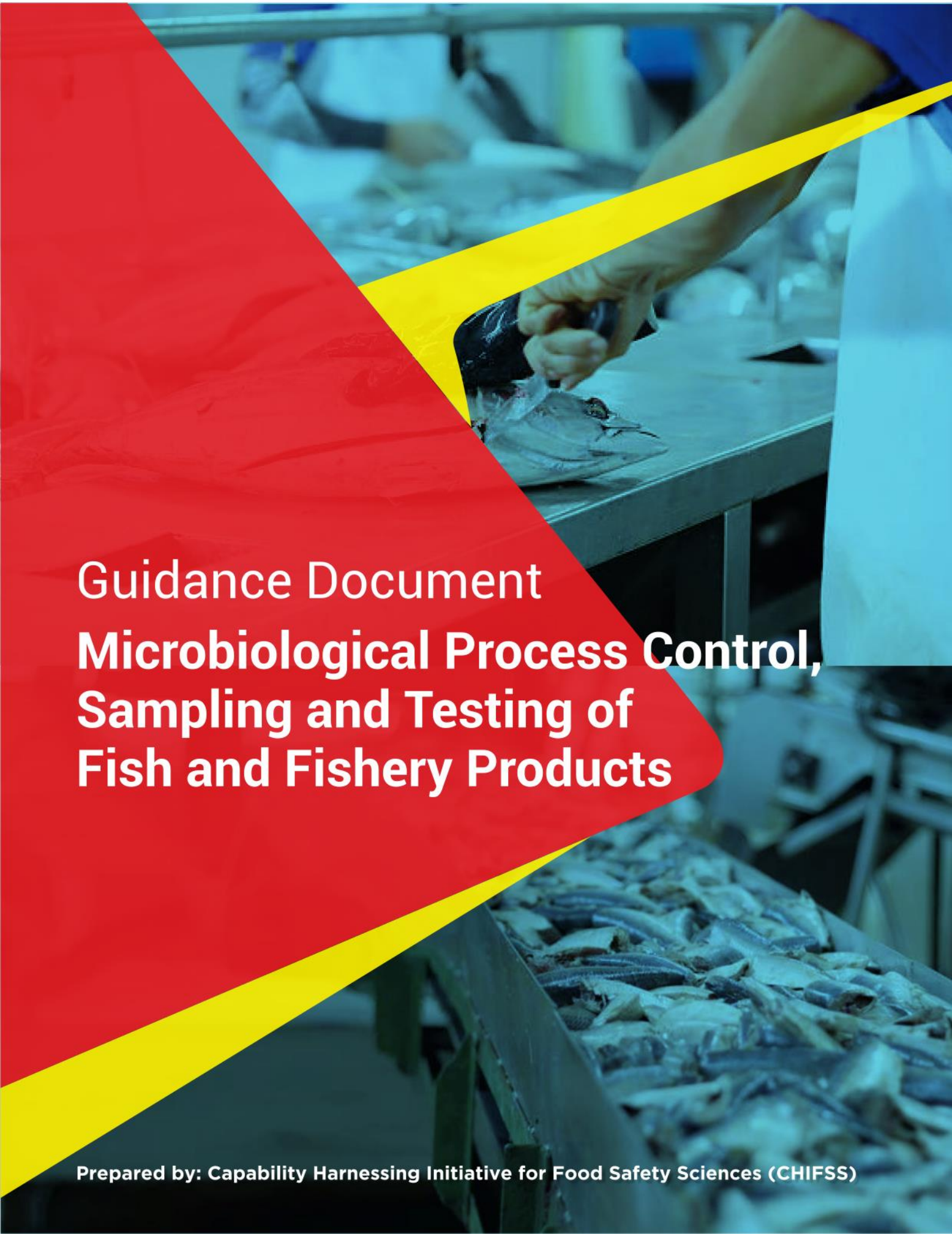

Guidance Document

Microbiological Process Control, Sampling and Testing of Fish and Fishery Products

Prepared by: Capability Harnessing Initiative for Food Safety Sciences (CHIFSS)

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA
Inspiring Trust, Assuring Safe & Nutritious Food
Ministry of Health and Family Welfare, Government of India

CHIFSS
Capability Harnessing Initiative for Food Safety Sciences

GUIDANCE DOCUMENT
ON
MICROBIOLOGICAL PROCESS CONTROL SAMPLING AND TESTING FISH AND FISHERY PRODUCTS

Prepared by : Capability Harnessing Initiative for Food Safety Sciences (CHIFSS)

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Acknowledgement

The ***Guidance Document on "Microbiological Process Control, Sampling and Testing for Fish and Fishery Products"*** has been prepared with the aim to build capacity and help in bringing together the information on recommendations/guidelines for operational control measures in Poultry processing related to microbiology of indicator organisms and pathogens significant to process hygiene and food safety.

This document is prepared by **CHIFSS (Capability Harnessing Initiatives for Food Safety Sciences).**

"CHIFSS aims to accomplish a foods operations regime in India, which embodies the principles of food safety sciences and is positioned on risk-based food safety approaches".

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FOREWORD

It gives us immense pleasure to release this Guidance Document on "Microbiological Process Control, Sampling and Testing for Fish and Fish products" given the complexities in the food processing sector, huge demand for safe fish and its products and rising consumer concerns about food safety. It is important to build confidence among consumers by developing objective and transparent mechanisms for setting food safety standards.

This is particularly relevant to microbial safety of fish and fish products. We congratulate CHIFSS on this pioneering effort. It ensures this will enable capacity building among all relevant stakeholders, including MSMEs driving the national food safety agenda.

FSSAI

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Purpose of the document

India has witnessed impressive growth in recent years in fisheries and aquaculture The country is rich in aquatic genetic resources distributed from deep sea to the cold Himalayan rivers sharing about 10% of the global aquatic biodiversity. The capture fisheries production in the country has more or less stabilized but the growth in inland aquaculture has been overwhelming during the past three decades. Thus the fishery sector can be considered to be the 'sunrise sector' with potential scope of employment, revenue and food security.

Freshwater aquaculture contributes to about 85-90% of the farmed fish. In 2018-19, India was the second largest producer of fish in the world with a total production of 13.4 million t comprising 3.7 million t from marine and 9.7 million t from inland sector. Several technological advances enabled the country to significantly enhance production and productivity particularly in the fresh and brackish water aquaculture sectors. Major constraints in capture fisheries production are depleted stocks due to habitat degradation, overexploitation, poor governance, climate change, in addition to harvest and post-harvest losses. The key problems in culture fisheries include low average productivity due to low adoption of technology, disease prevalence, non-availability of quality seed and cost-effective feed for desired species. Cutting edge translational research and technology upscaling and dissemination along with improved governance, fisher and farmer centric fisheries policy support and large-scale capacity building in both aquaculture and marine fisheries sectors are the key strategies for achieving the targets of blue revolution in the country (Lakra and Gopalakrishnan, 2021).

In view of this, microbial safety and relevant practices of fish and fish products is an important aspect in the context of upholding the quality and increasing consumption and production. Generally, the bacteria originating from fishes following harvest, the storage/processing area, personnel health and hygiene and the equipment used can be a source of contamination of fishes, and subsequently in the processed products. Fish processors may also establish process hygiene criteria and microbiological guidelines for their operations as well as setting microbiological specifications for raw materials and ingredients or finished products. The unhygienic conditions of the boats/vessels which serve as temporary storage prior to transportation and pest infestation of yards frequently serving as storage areas are other important sources of entry and proliferation of microorganisms.

Microbiological criteria require to be established for examining raw materials, in-

process and environmental samples and food products that may be collected at different points during the processing steps. The microbiological criteria stated by FSSAI includes food safety criteria applied to determine the safety of a lot/batch and relevant criteria applied to verify hygiene measures or control of process which are applied at a specified point in the manufacturing process.

Effective implementations of GMP and GHP are the primary factors which can affect the growth of microorganisms in fish meat and fish product processing. Microbial contamination may occur from equipment surfaces, water, air, personnel health and hygiene as well as from the immediate environment. Moreover, the microorganisms can easily become established in processing environments when favorable conditions arise during storage. In particular, it is necessary to design equipment for ease of maintenance and clean ability. Bacterial contamination by equipment surfaces can take place early in the process. Cross contamination between harvested fish may occur by direct contact or through contact with contaminated surfaces. Efficient control over hygiene, cleaning and sanitation and product handling and storage are extremely critical to achieve food safety. It is also essential that cleaning and sanitation procedures are established, and their ongoing effectiveness monitored and verified.

Interventions in the storage/processing area or plant cannot always completely remove microorganisms. However, there are some process control measures available which are responsible for sanitization of harvested fish such as maintaining proper temperature and implementing a balanced and operational HACCP system to achieve a desired level of acceptance. Refrigeration conditions are of absolute importance, and an interruption of the refrigeration/cold chain can accelerate microbial growth.

In line with the aforementioned deliberations, this document helps in bringing together the information on suggested operational control measure recommendation/ guidelines relevant to fish processing microbiology (indicator organisms and pathogens) crucial for process, hygiene and food safety.

Chapter 1 – Brief about Microorganisms relevant to Fish

Microorganisms relevant to fish are broadly divided into Hygiene/ Utility/ Environmental indicators such as - *E. coli*, *S. aureus* and Pathogens- *Vibrio* spp, *Salmonella* spp, *Listeria monocytogenes*, *Clostridium botulinum*, *Yersinia and Aeromonas* spp. Total aerobic counts (APC)/total viable count (TVC)/heterotrophic bacteria count and yeast/mold counts are important to indicate the general load/burden of microorganisms in fish, fish products, contact surfaces, water/ice used.

Microorganisms: Utility, indicator and pathogens

Utility - Some microbiological tests provide information regarding general contamination, incipient spoilage or reduced shelf life e.g. microscopic counts, yeast and mold counts, aerobic plate counts, or specialized tests such as for psychotropic organisms. Utility tests are not related to health hazards but rather to economic and aesthetic considerations, therefore the level of concern is low. Evidence should support the use of a utility test for the intended purpose. For example, evidence should support the use of a total aerobic count as measure of incipient spoilage. Such tests may be useful indicators of product quality. The microbiological profile in general reflects the quality of the immediate environment.

Indicator - Microorganisms that are not normally harmful, but may indicate the presence of pathogenic microorganisms may be used as indirect indicators of health hazard like *Enterobacteriaceae*, generic *E. coli*, etc. It is important to recognize that relationships between pathogen and indicators are not universal and are influenced by the product and process. Indicator organisms may be useful in other situations, e.g., for assessing the efficiency of cleaning and disinfection or in investigational sampling. Some of the examples of use of indicator organism are as follows: *E. coli* in water indicates recent faecal contamination, and *S. aureus* in cooked foods can indicate contamination from the human skin or nose. Other examples like high numbers of mesophilic spore-forming bacteria in low-acid, shelf-stable canned foods indicate probable under-processing when it is certain the container is not a leaker; the presence of *Enterobacteriaceae* or coliforms in some properly pasteurized foods indicates re-contamination after heat processing; Because of the uncertain relationship between indicators and specific pathogens, the level of concern is

moderate and it is inappropriate to apply sampling plans with a high stringency for indicator microorganisms.

Pathogens - The microorganisms associated with foodborne illness are considered as pathogens. These may be bacterial pathogens and their associated toxins or toxic metabolites, viruses, parasites and toxigenic fungi. The risks associated with microbial hazards vary greatly, ranging from quite mild symptoms of short duration to very severe, life- threatening illnesses. When deciding on the level of concern, health hazards generally fall into three categories

Moderate hazards Moderate hazards are rarely life-threatening, do not result in sequelae, are normally of short duration, and cause symptoms that are usually self-limiting but can result in severe discomfort. Some microorganisms can be both severe hazards for specific populations and mild hazards for the general population. For instance, *L. monocytogenes* can cause abortion and/or stillbirths

in pregnant women, life-threatening disease among immune compromised people, but only cause no symptoms or a mild flu-like illness and/or diarrhoea of short duration in the general population.

Serious hazards, incapacitating, but not life-threatening These hazards result in disease of moderate duration, and do not normally cause sequelae. Some pathogens such as *C. jejuni* and other thermophilic campylobacters occur most commonly in the lower, moderate category of hazard, but some strains of *C. jejuni* cause severe illness, i.e., Guillain-Barré Syndrome (GBS) in susceptible persons.

Severe hazards, life threatening These microbial hazards can result in substantial chronic sequelae or the effects can be of long duration, can affect either the general population, or may be specific to populations at high risk. Factors influencing the development of illness in high-risk populations include specific host susceptibility to infection such as listeriosis in pregnant women.

For a more comprehensive on major microbial pathogens and toxins associated with foods in relation to their impact to public health, their frequency of involvement in disease, the types of foods that have served as vehicles and significant factors contributing to disease, ICMSF's Book 72nd edition (ICMSF, 2018) can be referred.

Process Hygiene Indicator/Utility / Environmental

1. Yeast and Mold

Morphology

Yeast is large (5 to 8 µ), single-celled organisms that rarely form filaments. Most yeasts reproduce by the asexual process of budding. The molds form large multicellular aggregates of long branching filaments, called hyphae. There are vegetative hyphae and reproductive hyphae.

Growth and survival criteria

Most of the yeast and molds are aerobes and grow in a wide range of pH (2 - 9), enabling them to survive in very acidic environments, such as fruit juices and pulps. The temperature range for the growth of yeasts is 0 - 47°C. Many species are xerotolerant (a_w as low as 0.65).

