hyroxide soln.

Procedure :-

1) Assemble as shown in the figure.
2) Take 40ml of neutralized Hydrogen peroxide soln. in each U- tubes.
3) Place 100gm of sample (homogenized) in 3 necked flasks.
4) Add 75ml of 1:2 HCl
5) Add 325ml DW.

6) Start water flow and gas flow.
7) Then switch on heater. Reflux the sample for 30 min.
8) Sulphur dioxide in the sample gets entrapped in hydrogen peroxide in U-tubes
9) Remove and transfer into a flask and titrate against 0.1N NaOH.

Calculation:- 1 ml of 0.1N NaOH = 3.203 mg of SO_2

13. AN INTRODUCTION TO HACCP CONCEPT IN SEAFOOD INDUSTRY

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On December 18, 1995, The Food and Drug Administration (FDA) published as a final rule 21 CFR 123, "Procedures for the Safe and Sanitary Processing and Importing of Fish and Fishery Products" that requires processors of fish and fishery products to develop and implement Hazard Analysis Critical Control Point (HACCP) systems for their operations. The regulation became effective December 18, 1997.

Hazard Analysis and Critical Control Point (HACCP) system is a management system in which food safety is addressed through the analysis and control of biological, chemical, and physical hazards from raw material production, procurement and handling, to manufacturing, distribution and consumption of the finished product. In other words, HACCP is applied throughout the food chain from primary production to final consumption and its implementation should be guided by scientific evidence of risks to human health. Hence, HACCP is the application of common sense and scientific principles to food preparation. Apart from enhanced food safety, implementation of HACCP can provide other significant benefits. One of the advantages of HACCP programme is that it permits lesser destructive sampling than the traditional inspection system. Also, it is capable of accommodating change, such as advances in equipment design, processing procedures or technological developments. Based on the mandatory requirements from the importing countries including USA and European Union, the Expert Inspection Council of India has also formulated HACCP procedure for Indian Seafood Industry and now HACCP has become the guiding principle for Indian Food Industry.

Components of HACCP Plan

There are twelve important components for a HACCP plan/manual. They are

1) Quality Policy
2) Organisational chart
3) Organisational narrative
4) HACCP Team, duties and responsibilities of the members
5) Description of products and end use
6) For each similar product group there should be:
    a) Process flow chart, Hazard analysis worksheet

Process flow chart

A process flow chart is a schematic and systematic representation of the sequence and interactions of steps involved in a process. Flow charts are needed for the implementation of the quality assurance programme based on HACCP system in a production line. Flow chart should contain all the steps in a production process with sufficient details so that the CCP with respect to each possible hazard can be easily identified. The HACCP worksheet and HACCP plan form are prepared based on process flow-diagrams. A model of flow chart is attached as Annexure 1&II.

HACCP worksheet

The HACCP worksheet addresses the first two principles of HACCP. The worksheet should essentially contain the name and address of the production unit, name of the product, indented use of the product, target consumers and method of storage and distribution.

b) HACCP plan forms for each CCP

Every processor shall have and implement a written HACCP plan whenever a hazard analysis reveals one or more food safety hazards that are likely to occur. The HACCP plan form is a tool which helps to manage each CCPs. The plan form addresses the last five principles of HACCP. A HACCP plan form typically contains 10 columns listing the details of CCPs identified, significant hazards at each CCPs, critical limits, monitoring (such as what, how, frequency and who), corrective actions, records and verification. Like worksheet, plan form also should contain details like name and addresses of the production unit, name of the product, intended use of the product, target consumers and method of storage and distribution. It should be specific to: (1) Each location where fish and fishery products are processed by that processor; and (2) Each kind of fish and fishery product processed by the processor.

7) Record keeping procedure

8) Good Manufacturing Practices (GMP)

9) Sanitation Standard Operating Procedure (SSOP)

10) Verification Procedure (HACCP Team)

11) Recall Procedure

12) Labels/Specifications

The HACCP concepts and the system work based on the seven principles of HACCP. They are

(1) **Conduct hazard analysis**: ***List the natural hazards reasonably likely to be associated with the species you process.***

Hazards in seafood may be biological, chemical or physical hazards. These hazards can be introduced both within and outside the processing plant environment, including that can occur before, during, and after harvest. FDA has developed "Fish and Fishery Products Hazards and Control Guide," a guide of species and the hazards normally associated with them including toxins, microbiological growth, and chemical contamination.

Determine Critical Control Points (CCPs): ***At what point can a procedure be applied to prevent, eliminate or reduce the hazard***?

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

2) **Determine Critical Limit**: ***Establish the minimum or maximum limit needed to prevent, eliminate or reduce the hazard to an acceptable level.***

A critical limit is defined as the maximum and minimum value to which physical, biological or chemical parameters must be controlled at a critical point to prevent, eliminate or reduce to an acceptable level the occurrence of the identified food safety hazard.