Public health significance

Many yeasts and molds naturally occur in the environment. Contamination may occur during processing, packaging or storage of raw materials or finished products. It is important that manufacturers are aware of possible routes of contamination in order to minimize the risk of contaminated products. Potential sources of contamination include air, water, raw material equipment, and fish coming in contact with the spores through contaminated environment. The predisposing factors for flaring spore generation and dissemination in the air/environment include warm environment, humidity, poor ventilation and sanitation of the storage area.

The toxins produced by molds are called mycotoxins. Among foodborne molds *Aspergillus flavus* (A. flavus) and *A. parasiticus* are important due to their ability to produce aflatoxins. Ochratoxin, patulin, zearalenone, T-2 toxin are the other important mycotoxins significant to public health. These toxins are known to damage liver, kidney and impair the normal functioning of immune system in humans and animals.

Associated foods - Grains, nuts, beans, and fruits, dried fish and fish products, meat, poultry products etc. with low water activity.

Control Measures

- Decontamination of packaging is usually achieved using heat, UV irradiation, hydrogen peroxide or gamma irradiation.

- Heating, sterilizing, pasteurizing, drying, the addition of preservatives.
- Keeping the unit proper ventilated and to keep it dry with a dehumidifier or exhaust fans, if essential
- Air handling unit, if essential with High-Efficiency Particulate Air (HEPA) filters
- Fumigation of the section with disinfectants.
- Proper cleaning and sanitizing, hand washing, cleaning in place of equipment, should also be taken care of to avoid the product getting contaminated with yeast and mold from the equipment or workers' hands.

2. E.coli

Morphology

Escherichia coli are rod-shaped, facultative anaerobic, Gram-negative bacteria. Based on presence of virulence genes, disease syndrome, on their effect on certain cell cultures and serological reactions, at least six pathotypes of *E. coli* have been recognized: enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC) or Shiga toxin producing *E. coli* (STEC), enteroinvasive (EIEC), enteropathogenic (EPEC), enterotoxigenic (ETEC) and diffusively adhering (DAEC).

Growth and survival criteria

Shiga toxins (Stx) production by STEC occur at all temperatures that supports growth, although less toxin is found when cells are grown at 21℃ than at 37℃. The optimum pH range for *E. coli* STEC growth is pH 6 - 7 with the minimum pH for growth being 4.4. The minimum water activity at which *E. coli* STEC can grow is the 0.95.

Public health significance

Although most of *E. coli* are non-pathogenic, certain strains of STEC (e.g. *E. coli* O157:H7) causes life threatening disease in humans *E. coli* O157:H7 is transmitted to humans primarily through consumption of contaminated foods, such as raw or undercooked ground meat products, fishes and raw milk. Faecal contamination of water and other foods, as well as cross- contamination during food preparation (with beef and other meat products, fishes contaminated surfaces and kitchen utensils), will also lead to infection. The fish could have been contaminated by an infected handler or during storage and processing. Symptoms of STEC infection include abdominal cramps, bloody diarrhea, vomiting and fever. The illness develops over 3-8 days, with many patients improving in 10 days. However, in some patients more severe symptoms can occur *viz*. including hemorrhagic colitis (HC), hemolytic anemia,

thrombocytopenia, kidney failure and death. Children under five years of age and the elderly are more susceptible to infection and the development of serious illness. Enteroinvasive *E. coli* (EIEC) causes diarrhea. Enteropathogenic *E. coli* (EPEC) strains generally do not produce enterotoxins, although they can cause diarrhea. Enterotoxigenic *E. coli* (ETEC) strains produce heat stable and heat-labile enterotoxins causing diarrhea in both children and adults. ETEC strains are the leading causes of travelers' diarrhea.

Associated foods

Inadequately processed ground beef (hamburger patties), comminuted meat (e.g. salami), raw dairy products, fresh produce such as vegetables, sprouted seeds, fishes and seafoods.

Control Measures

The main source for Shiga toxin-producing *E. coli* and entry point into the food chain is animal faeces. Control measures for Shiga toxin-producing *E. coli* are through the food chain and include preventing contamination of raw products at primary production by implementing good hygienic practices ensuring processing controls are adequate (e.g. cooking, pasteurization, fermentation, and control of pH, water activity). Preventing cross- contamination of ready to eat foods with raw foods and the processing environment.

3. Staphylococcus aureus

Morphology

Staphylococcus aureus is a Gram-positive, non-spore forming cocci, facultative anaerobic bacteria that belong to the genus *Staphylococcus*.

Growth and survival criteria

S. aureus can grow over a wide range of pH 4.0 –10 (Optimum, 6-7) temperatures 7 - 48°C (Optimum 37 °C), aw 0.83 - 0.99 (Optimum 0.98) and in presence of up to 20% NaCl. Staphylococcal enterotoxins are produced at a temperature range of 10-45 °C, pH 4-9.6, aw 0.85 - 0.99 and in presence of up to 10% NaCl. There are at least 23 types of Staphylococcal Enterotoxins (SE), among them five are major toxins (SEA, SEB, SEC, SED, SEE). The enterotoxins are heat stable and can withstand boiling temperature. The enterotoxins are also resistant to inactivation by gastrointestinal acids and proteases.

Public Health Significance

Staphylococcal food poisoning occurs following the ingestion preformed enterotoxins present in food. Generally, there will be rapid onset of symptoms, usually within 3 hours after ingestion which includes nausea, vomiting, abdominal cramps, and diarrhoea. While illness is acute, it is generally self-limiting, and recovery is rapid (within 2 days). *Staphylococci* are widespread in the environment and commonly occur on the skin and mucous membranes of warm-blooded animals. Humans are the main source of enterotoxin producing strains, with many healthy people (50% or more) carrying *S. aureus* as part of the normal microflora of the nose, throat or skin.

The most frequently involved factors that contributed to the outbreaks were the following

1. Extensive handling of foods followed by improper storage conditions for extended period.
2. Preparing foods far in advance of planned service
3. Infected persons' practicing poor personal hygiene
4. Inadequate cooking or heat processing
5. Holding food at a temperature between 4℃ – 60℃ over an extended period of time

Implicated foods

Apart from fish, meat, poultry, and egg products, raw milk, bakery products such as cream or custard-filled pies and sandwich fillings, foods high in starch and protein are known to favor staphylococcal enterotoxins production. Fish and fish products are vulnerable to S.*aureus* contamination and enterotoxins can occur in fishes.

Control Measures

i) Preventing unnecessary contact with ready to eat food

ii) Using gloves, tongs or other implements to handle food

iii) Hand washing whenever direct contact with food is likely to occur

iv) Avoiding sneezing, coughing or blowing over food or food contact surfaces

Food Safety Microorganisms

4. Salmonella spp.

Morphology

Salmonella spp. are members of the family Enterobacteriaceae. They are Gram-negative non- spore forming rod-shaped bacteria, generally motile.

Growth and survival criteria

The optimal growth temperature for *Salmonella* spp. is 35 to 43℃. Most serotypes do not grow at temperatures below 7℃ and over 50℃. *Salmonella* spp. will grow over a broad pH range; however, the optimum pH for growth is 7 -7.5. The minimum pH at which *Salmonella* spp. can grow is dependent on temperature, presence of salt and nitrite and the type of acid present. A minimum growth pH of 4.05 has been recorded. The optimum water activity for growth of *Salmonella* spp. is 0.99.

Public health significance

The primary habitat of *Salmonella* spp. is the intestinal tract of humans, animals such as birds, farm animals, reptiles, amphibians and occasionally insects.

There are a variety of opportunities for introduction and subsequent cross-contamination of *Salmonella* spp during poultry processing i.e. scalding and subsequent removal of feathers, evisceration of intestinal organs, and eventual immersion in a chiller tank. *Salmonella* Typhi, the agent causing typhoid fever, is the only *Salmonella* serovar for which humans are the only carrier. *Salmonella* spp. associated with gastrointestinal foodborne illness are termed non- typhoidal *Salmonella* (members of the species *Salmonella enterica*).

Salmonella spp. are transmitted via the consumption of contaminated food or water, as well as person-to-person contact or from direct contact with infected animals. Gastrointestinal illness results when *Salmonella* is able to invade the intestinal epithelial cells and infect the host, producing a heat-labile enterotoxin. Symptoms of salmonellosis usually start 12 to 36 hours after infection and include nausea, vomiting, diarrhea, cramps and fever, abdominal pain, headache, and chills. These symptoms are usually accompanied by prostration, muscular weakness, faintness, moderate fever, restlessness, and drowsiness.

Associated foods

i) Animal origin foods such as eggs (particularly raw egg dishes), poultry, raw meat, milk and dairy products, fish

ii) Vegetables and fruits (such as leafy greens, seed sprouts, melons,)

iii) Low moisture foods such as spices, peanut butter, chocolate.

Control Measures

Control of *Salmonella* includes

i) Prevention of cross-contamination (particularly of ready to eat foods)

ii) Maintaining temperature control to prevent the growth

iii) Prevention of faecal contamination.

iv) Cooked poultry meat and egg products to an internal temperature of 75°C.

v) Wash hands with soap and water after handling fishes.

5. Listeria monocytogenes Morphology

Listeria monocytogenes is a gram-positive, non-acid-fast, non- spore-forming bacterium, rod or coccobacillary shaped bacterium.

Growth and survival criteria

L. monocytogenes grows at low oxygen conditions and refrigeration temperatures (<5 °C). The nutritional requirements of listeriae are typical of those for many other Gram-positive bacteria. They grow well in many common media such as brain heart infusion, trypticase soy, and tryptose broths. At least four B vitamins are required-biotin, riboflavin, thiamine, and thioctic acid and the amino acids cysteine, glutamine, isoleucine, leucine, and valine are required. listeriae grow best in the pH range 6–8.

Public health significance

Listeria is widely prevalent in the environment viz., water, soil, decaying vegetation, sewage, silage, animal excreta, and contaminated foods. *Listeria* can be spread to people by several different methods. Eating food contaminated with the bacteria, such as through raw (unpasteurized) milk or contaminated vegetables, is often a source for cases. The bacteria may be passed from mother to foetus during pregnancy. *L. monocytogenes* causes listeriosis, which may be non-invasive (a mild form of the disease) or invasive. Invasive listeriosis is a relatively rare but often severe disease with fatality rates around 20-30%. Populations at risk include those with chronic

disease (e.g. cancer, diabetes, malnutrition, AIDS), pregnant women (fetuses or neonates infected in utero). Individuals infected with *L. monocytogenes* may exhibit mild flu-like symptoms such as fever and muscle aches, and sometimes gastrointestinal symptoms such as vomiting and diarrhea. In at-risk population groups manifestations of the more severe, invasive form of the disease include bacteremia, septicemia, meningitis, encephalitis. Abortion, premature birth, and stillbirth is often the consequence of listeriosis in pregnant females. When a newborn is infected at the time of delivery, listeriosis symptoms typically are those of meningitis.

Associated foods

Raw milk, soft cheeses, ice cream, fresh and frozen meat, poultry, delicatessen meats, cooked chicken, fish, seafood products, fruits and vegetable products, sprouts, pre-prepared salads and smoked seafood.

Control Measures - Control of *L. monocytogenes* in ready- to- eat foods includes

i. Minimizing the contamination of raw materials during primary production
ii. Using listericidal processes
iii. Restricting growth through limiting shelf life, maintaining the cold chain or product formulation.
iv. Proper design and maintenance of premises and equipment, process flow, and cleaning and sanitation programs.

6. Sulphite Reducing Clostridium

Production appears to require higher a_w values than the above minima.

Clostridium botulinum Morphology

It is a Gram-positive, spore forming (terminal or subterminal spores), anaerobic rod. The disease caused by consuming toxins produced by *C. botulinum* is called as botulism. Based on the serological specificity of their toxins, seven types are recognized: A, B, C, D, E, F, and G.

The nutritional requirements of these organisms are complex, with amino acids, B vitamins, and minerals being required. The proteolytic strains tend not to be favored in their growth by carbohydrates. It is generally recognized that the growth of *C. botulinum* does not occur at or below pH 4.5. The minimum a_w that permits growth and toxin production of types A and proteolytic B strain is 0.94, and this value is well established. The spores of C. *Botulinum* are resistant of heat, freezing and drying.

Public health significance

Food-borne botulism is caused by consuming food contaminated with the botulism toxin (neurotoxin). Symptoms of botulism may develop anywhere between 12 and 72 hours after the ingestion of toxin containing foods. In the adult form of botulism, preformed toxins are ingested. Symptoms consist of nausea, vomiting, fatigue, dizziness, and headache, dryness of skin, mouth, and throat, constipation, lack of fever, paralysis of muscles, double vision, and, finally, respiratory failure and death. The duration of the illness is from 1 to 10 or more days. Infants get viable spores from infant foods and possibly from their environment. High numbers of spores are found in the feces of infants during the acute phase of the disease. Vehicle foods are those that do not undergo heat processing to destroy endospores (e.g. Syrup and honey).

Associated foods ad implication for fishes

Fishes, Meat and Poultry Products, Home-prepared and home-canned vegetables, fruits and meats, Fermented meat, Smoked fish, seafood, honey. In fishes, the importance of *Cl. botulinum* lies in adequate thermal processing canning or others) in order to inactivate the pathogen.

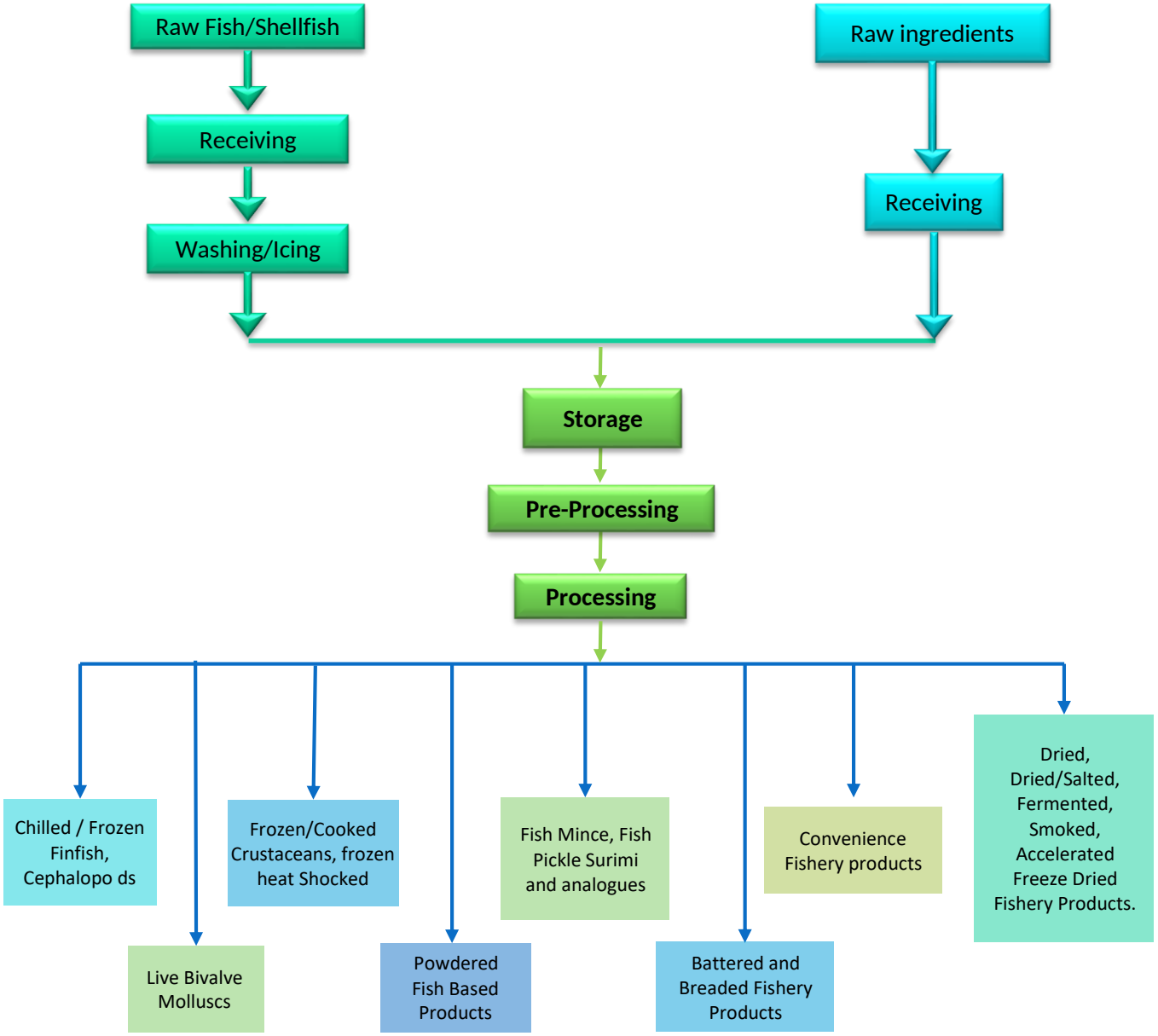
Control Measures

The control of *C. botulinum in* the food chain relies on the killing of organisms in foods that will support the growth of this bacterium, or the formulation of food ingredients and processes to prevent growth. Refrigeration will not prevent growth and toxin production unless the temperature is kept below 3℃.

The growth of *C. botulinum* strains does not occur at or below pH 4.5. *C. botulinum* produces heat-resistant spores. The growth of *C. botulinum* is inhibited by high temperature, acidification, dehydration, salination, certain food preservatives e.g. nitrite, ascorbates, polyphosphates, and competing microorganisms such as *Lactobacillus* spp. Since many cases of botulism are associated with home-preserved food, public education about the need for adequate heating, appropriate storage is important.

Chapter 2 – Process Control - Fish and fish product with respect to microbiological aspects

Process Flow Chart and Brief of Manufacturing Process



Primary packing, Secondary/Final packing----Storage, warehousing----Retail & Display

Manufacturing Process- in brief

All fish and shellfish deemed fit for human consumption should be handled properly with attention being paid to time and temperature control. Temperature is the single most important factor affecting the rate of fish and shellfish deterioration and multiplication of micro-organisms.

1. General manufacturing process

Note: *Care should be taken to avoid microbial contamination in all stages.*

1.1 Raw material receiving - All incoming raw materials including fish, shellfish as well as ingredients should be subjected to an examination for food safety hazards and defects based on existing food regulations by the concerned supervisor and recorded.

In case of raw fish, shellfish- the best method of assessing the freshness or spoilage of fish is by sensory evaluation techniques. Fish should be rejected if it does not conform to the raw material standards (For eg. fishes treated with formaldehyde, ammonia, gill rakers smeared with blood or any other coloured substances).

Every lot should be received from the approved supplier. For The supervisor should verify and record the standard information about the harvesting area, chemical treatments if carried out (for e.g., the presence of sulphites applied to the shrimps for preventing black spot) and transportation conditions. Temperatures of all incoming lots should be recorded.

Only ingredients, packaging material and labels complying with the existing food regulations and specifications of the processors should be accepted into the processing facility. For E.g. Packaging material used should be clean, sound, durable, sufficient for its intended use and of food grade material.

All these materials should be inspected on receipt by the supervisor. representative samples should be taken and examined to ensure that the product is not contaminated and meets specifications for use in the end product. Also, cleanliness and suitability of the transport vehicle that carry food products should be examined.

1.2 Storage - Raw fish/shellfish materials should be washed in chilled potable water and properly iced or moved to the chilled storage facility without undue delay if not immediately taken for processing. The fish and fish products should be kept between 0 °C and +4 °C.

The fish should be stored in shallow layers and surrounded by sufficient finely divided ice or with a mixture of ice and water such that damage from over stacking or overfilling of boxes will be prevented. The chill room should be equipped with a calibrated indicating/self- recording thermometer. Ingredients and packaging material should be properly protected and segregated to prevent cross-contamination and stored appropriately in terms of temperature and humidity. Systematic stock rotation plan should be in place to follow FIFO/FMFO/FEFO.

Pre-processing - Pre-processing operations vary with the species or according to product specifications. Each product has a specific pre-processing procedure. For example, pre-processing of shrimp/ prawn (depending on the product) includes processes such as washing, sorting, grading, beheading, peeling, deveining, etc. Pre-processing in fin fishes consists of washing, sorting, grading, gutting, beheading, de skinning, cutting, filleting, trimming etc. They shall be well iced or appropriately chilled in clean insulated containers, protected from dehydration and stored in appropriate areas within the processing facility.

Processing - Each product category has as specific mode of processing. Product Category as per Food Safety and Standards (Food Products Standards and Food Additives) Regulations,2011.

***Processing of freshwater fish.**

Briefly, important unit operations involved are as listed as follows:

- Grading,
- Slime removal,
- Scaling,
- Washing,
- Deheading,
- Gutting,
- Cutting Fins,
- Slicing,
- Filleting,
- Skinning and Meat bone separation

Stunning, Grading, Slime Removal, Scaling, Washing, De-heading, Gutting, Cutting Fins, Slicing, Filleting, St

It is to be noted that evisceration to be done if required, in case it is done, wastes to be disposed as per environmental regulations. Water used for all washing operations to be as per IS norms.

Odour management during processing

Fish and fish byproduct processing odors are objectionable can be controlled with afterburners, chlorinator-scrubbers, or condensers. Adequate odour management is desirable for fish processing units as a part of environmental norms.

- Afterburners are most effective, providing virtually 100 percent odor control, but they are costly from a fuel-use standpoint.
- Chlorinator scrubbers have been found to be 95 to 99 percent effective in controlling odors from cookers and driers. Condensers are the least effective control device.

Particulate emissions from the fish meal process are usually limited to the dryers, primarily the direct-fired dryers, and to the grinding and conveying of the dried fish meal. Because there is a relatively small quantity of fines in the ground fish meal, particulate emissions from the grinding, pneumatic conveyors and bagging operations are expected to be very low. Generally, cyclones have been found to be an effective means to collect particulate from the dryers, grinders and conveyors, and from the bagging of the ground fish meal.

Quality testing and Processing regulations to be considered

As a prerequisite for microbiological process controls, one should follow Pre-Requisite Program (PRP)/ GMP practices.

Recommended: HACCP implementation of fish product processing plant. (For Hazard Analysis and Pre-Requisite Program (PRP) / GMP – Please refer to: https://fssai.gov.in/cms/guidance-document.php)

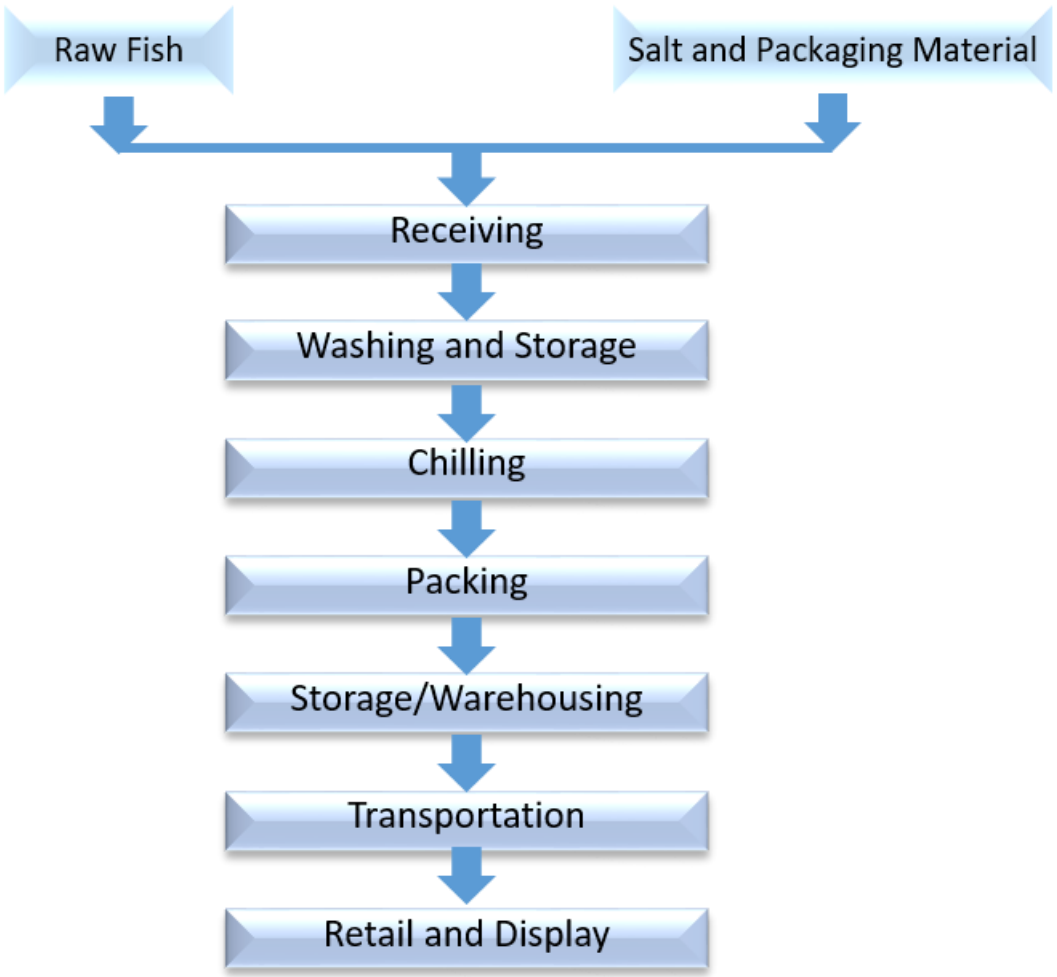
Other criteria to be considered

- Hygienic practices of fisherman: Hygienic status of immediate storage of fishes(boats/vessels) and personal hygiene of fisherman.
- Water quality of fish harvesting area and seasonal variations and adverse climatic conditions (after cyclones, floods, drought etc)