3) **Establish monitoring Procedures**: ***Establish reliable measuring and frequency of measurements at critical control points.***

This facilitates easy tracking of the operation and used to determine when there is a loss of control and a deviation occurs at a CCP in exceeding or not meeting a critical limit. Apart from that, monitoring provides written documentation for use in verification.

4) **Establish corrective actions**: ***Identify what you will do if any of the critical limits are exceeded or not met***

When the results of monitoring indicate a failure, corrective action must be taken in order to prevent a health hazard.

5) **Establish record keeping and documentation**: ***Most crucial part of HACCP Plan.***

Any record that deals with product safety, test results, process safety, research report, calibration records and inspection records must be approved, signed and dated.

6) **Establish verification procedures**: ***At least once a year, verify that your HACCP plan adequately controls food safety hazards and that it is being implemented***

effectively.

This includes initial validation of the HACCP plan, subsequent validation of the HACCP plan and verification of CCP monitoring as described in the plan

Pre-requisite programs for HACCP

Prerequisite programs cover all the activities which interact within and across various processes, that may influence the food safety outcomes of the product. Good manufacturing Practices (GMP) and Sanitation Standard Operating Procedures (SSOP) are the two important prerequisite programs needed for HACCP implementation.

Good Manufacturing Practices (GMP) are the procedures laid down for achieving safety from plant, machinery, personnel and other infrastructure used in the food production. GMP deals mainly with plant facilities, personnel hygiene, sanitary facilities, equipments and utensils, process control, chemical control, pest control etc.

Sanitation Standard Operating Procedures (SSOP) are written procedures that an establishment develops and implements to prevent direct contamination or adulteration of product. In other words, SSOP should describe all the procedures an official establishment will conduct daily, before and during operations, sufficient to prevent direct contamination or adulteration of products. It mainly deals with safety of water, condition and cleanliness of food contact surfaces, prevention of cross contamination, maintenance of hand washing, hand sanitizing and toilet facilities, protection from adulterants, proper labeling, storage and use of toxic compounds, control of employee health, exclusion of pests etc.

The other programs that are needed to have an effective HACCP implementation are product identification, tracking and recall, preventive maintenance, and education and training of employees.

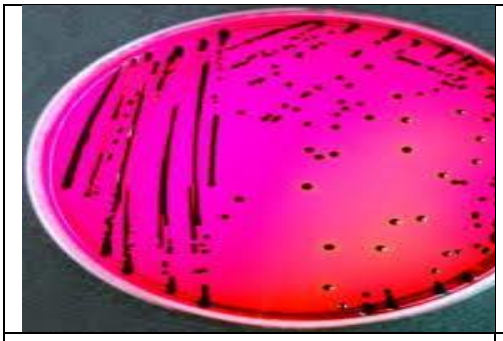
Special Considerations in HACCP Planning

Imports: State how you ensure that any imported products in your processing comply with HACCP regulations.

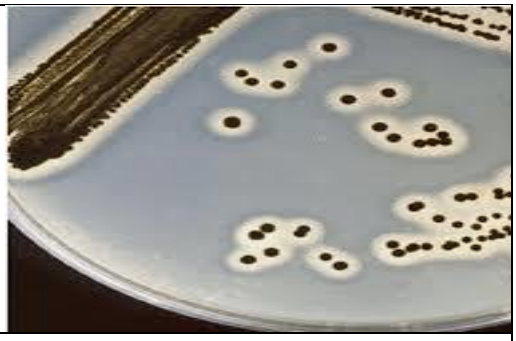
Biological toxins: State how your processing controls will prevent the development of the biological toxins over your product's shelf life. This is crucial in the case of certain fish species and specialized products such as canned and smoked products.

Harvest area: Explain how you verify that the product is received exclusively from approved waters. This is crucial in the case of filter-feeders such as shellfish.

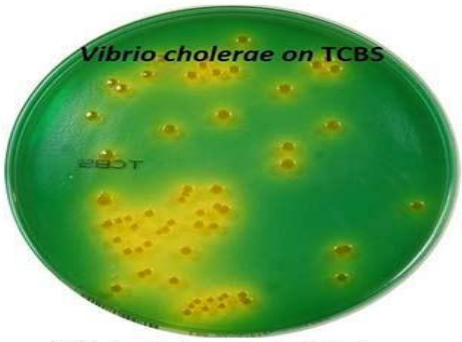
Colony characteristics of seafood pathogen