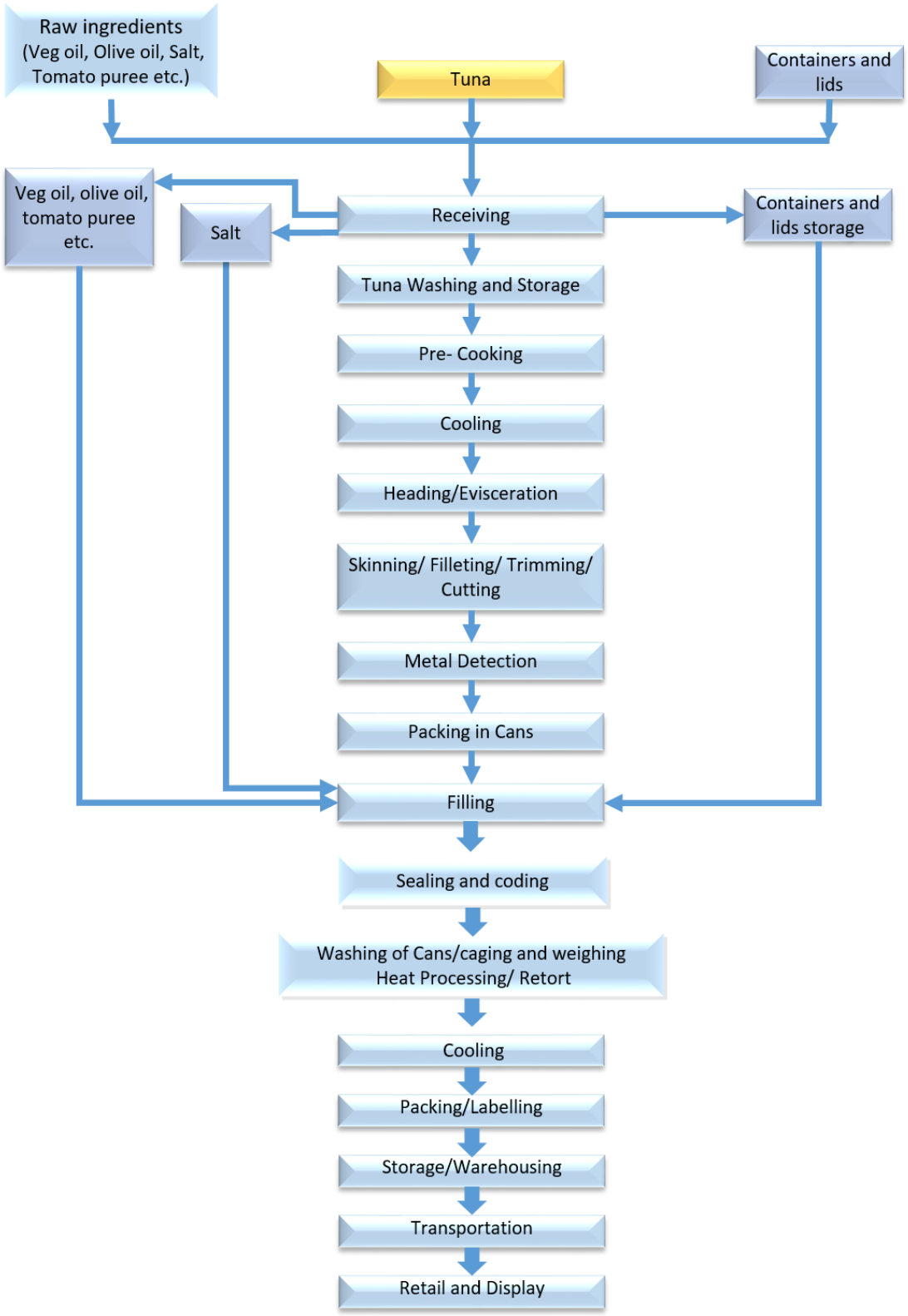
Since the manufacturing processes are specific to each fish product, the processing establishment should establish operation controls specific to the product to ensure safety. As examples two processes viz chilled fish and canned tuna (oil/brine/sauce) are depicted below for convenience of understanding.

Chilled Fish

Chilling is a process by which temperature of the fish is lowered to a point at temperature 0- 4-degree Celsius by means of heat withdrawal. Chilling should be regarded as a short-term storage method. However, it can increase the storage life of some fish by between 14-21 days.



Canned Tuna (Oil/Brine/Sauce)-



FSSAI Recommended Processes: **Canned fish**

The material used for the preparation of canned finfish shall be from sound fish of the species as given in the regulation and of a quality fit to be sold fresh for human consumption. Heads and gills shall be completely removed, scales and tail may be removed.

The fish may be eviscerated. If eviscerated it shall be practically free from visceral parts other than roe, milt or kidney. If not gutted, it shall be practically free from undigested feed or used feed.

Shrimp, crab meat, mussels and squid rings must all be prepared from sound species as given in the regulation and must be of a quality fit for human consumption and mussels and squid rings should have no evidence of spoilage and degradation.

Smoked fish, smoke-dried fish, and smoke-flavored fish

Smoking is a process of treating fish by exposing it to smoke from smoldering wood or plant materials. This process is usually characterized by an integrated combination of salting, drying, heating and smoking steps in a smoking chamber

Provided the wood or other plant material for the generation of smoke or smoke-condensates shall not contain toxic substances either naturally or through contamination, or after having been treated with chemicals, paint or impregnating materials and shall be handled in a way to avoid contamination.

Provided further, that smoking of fish shall be done in a manner that minimizes the formation of polycyclic aromatic hydrocarbons (PAH).

Smoking by regenerated smoke is a process of treating fish by exposing it to smoke which is regenerated by atomizing smoke condensate in a smoking chamber under the time and temperature conditions similar to those for hot or cold smoking

Smoke condensates are products obtained by controlled thermal degradation of wood in a limited supply of oxygen (pyrolysis), subsequent condensation of the resultant smoke vapors, and fractionation of the resulting liquid products

Hot smoking is a process in which fish is smoked at an appropriate combination of temperature and time sufficient to cause the complete coagulation of the proteins in the fish flesh; hot smoking is generally sufficient to kill parasites, to destroy non-sporulating bacterial pathogens and to injure spores of human health concern

Cold smoking is a process of treating fish with smoke using a time and temperature combination that will n

Salting is a process of treating fish with a salt of food grade quality to lower water activity in fish flesh and to enhance flavour by any appropriate salting technology (e.g., dry salting, brining, injection salting).

Drying is a process in which the moisture content in the fish is decreased to appropriate required characteristics under controlled hygienic conditions.

Packaging is a process in which smoked fish is put in a container, either aerobically or under reduced oxygen conditions, including under vacuum or in a modified atmosphere.

Storage *is a process in which smoked fish is kept refrigerated or frozen to assure quality and safety of the product*

Smoke drying is a process in which fish is treated by combined smoking and drying steps to such an extent that the final product can be stored and transported without refrigeration and to achieve a water activity of 0.75 or less (10% moisture content or less), as necessary to control bacterial pathogens and fungal spoilage.

Smoke flavours are either smoke condensates or artificial flavour blends prepared by mixing chemically defined substances in known amounts or any combination of both (smoke-preparations)

Smoke flavouring is a process in which fish or fish preparations are treated with smoke flavour. The smoke flavour can be applied by dipping, spraying, injecting, or soaking.

Edible Fish powder

Process requirements

Edible fish powder means the product prepared from non-oily white fish like sprats, either from a single species or their mixture. Fresh fish of edible quality which is normally consumed whole should be used for the Poisonous fish like marine snakes, elasmobranch fish with a high quantity of urea, oily fish and fish with black viscera are not considered suitable for preparation of edible fish powder. The fish need not be dressed but should be washed and cooked well for the preparation of the powder.

Frozen Minced Fish Meat

Process requirements

Clean and fresh fish which do not show any signs of degradation and spoilage as raw material) The fish shall be gutted; the tail, entrails, bones, tips, skin, head and other non- edible portion shall be removed and eviscerated. Fish shall be washed thoroughly with clean potable water to remove the blood.

he variety of fish used shall be specified, the fish shall be properly iced and maintained at a temperature not exceeding 5°C till transport to the freezing factory, fresh fish shall be washed to make free of all foreign matter preferably by eighth chilled potable water (5°C) having 5 mg/kg (ppm) of available chlorine and meat separated from fish in the wholesome condition, the material shall be quickly frozen at a temperature not exceeding -30°C in polyethylene wrappers and packed in waxed cartons in the minimum possible time.

The quickly frozen material shall be stored in the cold storage at a temperature not less than -23 °C

Freeze dried prawns (shrimps)

Freeze dried prawns (shrimps) means the product freeze dried prawns as defined below and offered for consumption. They shall be any edible species of the following types:

- peeled, non-deveined and cooked – head and shell removed completely and cooked
- peeled, deveined and cooked – head, shell and dorsal tract removed and cooked
- cooked and peeled – peeled after cooking

Processing

The raw material shall be prepared from clean, wholesome and fresh prawns, and shall not show any visible sign of spoilage. The colour of the raw material shall typically be of freshly caught prawns. The meat shall be firm and shall have the typical odour of freshly caught prawns. The material must be free from any discoloration and off odours

The water used in the processing of prawns shall be of potable quality and shall contain 5 mg/kg available chlorine. The maximum value for moisture content shall be 2.0 percent. The extent of rehydration shall be minimum 300 percent (IS IS 14949). Frozen clam meat needs to be processed in the same way.

A broad classification of fish processes has been suggested recently (CIFT, ICAR, 2018). These are listed below as:

1. **Live fishery products**
2. **Chilled fishery products**
3. **Vacuum packaged products**
4. **Modified atmosphere packaging**

For each of the above, FSSAI guidelines are required to be followed.

For frozen fish and fish products, dried, salted fish products. Smoked fish and retort pouching has been also highlighted. In addition, novel techniques which do not alter physiochemical properties, sensory and other characteristics of fish-like extrusion, Irradiation, Microwave, Infrared and radiofrequency-based treatments, ohmic heating, high hydrostatic pressure, ultrasound-based techniques, bio preservation methods and enzyme-based processes have been advocated as alternative methods to those conventionally used. Although, commercial status of the cited methods may require thorough optimization, it is worthwhile to realize the singular benefits for several of these.

Procurement and Quality inspection of fish after catch/harvest –

- Safe and healthy fish production starts at the harvesting point, and bio - security at plays a major role in the control of infection and contamination to the live fishes. Every single part of the production system can be made aware of its potential contribution in this respect. The storage area prior to transport boats/vessels) must be maintained in a hygienic condition.
- The normal microflora is present in the live fishes at site and upon receipt at processing house. Fish intended for consumption shall be in good health condition. Fish health examination shall be done by qualified veterinarian.
- Live fishes shall be transported hygienic transport systems. It is advisable not to use any damaged crates to avoid injury to fishes during transportation. The transportation vehicles shall be clean and free from contamination to avoid cross contamination of microorganisms.
- Preferably fish catchment history data sheet can be made available at processing site to get information on receipt.

Holding / Storage

- Freezing is needed to extend shelf life for long periods. This can be achieved by changing two parameters: first, a considerable decrease in temperature, and second, by freezing the water in the fish tissue. The second is of particular importance because water in the fish tissue acts as a solvent for many organic and mineral compounds which are a suitable environment for the growth of micro-organisms and also because they influence the biochemical processes. A temperature of about -10° C is a limit for growth of such micro-organisms. It must be noted that some moulds and yeasts multiply very slowly at -15 to -18° C.
- Fish should be frozen rapidly in order to produce the highest quality frozen products. Quick freezing implies a fast change from acroscopic temperature to -5° C. During this period (about 2 hours) the main changes take place in fish tissue. A faster freezing process is linked to the formation of smaller ice crystals which damage the cellular membranes to a lesser degree, especially if freezing takes place before *rigor mortis* sets in.
- Even properly frozen fish has limited storage life. Low temperatures inhibit processes of microbiological decomposition but do not protect against fat oxidation and loss of water. The stability of frozen fish depends on the initial quality of the raw material, the rancidity, the drying process and the storage temperature.
- A simple and cheap method which effectively prevents water loss of from fish tissue is glazing This method is used especially for freezing of whole fish or in fish/fillet blocks. Individual portions of fish or individual fillets are packed in plastic material characterized by low permeability of water vapour and oxygen. This prevents rancidity and loss of water.
- The storage temperature of frozen products is the next factor which influences the quality and stability of frozen products. Adequate temperature recording is required otherwise low quality of products results from such practice, particularly texture and flavour; fish becomes dry and very often discolored.

Grading

- Grading can be done by different methods such as manual or mechanical
- If mechanical graders are used, they must be appropriately sanitized

Removal of slime

Slime usually accumulates on the skin surface of dying fish is a protection mechanism against harmful conditions. In many freshwater species slime constitutes 2-3% of body weight. Slime excretion stops before *rigor mortis*. Slime creates a perfect environment for micro-organism growth and should be removed by thorough washing. Eel, trout and carp require special care with regard to slime removal. Even small amounts of slime, which frequently remain after manual cleaning, result in visible yellowish-brown spots (particularly in smoked eel). Slime can be removed from eel, trout and other freshwater species by soaking fish in a 2% solution of baking soda and then washing in a cylindrical rotating washer.

Scaling refers to removal of scales from fish body. Several freshwater species are routinely scaled; this is usually very labor-intensive when done manually.

- Tools for scaling are used, these are moved over the body of fish from tail fin towards the head, pulling out the scales.

- Several processes are used for removal of scales including washing with hot water and water jets, however none of these should damage the fish tissue or cause mechanical injury

Slicing of whole fish into steak: A traditional fish processing technique is to slice de-headed whole fish into steaks with a cut perpendicular to the animal's backbone. This processing technique is common in retail markets and the canning industry due to its high technical performance as compared to filleting and automated cutting into parts.

- Skinning: skinning is mostly manual and requires skill; carried out by a sharp knife and a flat metal or plastic board. The fillet is put skin-down on the sheet, the meat is held in the left hand, and the knife is drawn between the skin and the meat.
- Meat bone separation: For less valuable fishes, minced meat is often made from after de-heading, cleaning; the process can be automatic using separators.

Chilling of harvested fish

- Immediately after harvesting fishes should be chilled using immersion and/or air chilling systems to minimize microbial proliferation and handled in a manner to minimize microbial contaminants.
- The purpose of this control point is to establish a system that achieves a

consistent reduction in overall microbial load on fishes to improve food safety in regard to pathogens of concern in addition to improving product quality.

- To minimize the microbial contamination operator shall ensure below points -
  - Used water and ice must be potable i.e. meeting IS10500 norms.
  - Build-up of organic matter is minimized. For this multistage immersion chilling system can be used.
  - Harvested fishes during chilling can be sanitized with sodium hypochlorite at chlorine concentration varying from 50 to 100 ppm depending upon process control and appropriate validations. Other Sanitizers like chlorine di-oxide etc. can be used.
  - Ensure continuous overflow of water and replenishment of sanitizer to maintain effective and sanitary operational conditions.
  - Adjust operational temperature of chilling system (e.g. add chilled water or ice or combination of both to spin chiller).
  - Excess water and/or ice shall be removed from the fishes after immersion chilling to minimize cross contamination later in the food chain.

Weighing and Grading

- Grading of chilled fishes can be done manually or with automatic grading machine.
- After grading, fishes shall be shifted to the Chiller or ice shall be added to maintain the temperature till it is further process.

Cutting and Portioning

- Cutting and portioning consist of cutting of whole fish into pieces as per requirement and can be a manual or mechanized operation
- The processing hall temperature shall be maintained at an appropriate low temperature to maintain quality of the fish.
- The knives, cutting saws and other accessories used shall be properly cleaned and sanitized, preferably at regular interval and after use.
- All the storage containers, crates, and other storage facilities shall be cleaned and sanitized every day.
- Product shall be brought into the processing room progressively as needed and

shifted to the chiller or freezer immediately after processing, to maintain required product temperatures.

- Maintaining good personal hygiene practices and sanitization of hands is very important aspect in controlling cross contamination.

Deboning

- Process of removal of bones and cartilages from whole fish to obtain boneless fish meat.
- It can be done manually or semi-automatic or in automatic line.
- The temperature in premises for deboning and trimming should be controlled to appropriate low level.
- Temperature below 10^{o} – 12^{o}C to maintain quality
- Shift deboned fish products to the chiller as early as possible to maintain its temperature below 4°C.
- Sanitization/sterilization of knives and scissors used for deboning shall be done at regular interval, in breaks and after the end of production to avoid cross contamination. Sterilization shall be done above 82^{o}C temperature for minimum 2 minutes.
- Maintaining good personal hygiene practices and sanitization of hands is very important aspect in controlling cross contamination.

Processed fish Products –

- The safety and suitability of fish products shall be ensured through adherence to efficient food handling controls and good hygiene practices that prevent or minimize contamination and further growth of pathogenic microorganisms.
- For storing fish or fish products wood, ferrous, brass and galvanized metals are to be preferably avoided.
- Majority fish products are perishable and must be refrigerated or frozen.
- The possible microbial hazards include *Salmonella* spp., *Listeria monocytogenes*, *Campylobacter* spp. *and Clostridium* spp.
- Control of *Salmonella* spp., *Listeria monocytogenes* and *Campylobacter* spp. involve use of validated cooking procedures and prevention of recontamination.
- Recontamination is managed through effective application of GHP and chilling

and storing of cooked fish products (at < 4^0C) at a rate that prevents unacceptable multiplication of microorganisms and pathogens.

Enable below process control measures during further processing and handling of processed fish product -

- The harvested fishes and other refrigerated ingredients if any shall be stored at or below 4°C and limiting the time that they are out of the Chiller or freezer.
- Minimize product handling as much as possible.
- Water and Ice used should conform to IS
- To minimize the growth of bacteria during processing and to assist in maintaining chill product temperatures, it is strongly recommended that fish duct processing areas are to be operated at low temperature. HACCP implementation is highly desirable.
- Ensuring that all equipment is thoroughly cleaned and sanitized before and after use.
- Ensuring that personal protective equipment (e.g. gloves, sleeves, aprons etc.) that may contact the product is clean and/or sanitized or changed as often as necessary to minimize contamination;
- Food handlers shall maintain a high degree of personal cleanliness and, where appropriate, wear suitable protective clothing, head covering and footwear.
- There shall be restricted entry to processing area to authorized persons only.
- Personnel shall always wash their hands as personal cleanliness may affect food safety.
- The chilled products shall be maintained at or below 4^0C. While frozen products should be kept at or below -18^0C temperature as soon as possible after processing.
- Monitoring of processing environment and equipment surfaces for organisms such as APC, *E. coli*, or may be for *Listeria monocytogenes* to verify the effectiveness of cleaning and sanitation programme.
- Only medically fit person shall allow to work in the food processing. Any person having illnesses or symptoms of illnesses (Diarrhea, vomiting, jaundice and persistent sneezing or coughing, discharges from natural body openings and skin lesions such as infected wounds, sore, boils, burns, blisters, cuts or open

wounds) shall not be allowed to work in food processing areas.

- A sanitization record for workers is to be maintained, contact surfaces should be preferably monitored using ATP bioluminescence rapid assays (kits can be used) at least twice a week to ensure efficacy of sanitizing of the surfaces and avoidance of biofilms (storage and processing surfaces).

Primary Packing

- Processed material is weighed and packed in primary packing material which should confirm FSSRs (Packaging and Labelling) and Legal metrology (Packaged Commodities).
- Packing material shall be of food grade.
- The packing material should be stored and handled in way to minimize the contamination.
- Maintain product temperature below 7^0C during primary packing.

Freezing

- Freezing is done to control hazards associated with microbiological growth to maintain minimal pathogenic loads and preserve product quality.
- In Freezing core temperature of the product should be attained at or below –18°C.
- For preparing ice flakes, ice generators or ice generators can be used. Usually, 1kg of ice flakes suffices for 4 kg of fish.

Metal Detection

- All finished products shall be passed through metal detector.
- Metal detector shall be calibrated/ verification at frequency appropriate to assure food safety.
- Recommended Metal detection sizes are listed below:
  - Chilled Products: Fe – 3mm, Non-Fe – 3.5mm and SS 4.5mm
  - Frozen Products: Fe – 1.5mm, Non-Fe – 2mm and SS 2.5mm

Secondary Packing

- Frozen fish / fish products after passing through metal detector shall be packed in suitable container.
- Secondary Packing material shall be secure to prevent spoilage and

contamination during transit and storage.

- Products must be transferred without unnecessary delay to freezer / cold storage to ensure that product temperatures are maintained at or below – 18°C; and in a manner that minimizes any potential microbial proliferation and contamination or damage to products during secondary packaging.

Storage

Chilled Storage-

Inadequate temperature leads to microbial contamination. Frozen fish and fish products shall be stored in the chiller at or below 4^0C.

Cold Storage-

- Frozen fish and fish products shall be stored in cold store at or below minus 18 degrees Celsius till dispatch to avoid microbial growth.
- Cold store temperature shall be maintained at or below minus 18 degrees Celsius except during defrosting cycle.
- FIFO / FMFO/FEFO method shall be followed in the cold storage for dispatch of product.

Quality Evaluation

- Finished products are tested in internal / external laboratory as per the sampling plan identified by the processing plant, for microbiological and applicable physio-chemical parameters as defined by FSSAI.

Loading, Dispatch and Transportation -

- The product temperature shall be maintained at or below minus 18°C for frozen at and or below 4°C for chilled products in any part of the cold chain, during storage, transport, distribution, and merchandising in retail stores.
- Chilled and frozen products should be dispatched and loaded into refrigerated vehicles without unnecessary delay after removal from the storage to ensure that required product temperatures are maintained. Products should be adequately protected from the elements and environmental contaminants prior to and during loading, dispatch and transportation.
- Sensitivity of the metal detector shall be always less than 7.0 mm for any kind of metal.
- Dispatch vehicle shall be checked for presence of any contaminants, cleanliness,

unacceptable odour and proper working of refrigeration system before loading. Dispatch vehicles shall be cleaned and sanitized using appropriate cleaning and sanitizing agents regularly to maintain the hygiene standard.

- All the transportation systems are expected to maintain the temperature of the processed fish and fish products within close limits to ensure its optimum safety and recommended shelf life.
- Refrigerated transport units must be designed, constructed and equipped to ensure that the specified temperatures are achieved and maintained throughout transportation.
- Temperature-measuring devices used to measure critical temperatures must be calibrated and located to measure the internal temperature of a transportation unit.
- Ensure proper air circulation is available to maintain product temperature during transportation.
- While loading in the refrigerated containers, the temperature in the container must be brought to -12^{0}C (Pre-cooling) further pre-cooling temperature shall be at or below 4^{0}C.
- The containers shall be clean and disinfected before loading.
- Conveyances and/or containers used for transporting shall be kept clean and maintained in good repair condition and shall be designed and constructed to permit adequate cleaning and/or disinfection.
- Preferably, unpacked Fresh / Chilled / Frozen fish shall not be transported with other food products to avoid cross contamination.
- Where conveyances and/or containers are used for transporting anything in addition to foodstuffs or for transporting different foods at the same time, there shall be, where necessary, effective separation of products to prevent cross-contamination.
- Fresh fish meant for immediate sale need to be stored in cool conditions. It can be transported in suitable a hygienic and sanitary condition in clean containers with covers to the retail shops/selling units with adequate precautions to ensure that no contamination or deterioration takes place.

Outsourcing of raw fish -

- Outsourced fish shall only be procured from a FSSAI licensed facility. The disease and transportation history of fishes shall be ensured.
- The fish shall be transported to the selling/processing unit under hygienic and sanitary conditions. It shall be transported in a clean insulated refrigerated container with covers (lids) with precautions to ensure that no contamination/ cross contamination or deterioration takes place and at appropriate temperature (at or below 4℃ or below -18℃.)

Chapter 3 – Purpose/aim of micro sampling and testing and Determination of Batch/Lot

Purpose/aim of micro sampling and testing

a. To verify food safety management system/process control
b. To check the compliance of individual batches
c. To verify the compliance with the criteria laid down in National Regulation
d. To obtain general information on the microbiological status of certain products placed on the market
e. Monitoring and surveillance
f. To investigate suspected food-borne outbreaks, customer complaints
g. Improvement in product with respect to food safety.

Determination of Batch/Lot

A lot is a quantity of food or food units produced and handled under uniform conditions (CAC, 2004). A lot should be composed of food produced with as little variation as possible for a given process or commodity as the acceptability of the lot is dependent on the testing of sample units of a product drawn from the lot.

If a consignment consisting of a mixture of production batches is treated as one lot, there are chances of rejection of lot even if only few of the production batches within the lot may be of poor quality and the whole lot will be affected by this decision. This increases producer's risk and can have severe consequences. Treating the individual production batches as lots, and coding appropriately, permits more precise identification of poor-quality food and can result in the rejection of fewer units from the whole consignment although may incur more expenses for an increased analysis. It is therefore helpful to give identifiable code numbers to batches (lots) of food produced over short time periods (e.g. a day or part of a day), for particular processes.

Fish processor has to assign the batch number of harvested lots of fish, packing materials, incoming raw material and finished goods. This is essential for traceability.

To decide on batch/Lot number, below guidelines can be referred.

- **Batch/lot for Raw Ingredients** – suitable batch/lot coding system shall be followed by Processor for all incoming raw ingredients for its tracing in finish product.
- **Batch/lot for Packing Material** – suitable batch/lot coding system shall be followed by processor for all incoming packing material for its tracing in finish product.
- **Batch/Lot for Finished Goods (Processed Fish Products)** suitable batch/lot coding system shall be followed by processor for all finish goods for its further tracing in food Chain.

Batch/lot size may be varying from plant to plant depending upon capacity of processing machinery used.

A *representative sample* reflects, as far as is possible, the composition of the lot from which it is drawn. While drawing the sample (s), it is important to avoid bias and to draw a sufficient number of sample units to confidently make a judgement about a lot.

Sampling at random is the universally recognized way of avoiding bias. The units (cartons or containers, particular weights of solid, or volumes of liquid) for testing are selected by using random numbers. There is, of course, no guarantee that a random sample has characteristics identical with those of the lot, but the randomness of sampling is the basis for calculation of the probability that a sample will give a certain result and results in a better chance of encountering all the variation in the lot.

Chapter 4 – Sampling Plan (two class/three class), ICMSF cases, ICMSF tool

Sampling Plan is the planned procedure which enables one to choose, or draw separate samples from a lot, in order to get the information needed, such as a decision on compliance status of the lot. (Codex, 2004) Sampling plans are required to ensure that fair and valid procedures are used when testing the food for compliance with a particular standard. No sampling plan can ensure that every item in a lot conforms; however, these plans are useful for guaranteeing an acceptable quality level. This guideline explains the sampling plans proposed by international commission on Microbiological Specifications for Foods (ICMSF, 1986), which are being widely adopted and used by codex and national regulatory agencies for microbiological testing. For detailed statistical aspects of the sampling plans refer ICMSF's Book 7, 2nd edition (ICMSF, 2018). The sampling plans described in these guidelines may be implemented either by producers and/or traders for self-inspection and also by governmental regulatory authorities to check the appropriateness of the sampling plans implemented by the industry. It is recommended that the different parties concerned with sampling come to an agreement on the implementation of the same sampling plan for the respective controls.

Sampling plans should include the sampling procedure and the decision criteria to be applied to the result, based on examination of a prescribed number of sample units and subsequent analytical units of a stated size by defined methods. A well-designed sampling plan defines the probability of detecting microorganisms in a lot, but no sampling plan can ensure the absence of a particular organism from an entire lot. Sampling plans should be administratively and economically feasible.