Salmonella in XLD

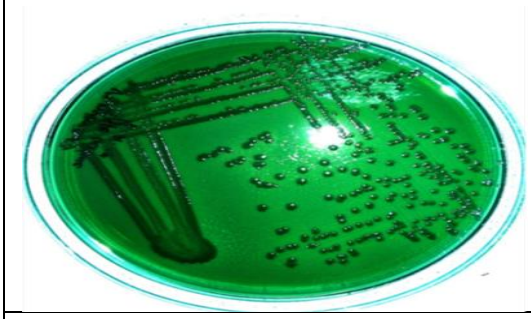


Staphlococcus aureus in BP

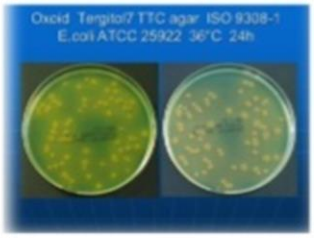


Vibrio cholerae **on TCBS Agar**

Vibrio parahaemolyticus **on TCBS Agar**



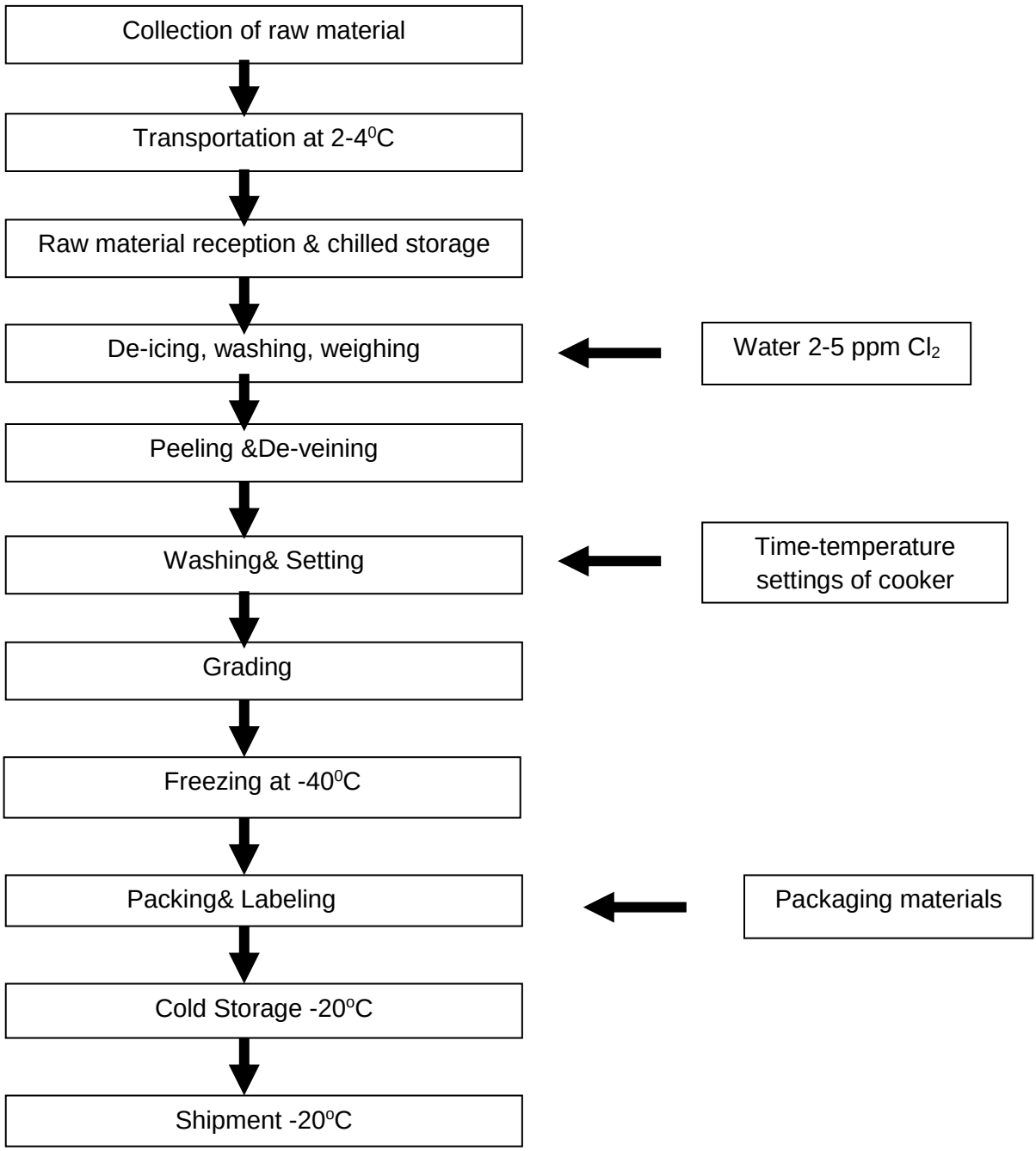
Vibrio mimicus **on TCBS Agar**



E.coli **on T7**

ANNEXTURE -I

PROCESS FLOW DIAGRAM FOR PEELED AND COOKED PRAWN



PROCESS FLOW DIAGRAM FOR PRODUCTION OF FROZEN SQUID WHOLE CLEANED