In developing a sampling plan, several factors should be taken into consideration including properties of food, production processes, and storage conditions of the final products, associated risks, targeted consumers and practical limitations. Each food product should be considered individually. A comprehensive sampling plan includes the following elements:

- The microbe or group of microbes of concern or interest.
- Number of samples to be tested (n);
- Testing method(s);

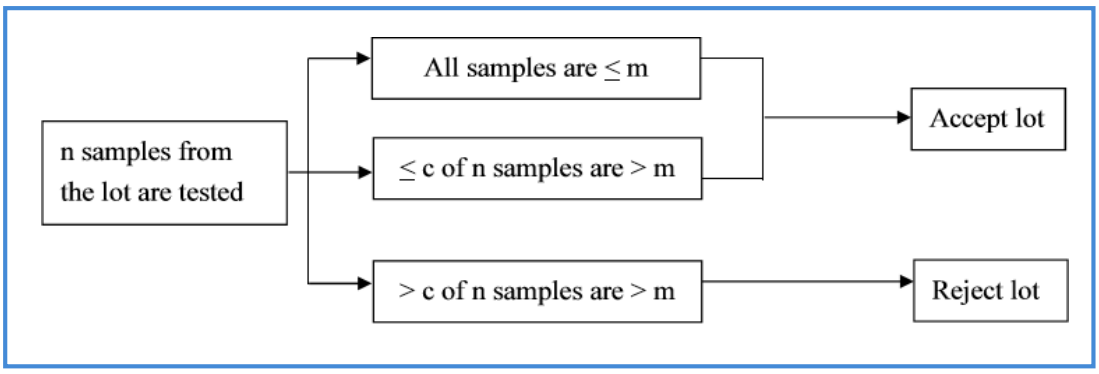
- Microbiological limit(s), m and M
  a. Acceptable (≤ m)
  b. Marginally acceptable (> m and ≤M)
  c. Unacceptable (> M);
- Number of samples which fall into each category of microbiological limit (i.e. acceptable / marginally acceptable / unacceptable).

Two-class attributes Plans

A simple way to decide whether to accept or reject a food lot may be based on some microbiological test performed on a certain number of sample units (n). This will usually be a test for the presence (positive result) or absence (negative result) of an organism. Concentrations of microorganisms can be assigned to a particular attribute class by determining whether they are above (positive) or below (negative) some preset concentration.

The decision-making process is defined by two numbers. The first is represented by the letter n and defines the number of sample units required for testing. The second number, denoted c, is the maximum allowable number of sample units yielding unsatisfactory test results, for example, the presence of the organism, or a count above the defined concentration, denoted m, which in a two-class plan separates good from defective units. E.g. The sampling plan n = 5, c = 0 and m= absence/25 g means that 5 sample units each of 25 grams are taken and tested; if none of the samples showed presence of the organisms, the lot is accepted (with respect to this characteristic); but if 1 or more of the 5 samples show the presence of the organism, the lot is rejected. Similarly, n = 5, c = 0 and m= 100/10 g means that 5 sample units each of 10 grams are taken and tested; if none of the samples showed counts of the organisms above 100, the lot is accepted; but if 1 or more of the 5 samples show the counts of organism above 100, the lot is rejected

The performance of the sampling plan depends upon *n* and *c*. The larger the value of *n* at a given value of *c*, the better is the distinction between acceptable and unacceptable lots. Thus, compared with *n* = 10, *c* = 2, the plan *n* = 15, *c* = 2 is more stringent, while the plan *n* = 5, *c* = 2 is more lenient. On the other hand, for a given sample size *n*, if *c* is decreased, the plan becomes more stringent. Conversely, if *c* is increased, the plan becomes more lenient and will more often pass food lots with unacceptable quality.



Three-class attributes plan

Three class attributes plans are defined by the values n, c, m and M and are applied to situations where the quality of the product can be divided into three attribute classes depending upon the concentration of micro-organisms within the sample:

- ***Good or acceptable quality***: Where the counts of microorganisms do not exceed the value, m.
- ***Marginally acceptable quality:*** A certain number of sample units/ items (denoted by c) may have a count of microorganism which exceeds m, but which is less than M (such concentrations are undesirable, but some can be accepted, the maximum number acceptable being denoted by c).
- ***Unacceptable quality:*** If any sample shows concentration of microorganisms above the value M

E.g. The sampling plan n = 5, c = 2, m= 1000 CFU/g and M=10000 CFU/g means that 5 sample units each of 1 gram are taken and tested; if all the samples show counts below 1000 CFU/g, the lot is acceptable; if two of the samples (c=2) showed counts of the organisms between 1000 and 10000, the lot is marginally acceptable; but If any sample shows the count above 10000 CFU/g, or more than 3 samples show counts between 1000 and 10000 CFU/g, the lot is rejected. or more of the 5 samples show the presence of the organism, the lot is rejected.

The sampling plans are independent of lot size if the lot is large in comparison to sample size. The relationship between sample size and lot size only becomes significant when the sample size approaches one tenth of the lot size, a situation rarely occurring in the bacteriological inspection of foods. The choice of sampling plans should take into account

(i) Risks to public health associated with the hazard (severity of the illness and likelihood of occurrence of the hazard through a particular food). Greater the risk, the more stringent should be the sampling plan;

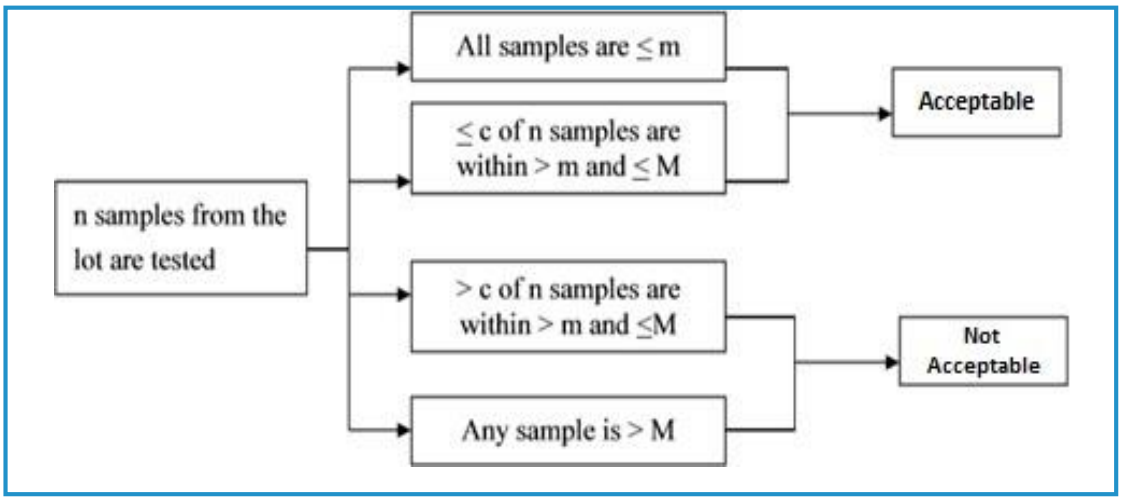
(ii) The susceptibility of the target group of consumers e.g. if consumers are very young or old, immune-compromised, etc.;

(iii) The heterogeneity of distribution of microorganisms

(iv) The conditions under which the food is expected to be handled and consumed after sampling;

(v) The acceptable quality/safety level and

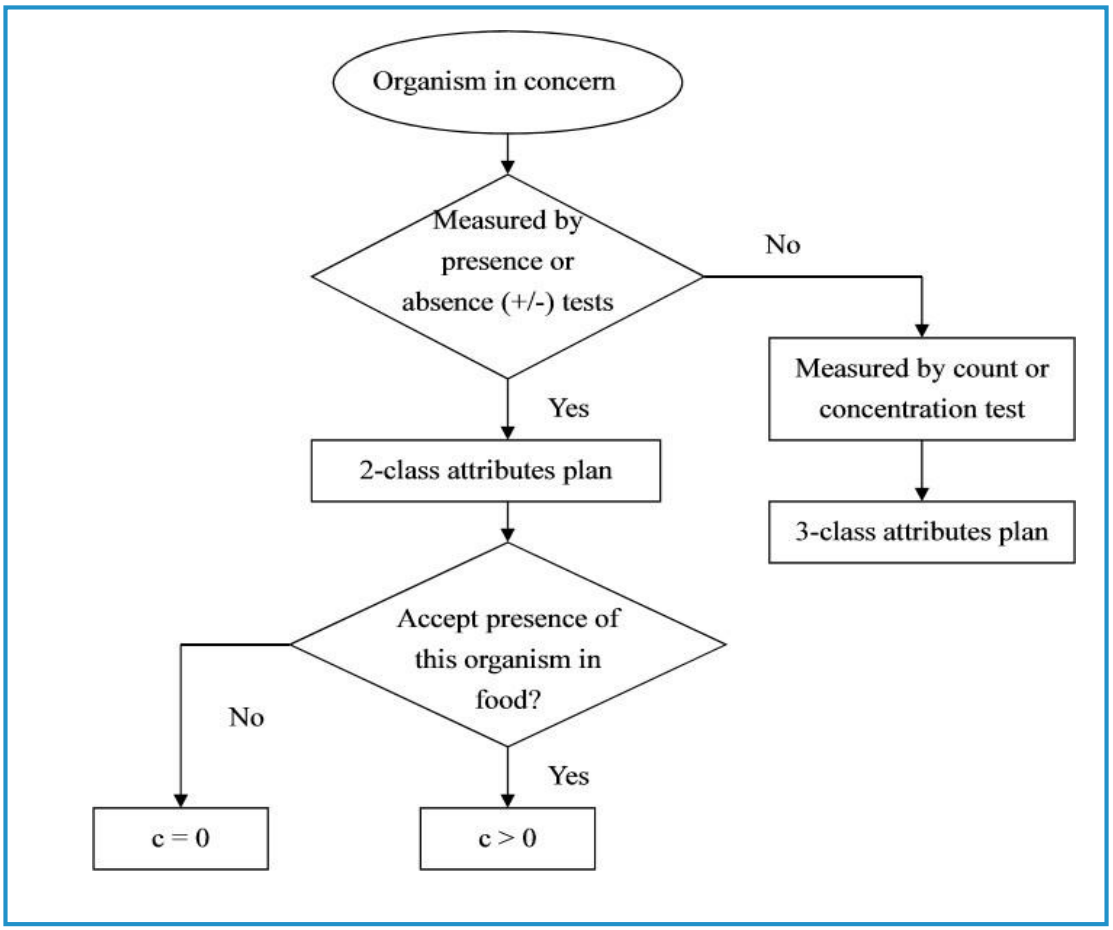
(vi) The desired statistical probability of accepting or rejecting a non-conforming lot.



Choice of sampling plan

In general, a two-class attributes plan is preferred when the organism of concern is not permitted in food sample. If the number of microbes in a unit-volume is allowable, a three- class attributes plan is usually adopted. The following decision tree shows how to choose an appropriate sampling plan for a specific application.

Note: To enhance food safety and improve food quality, more stringent microbiological limits (by decreasing values of m and/or M) should be adopted. By changing the value(s) of c and/or n, the stringency of sampling plan can also be adjusted.



ICMSF cases

ICMSF classifies 15 different 'cases' of sampling plans taking above factors into consideration. Table classifies 15 different cases of sampling plans on a two-dimensional grid taking into account these factors. The stringency of the sampling plan increases with the type and degree of hazard from a situation of no health hazard but of utility only, through a low indirect health hazard (as implied by the presence of

indicator organisms), to direct health risks related to disease of moderate or severe implication. The stringency of the sampling plan also changes according to the conditions under which the food is expected to be handled. Hazards may remain unchanged, be reduced by cooking, or increase because of subsequent growth of microorganisms. The most lenient plan is case 1. Stringency increases from left to right and from top to bottom of the table, so that case 15 is the most stringent.

Table: Suggested sampling plans for combinations of degrees of health concern and conditions of use (15 cases).

Degree of concern relative to utility and health hazard	**Conditions in which food is expected to be handled and consumed after sampling, in the usual course of events**		
	Conditions reduce degree of risk	***Conditions cause no change in risk***	***Conditions may increase risk***
Utility; (e.g. general contamination, reduced shelf life, spoilage)	Increase shelf-life Case 1 3-class n=5, c=3	No change Case 2 3-class n=5, c=2	Reduce shelf-life Case 3 3-class n=5, c=1
Indicator; Low, indirect hazard	Reduce hazard Case 4 3-class n=5, c=3	No change Case 5 3-class n=5, c=2	Increase hazard Case 6 3-class n=5, c=1
Moderate hazard; not usually life threatening, usually no sequelae, normally of short duration, symptoms are self-limiting, can be severe Discomfort	Case 7 3-class n=5, c=2	Case 8 3-class n=5, c=1	Case 9 3-class n=10, c=1
Serious hazard; incapacitating but not usually life threatening, sequelae rare, moderate duration	Case 10 2-class n=5, c=0	Case 11 2-class n=10, c=0	Case 12 2-class n=20, c=0
Severe hazard; (a) the general population or (b) restricted populations, causing life threatening or substantial chronic sequelae or illness of long Duration	Case 13 2-class n= 15, c=0	Case 14 2-class n=30, c=0	Case 15 2-class n=60, c=0

More stringent sampling plans would generally be used for sensitive foods destined for susceptible populations.

ICMSF tool

The ICMSF has developed in 1998 a freely downloadable Excel spreadsheet, in which two- class and three-class sampling plans can be evaluated (Microbiological sampling plans: a tool to explore ICMSF recommendations http://www.icmsf.org/main/software_downloads.html). OC curves are presented both for the proportion defective samples and for the mean of the concentration distribution. The Lognormal distribution is used to describe the microbial distribution. Furthermore, the Poisson-lognormal distribution is included for two-class sampling plans where the microbial method has an enrichment step. Both geometric means and arithmetic means are reported. All quantitative data are easily available since it is a spreadsheet, and the program are flexible in the type of scenario that needs to be evaluated.

Chapter 5 – Sample Collection Procedure

In routine testing, the adequacy and condition of the sample or specimen are of primary importance. If samples are improperly collected and mishandled or are not representative of the sampled lot, the laboratory results will be not the true indicatives. Because interpretations about a large consignment of food are based on a relatively small sample of the lot, established sampling procedures must be applied uniformly.

In suspected or affected lot, a true representative sample is essential when pathogens or toxins are sparsely distributed within the food or when disposal of a food shipment depends on the demonstrated bacterial content in relation to a legal standard.

The number of units that comprise a representative sample from a designated lot of a food product shall be statistically significant.

Steps to be followed in Sample collection procedure

Pre-sampling preparation and aseptic Techniques

a) Extraneous organisms from hands, clothing, sampling equipment, or the processing environment may contaminate samples and may lead to erroneous analytical results. Aseptic sampling techniques should be followed to ensure accurate results that are representative of the product and process.

b) Before beginning sample collection, it is important to assemble sampling supplies/tools, such as sterile gloves, Sterile SS scissors, forceps, spatulas, spoons, polythene bag or container, sterile sampling solutions, sanitizing solution etc. Sterile sampling solutions, such as Butterfield's phosphate diluent (BPD) or buffered peptone water (BPW), should be stored according to the manufacturer’s instruction at room temperature; however, at least the day before sample collection, check such solutions for cloudiness and do not use solutions that are cloudy or turbid or that contain particulate matter.

c) An area should be designated as a staging site for preparing the sampling supplies. Sanitizable surface, such as a stainless-steel table or wheeled cart, can be used. A small plastic tote may also be useful for transporting sampling supplies/tools to sample collection sites.

d) Sterile gloves should be used when handling fish samples or sterile sampling equipment during the sample collection process and care should be taken to prevent contamination of the external surface of the gloves prior to or during the sample collection process.

e) Whenever possible, submit samples to the laboratory in the original unopened containers. If products are in bulk or in containers too large for submission to the laboratory, transfer representative portions to sterile containers under aseptic conditions

f) Use containers that are clean, dry, leak-proof, wide-mouthed, sterile, and of a size suitable for samples of the product. Whenever possible, avoid glass containers, which may break and contaminate the food product.

g) Take care not to overfill bags or permit puncture by wire closure.

h) Identify each sample unit (defined later) with a properly marked strip of masking tape. Do not use a felt pen on plastic because the ink might penetrate the container.

i) Whenever possible, obtain at least 100 g for each sample unit.

j) Deliver samples to the laboratory promptly with the desired storage conditions maintained as nearly as possible.

k) Make a record for samples of the times and dates of collection/arrival etc.

l) Salted/ dried fish that are not perishable and are collected at ambient temperatures need not be refrigerated.

m) Transport frozen or refrigerated products in approved insulated containers of rigid construction so that they will arrive at the laboratory unchanged.

n) Always Keep frozen samples solidly frozen below -18°C.

o) Cool refrigerated samples shall be maintained at 0-4°C and transport them in a suitable refrigerant capable of maintaining the sample at 0-4°C until arrival at the laboratory. Do not freeze refrigerated products. Unless otherwise specified, refrigerated samples should not be analyzed more than 36 h after collection.

How Sample is to be taken and random sampling achieved

In order to obtain a true microbiological flora of fish and fish products it is necessary to take representative samples from the surface as well as the deeper portions.

Sampling can be of after chilling, raw materials, in process samples, environmental samples and finished good samples

- No single sample taken from a fish or other large piece of fish can be truly representative of the whole but equally it is impracticable to analyze the entire fish unit. However, attempts should made to sample lots using whole samples
- In regard to precut fishes select parts from different portions to be representative of the lot. Transfer the samples to sterile containers or polythene bags of convenient size.
- To have a representative composite sample of whole fish, below methods can be followed
    i. **Destructive Method** - It shall be collected from neck skin, meat portion (breast and leg) and wings. Microbiological samples should be done of suspected or affected part/carcass with sterile equipment.
    ii. **Non-destructive Method** - Non-destructive sampling can be achieved by the swab or rinse technique.
- Swab technique - Surface sampling units shall be taken by wiping with large moist swabs over the entire unit or selected areas- or by defining areas using a template.
- Rinse technique – Dip the entire fish or specimen in sterile polythene bag containing sterile 0.1 % peptone water and mix well for at least a minute to collect the surface micro flora of the fish. Then carefully collect the sample from the bag.

Raw Materials – Raw materials are often the major source of microbial contamination that is introduced into the manufacturing process. Raw materials of natural origin, such as animal and plant source, support an extensive and varied micro flora. Collect the representative random sample aseptically from the original container in a sterile polythene bag or container. Test raw material for the applicable parameters as per the regulation/customer requirement or get the certificate of conformance from the RM supplier.

Environmental Monitoring - To have a sample for hygiene and environmental monitoring, the plan shall be developed by the establishment which can depend on size of operations, risk assessment, production volume, process/ equipment's involved and cleaning and sanitation schedule. Environmental monitoring plan shall

also consider the fulfilment of the indicator microbial or environmental microbial requirement of finished goods. Based on sampling plan, swab sample and air samples may be collected after completion of cleaning and sanitation procedure.

Finished Goods -

- The number of sampling units to be taken in order to obtain a primary sample which is as representative as possible of the consignment or lot(s) shall be in accordance with the sampling plan specified in the contract or otherwise agreed between the parties concerned or as per regulations.
- Deciding of how many numbers of packing cases to be considered for Microbiological tests for products (Reference - IS 5404-1984. Methods for Drawing and Handling of Food Samples for Microbiological Analysis)

No. of packing cases in the lot (N)	**No. of packing cases to be opened (n)**	**No. of packing cases in the lot (N)**	**No. of packing cases to be opened (n)**
Up to 8	2	101 to 150	6
9 to 25	3	151 to 300	7
26 to 50	4	301 and above	8
51 to 100	5	....	....

Table 1: Selection of Packing cases for Microbiological tests for products

- Selection of packing case numbers to be opened by Random Sampling Methods (Simple or systematic random sampling) as below

  i. **Simple Random Sampling -** As example, consider 1000 cartons of chicken/ chicken products are of one lot and we have to collect random sample. In this case N=1000 (Carton Number 1 to 1000)

  As per above table we have to collect 8 random sample from anywhere in same lot.

  ii. **Systematic random sampling -** In above case only the exact box for sampling shall be derived by using

  Sampling interval k = N / n = 1000 / 8 = 125

  Sample should be collected with interval of exact 125 numbers to get the 8 sampling boxes.

iii. **Stratified Random Sampling -** Stratified random sampling is advised to be used where lot is of very big size and consisting of many number of batches from different production dates. It is also, possible, and maybe desirable, to use a *stratified random sampling* approach, i.e. drawing at random a given number of sample units from each *stratum* (e.g. each sub-lot or batch). The proportion of sample units from each stratum should correspond to the proportion of units in that stratum. This means, that if each stratum contains the same quantity of product as every other stratum the numbers of sample units per stratum should be the same, otherwise they should differ according to the proportion of the lot contained in the various strata that are being assessed. In case of above example, 1000 cartons may be divided into 8 strata, each containing 125 cartons and then from each strata select one sample random sample. Stratification is a method for handling known sources of variation and may be used where one has prior knowledge that the consignment is potentially not of uniform quality i.e. representing, for example, different days of production from the same plant, different plants of the same company, or from different suppliers. e.g. Fishes destined for processing originating from different farms/different supplier, the results for different strata should be assessed separately and then pooled if they appear to be homogeneous.

iv. Online software's also available to do the random sampling and link of same are as follow

1. Epitools: https://epitools.ausvet.io/randomnumbers
2. Startrek: https://stattrek.com/statistics/random-number-generator.aspx)

- Make a combined sample from all the opened packing cases as a final composite sample for analysis and submit for the in-house testing as a representative of the lot.

Locations and Frequency within the Process Where Samples to be Collected (Small/Medium/ Large Producers)

- Drawing of samples for checking microbiological compliance as well as monitoring hygiene environment and processing conditions –Samples of raw material as well as of the processed and packaged foods may be drawn at various stages of handling and processing in the factory for microbiological examination to check the hygiene environment and processing efficiency. Such examination should

however be supplemented with inspection of the premises, processing equipment and personnel hygiene.

- Sample collection frequency shall be based on risk assessment, available best process control measures and quality plan of each organization.
- FBO shall develop their own sampling and testing frequencies to ensure compliance with the applicable microbiological requirements.
- Suggested locations and frequency of collection of different samples are described below:
  - ✓ **Harvested fishes (chilled or stored under refrigeration) -** Establishment size, sampling frequency and sampling location in the below table

Establishment Size, Sampling Frequency and Sampling Location		
Establishment size	**Defined as**	**Minimum Sampling frequency**
Small	1000 fishes or less / day	One Sample / week
Medium	Line Speed: 2000 fishes/hour	Two Sample / week
Large	Line speed: 4000 fishes / hour	Daily One sample

  - ✓ **Raw materials** – Collect samples from the original container of the raw material on receipt from storeroom. If possible, collect from the online distribution point to study the microbial load in raw material at the time of production. Random sampling frequency shall be established by organization depending on risk group of ingredients and supplier status in market.
  - ✓ **In process samples** – In process sample collection point depends on the risk level, process steps and quality plan of each organization. Generally, in process samples are taken to know the level of indicator organisms and collected at defined critical steps in processing to know effectiveness of control step e.g. critical steps in fish processing are water and ice quality, wash after evisceration, chilling of cut fishes chillers, cooking, chilling of cooked fish, freezing, cold storage etc.
  - ✓ **FG samples** – Generally from FG stores either chill room or cold store. Each batch or lot should be sampled as per random sampling method as described in above document.

- ✓ **Hygiene and environment monitoring samples** – Collect swabs from both food contact and non-food contact surfaces including hand swabs for effective monitoring of hygiene and environment. Test hygiene indicator organisms for food contact surfaces samples and pathogens in both food contact and non-food contact surfaces.

- All the cases/containers in a single consignment of the material of the same type and belonging to the same batch of manufacture shall constitute a lot. If a consignment consists of different batches of manufacture the cases/containers of the same batch shall be separated and each such group shall constitute a lot.
- For ascertaining conformity of the material to the microbiological requirements of a specification, samples shall be tested from each lot separately. These cases/containers shall be selected at random from the lot as specified above in the random sampling procedure.

How samples are to be handled /stored to ensure sample integrity

Packaging, Storage and Transportation

Samples should be received or placed in a specified storage area in which environmental conditions are monitored and recorded.
The sample must be:

1. Collected in the correct, intact, container, device or non-expired transport media.
2. Transported under the correct conditions.
3. Processed/handled according to approved laboratory procedure.
4. In sufficient quantity to perform testing.
5. Received within acceptable time limitation (specific criteria to be determined by each laboratory).

a) If the units are packed in an airtight container, no further packing is required. If the units are not so packed, pack each sampling unit in a suitable sterile sample container, close it securely so as to avoid environmental contamination, seal it and label it.

b) The samples of foods drawn shall be transported to the laboratory for testing as quickly as possible, preferably within 24 hours, taking adequate precautions (e.g. temperature control, etc.) to prevent any change (increase or decrease) in the original microbial flora of the food.

- **Frozen fish and fish products samples** - The samples shall reach the laboratory in a frozen condition and at the temperature laid down by any legislation in force or in any case at a temperature of minus 18°C or lower. Store the samples in the freezer. Preferably each lot representatives are to be tested.
- **Ambient storage fish and fish product samples** - Apparently normal samples shall be stored, protected from direct sunlight or other sources of heat, at a temperature not exceeding 25°C.
- **Swabs samples** - Store the swabs samples in the refrigerator on receipt. The samples shall be examined as soon as possible, but in any case, on the day of receipt.

c) Preservatives shall not be added to sample units intended for microbiological examination.

d) Visibly defective samples shall be placed in sealed containers (for example plastic bags), so as to avoid environmental contamination and shall be stored in the refrigerator.

Labelling of Sample

- Immediately before or after the sample is taken, label the container.
- Proper labelling of samples is essential for tracking and future correspondence.
- Numbers of sample collected and make a record to identify the same.

Handling of Samples - Handle samples so as to avoid any risk of contamination and cross contamination, taking the following precautions:

- ensure that the working area is clean and dust-free; do not subject samples to direct sunlight.
- clean the work surface with a suitable disinfectant both before and after testing.
- Sterilize containers, trays, apparatus, work areas etc., and instruments for handling and opening packs or cans in advance.
- Also sterilize the hands of a person who will be handling the samples before touching them.

Thawing of Frozen Samples - Thawing in the refrigerator - Thaw the unopened samples in the refrigerator until thawing is complete, but for not longer than 24 h. When samples need more than 24 h to thaw, one of the following methods may be used if the sample received is frozen:

- Partially thaw it for 18 hours in a refrigerator at 2 to 5°C.
- If the frozen sample can be easily comminuted, proceed without thawing.
- With easily thawed material, thaw in an incubator at 35°C for not more than 15 minutes.

Opening of sample package

- Disinfect the packages over such a part of the exterior that contamination is avoided on opening. However, when the packaging or wrapping material is very thin and could be damaged by the cleaning process (wrapped portions of fish on trays) this procedure shall be omitted. Disinfection should be carried out very carefully.
- When the packaging can be removed without any risk of contamination, cleaning and disinfection are not necessary.
- All operations during and after opening shall be carried out under aseptic conditions preferably without interruptions; if interruption is unavoidable, it shall be as short as possible. During the whole of any interruption, the product shall be stored in the refrigerator.

Post- analysis Handling of Samples –

- Samples shall be properly stored and handled till the finishing of complete analysis and its interpretation.
- Once the complete analysis is over and results are communicated to the concerns, such samples can be disposed off with suitable disposal method so as to avoid its re-entry into food chain to avoid the cross contamination.
- If not disposed off immediately after completion of analysis, samples can be stored till the shelf life of the product if required for reference with proper labeling.

Chapter 6 – Sample analysis/ Analytical method used to Fish and fish Products samples

The section below describes the methods for microbial analysis of fish and fish product samples. The section has covered the methods for analysis of process hygiene indicator organisms such as- *Escherichia coli*, *Staphylococcus aureus* (Coagulase +ve)) in part I and food safety related microorganisms (*Salmonella* spp., *Listeria Monocytogenes*, Sulphite Reducing Clostridia, and *Clostridium botulinum*) in part II. Details of methods are as per IS standards specified in microbial regulation of food safety and standard.

Part I: Process Hygiene indicator organisms

The most widely accepted microbiological criteria for chilled and frozen raw fish are those set for aerobic plate counts (APC) at 25 °C and E. coli (ICMSF). An increase of APC to levels in excess of 106 cfu/g is usually indicative of inadequate refrigeration, long storage under refrigeration or one of the formers prior to freezing. Faecal coliform counts may be used instead of *E. coli* counts where this method is preferred. For fish of doubtful microbiological quality, it is desirable to test for *Salmonella* and *V. parahaemolyticus*

Aerobic Plate Count

The enumeration of microorganisms by counting the colonies grown in solid medium after aerobic incubation at 30^0 C or 35 ± 2^0C. This standard is applicable to products intended for human consumption according to IS: 5402/ISO 4833 /FSSAI-Microbiology of food 2017 as below.

Tools and Equipment

Refer Annexure A: General tools and equipment needed for sample analysis/ analytical method for microbial analysis

Medium

a. Plate count agar
b. Buffered peptone water 0.1 %

The analyst can use ready-made media as given above or prepare the media as per protocol given in IS/ISO method.

Procedure

a. Aseptically weigh fish and fish products sample in the ratio 1: 9 i.e. minimum 10 g of sample in 90 ml of buffered peptone water as primary dilution. Homogenization of sample can be done with stomacher, sterile scissor and forceps, mixing in sterile bag/conical flask etc.

b. Solution obtained by mixing a measured volume of the primary dilution with a nine-fold volume of diluent and by repeating this operation with further decimal dilution series. Serial dilution of -1, -2, -3 or further depending based on sample category.

c. Pipette 1 ml of the dilutions which have been selected for plating into Petri dish. Pour about 12 to 15 ml of the plate count agar at 44 to 47 ^{0} C into each Petri dish. The time elapsing between the end of the preparation of the initial suspension and the moment when the medium is poured into dishes shall not exceed 45 min. Plating shall be made in duplicate.

d. Carefully mix the inoculum with the medium by rotating the Petri dish and allow the mixture to solidify by leaving the Petri dishes standing on a cool horizontal surface.

e. Invert the Petri dishes and place them in the incubator at 30 ^{0}C±1 ^{0}C for 72 h±3h or 35±2^{0}C for 48 hours.

Counting of Colonies

a. After the specified incubation period count the colonies on the plates using the colony counting equipment if necessary, examine the dishes under subdued light. It is important that pinpoint colonies should be included in the count, spreading colonies shall be considered as single colonies.

Method of calculation

a. For a result to be valid, it is generally considered necessary to count the colonies on at least one dish containing at least 10 colonies. Maximum number of counts for total colonies present: 300 per dish (90 mm dish).

b. Calculate the number *N* of microorganisms present in the test sample from two successive dilutions using equation.

$$\mathrm{N} = \frac{\sum C}{(n1+0.1n2)\, d}$$

Where.

$\sum C$: sum of the colonies on the four dishes retained from two successive dilutions, at least two of which contains a minimum of 10 colonies;

n1: the number of plates counted in the first dilution.

n2: the number of plates counted in the second dilution

d: dilution corresponding to the first dilution retained

[d = 1 when the undiluted liquid product (test sample) is retained].

c. Round off the calculated result to two significant figures. When doing this, if the third figure is less than 5, do not modify the preceding figure; if the third figure is greater than or equal to 5, increase the preceding figure by one unit.

d. Report the result as the number *N* of microorganisms per milliliter (liquid products) or per gram (other products).

e. Example: Counting has produced the following results:

at the first dilution retained (10^{-2}): 168 & 170 colonies;

at the first dilution retained (10^{-3}): 18 & 17 colonies.

- $\sum C = 168 + 170 + 18 + 17 = 373$
- 2
- n2 = 2
- d = 10^{-2}

$$N = \frac{373}{\left(2 + (0.1 \times 2)\right) \times 10^{-2}} = \frac{373 \times 10^{2}}{2.2} = 16955$$

Rounding off the result as specified above, the number of microorganisms is 17000 per gram of product.

f. If there are no colonies on plates from the initial suspension, the number of aerobic bacteria per gram of product should be reported as fewer than 10.

Yeast and Mould count

The enumeration of yeast and mould in fish and fish products intended for human consumption by means of the colony count technique at 25^0 C according to IS:5403:1999 can be preferably followed.

Tools and Equipment's

Refer Annexure A: General tools and equipment needed for sample analysis/ analytical method for microbial analysis

Medium

a) Dichloran Rose Bengal chloramphenicol agar.

b) Yeast extract dextrose chloramphenicol agar

c) Buffered peptone water 0.1%

Procedure

a) Aseptically weigh fish and fish products sample in the ratio of 1:9 i.e. 25 g of sample in 225 ml Buffered peptone water 0.1% as primary dilution. Serial dilution of -1, -2, -3 or further depending based on sample category.

b) Transfer 1ml the initial suspension on the surface of the two sterile Petri dishes. Repeat the procedure with other dilutions if count expecting in higher dilution.

c) Pour about 15 ml of the Dichloran Rose Bengal Chloramphenical Agar medium or any other recommended medium into each petri dish. The time elapsing between the end of the preparation of the initial suspension and the moment when the medium is poured into the dishes shall not exceed 15 min.

d) Carefully mix the inoculum with the medium and allow the mixture to solidify by leaving the Petri dishes to stand on a cool horizontal surface.

e) Prepare a control plate with 15 ml of the medium to check its sterility.

f) Place the inoculated and control plates in upright position in the incubator at 25^0 C.

Counting of colonies

a) Preliminary counting of colonies on each plate shall be done on 3rd day and final count on 5 days of incubation. After 5 days plates showing no colonies shall be retain additional 2 days.

b) If parts of the plates are overgrown with moulds or if it is difficult to count well- isolated colonies retain the counts obtained after 3 days of incubation. In this event, record the incubation period of 3 days in the test report.

c) Carry out a microscopic examination in order to distinguish according to their morphology the colonies of yeast from colonies of bacteria.

Calculation

a) Use dishes containing fewer than 150 colonies.
b) Calculate the number (N) of microorganisms per gram or per milliliter of product using the following equation;

$$N = \frac{\sum C}{(n1 + 0.1n2)\, d}$$

Where

$\sum C$: the sum of the colonies counted on all the dishes selected;

n1 : the number of plates counted in the first dilution;

n2 : the number of plates counted in the second dilution

d : the dilution from which the first counts were obtained (for example 10^{-1}).

Example : Counting has produced the following results:

at the first dilution retained (10^{-2}): 3 & 4 colonies;

at the second dilution retained (10^{-3}): 3 & 2 colonies;

$$N = \frac{3 + 4 + 3 + 2}{\big(2 + (0.1 \text{ x } 2)\big) \text{ x } 10^{-2}} = \frac{12}{0.022} = 545$$

Rounding off the result as specified above, the number of microorganisms is 550 per gram of product.

If there were no colonies on plates from the initial suspension, the number of yeasts and moulds per gram of product should be reported as less than 10.

Escherichia coli

This standard IS: 5887 (Part 1) prescribes methods for isolation, identification and enumeration of *Escherichia coli*.

Tools and Equipment's

Refer Annexure A: General tools and equipment needed for sample analysis/ analytical method for microbial analysis

Medium

- Buffered peptone water 0.1%
- Levin Eosin-Methylene blue agar / Tergitol 7 agar / MacConkeys
- Triple sugar iron (TSI) agar
- Urea broth
- Tryptone broth
- Buffered glucose (MR-VP) medium
- Phenol red sucrose broth
- MacConkey broth
- Simmon's citrate agar

Procedure

a) Ascetically weigh fish and fish products sample in the ratio 1:9 i.e. 25 g of sample in 225 ml buffered peptone water as primary dilution. Make subsequent serial tenfold dilution.
b) Transfer 0.1 ml of inoculum from appropriately selected dilutions on the surface of L- EMB agar plates.
c) Carefully spread the inoculum as quickly as possible over the surface of the agar plates using the spreader. Allow the plates to dry with their lids on for about 15 min at laboratory temperature.
d) Invert the Petri dishes and place them in the incubator at 35 or 37°C for 24 hours.

Identification of *Escherichia coli*

1. **Cultural examination:** Morphological characteristics of colonies to be checked on Levin eosin-methylene blue agar after 24 hrs of incubation at 35 or 37°C. The suspect colonies show typical metallic green sheen with a dark centre.

2. **Microscopic examination:** A single colony of each isolate is fixed on a clean slide to study gram stain, under light microscope. *E. coli* is Gram negative rod-shaped bacterium.

3. **Biochemical test:** The suspected isolates are subjected to the biochemical tests as mentioned bellow in the table.

Test	Incubation period (hours)	Incubation (°C)	Reaction after incubation
TSI H_2S production	48	37	Negative
Urease	18-24	37	Negative
Indole test	48	37	Positive
Methyl red	48	37	Positive
Voges-Proskauer test	48	37	Negative
Sucrose : acid and gas production	18	37	Positive
Growth : MacConkey broth medium at 44°C acid and gas production	48	44	Positive

Counting colonies and expression of result

a) The number of viable colonies of *E. coli* per gram of sample shall be determined by counting the colonies from the plates multiplying by dilution factor and total should be divided with mass/weight of sample taken for analysis.

b) calculate the number (N) of microorganism per g or milliliter of product using following equation;

$$\mathrm{N\ (cfu/g)} = \frac{\sum a}{\mathrm{V\ x\ d}}$$

Where

∑ a : The sum of total number of colonies identified on all the petri plates selected

V : Total volume (in milliliter) of inoculum taken for plating on all petri plates

d : The dilution from which inoculum taken for plating.

Example: Counting has produced the following results:

at the Initial suspension retained: 4 colonies in all plates and sample cultured 1 ml;

$$\mathrm{N\ (cfu/g\ )} = \frac{4}{1\mathrm{ml\ x\ }10^{-1}} = 4\ x\ 10 = 40$$

If there were no colonies on plates from the initial suspension, the number of *Escherichia coli* per gram of product should be reported as less than 10.

Enumeration of E. coli by MPN method

Media and Reagents

1. Brilliant green lactose bile (BGLB) broth, 2%
2. Lauryl tryptose (LST) broth
3. Lactose Broth
4. EC broth
5. Levine's eosin-methylene blue (L-EMB) agar
6. Tryptone (tryptophane) broth
7. MR-VP broth
8. Koser's citrate broth
9. Plate count agar (PCA) (standard methods)
10. Butterfield's phosphate-buffered water or equivalent diluent
11. Kovacs' reagent
12. Voges-Proskauer (VP) reagents
13. Gram stain reagents
14. Methyl red indicator
15. Violet red bile agar (VRBA)
16. VRBA-MUG agar
17. EC-MUG medium
18. Lauryl tryptose MUG (LST-MUG) broth
19. Peptone Diluent, 0.5%

MPN - Presumptive test for *E. coli*

Weigh 50 g of fish/fish products into sterile high-speed blender jar. Frozen samples can be softened by storing for <18 h at 2-5°c, but do not thaw. Add 450 mL of Butterfield's phosphate-buffered water and blend for 2 min. If <50 g of sample is available, weigh portion that is equivalent to half of the sample and add sufficient volume of sterile diluent to make a 1:10 dilution. The total volume in the blender jar should completely cover the blades.

Prepare decimal dilutions with sterile Butterfield's phosphate diluent or equivalent. Number of dilutions to be prepared depends on anticipated coliform density. Shake all suspensions 25 times in 30 cm arc or vortex mix for 7 s. Using at least 3 consecutive dilutions, inoculate 1 mL

Add aliquots from each dilution into 3 LST tubes for a 3 tube MPN analysis (other analysis may require the use of 5 tubes for each dilution; See IV). Lactose Broth may also be used. For better accuracy, use a 1 mL or 5 mL pipet for inoculation. Do not use pipets to deliver<10% of their total volume; eg. a 10 mL pipet to deliver 0.5 mL. Hold pipet at angle so that its lower edge rests against the tube. Not more than 15 min should elapse from time the sample is blended until all dilutions are inoculated in appropriate media. Incubate LST tubes at 35°C± 0.5°C. Examine tubes and record reactions at 24 ± 2 h for gas, i.e., displacement of medium in fermentation vial or effervescence when tubes are gently agitated. Re-incubate gas-negative tubes for an additional 24 h and examine and record reactions again at 48 ± 3 h. Perform confirmed test on all presumptive positive (gas) tubes.

MPN - Confirmed test for coliforms

From each LST or lactose broth tube (showing gas evolution) transfer a loopful of suspension to a tube of BGLB broth, avoiding pellicle if present. (a sterile wooden applicator stick may also be used for these transfers). Incubate BGLB tubes at 35°C ± 0.5°C and examine for gas production at 48 ± 3 h. Calculate most probable number (MPN) of coliforms based on proportion of confirmed gassing LST tubes for 3 consecutive dilutions.

MPN - Confirmed test for fecal coliforms and *E. coli*

From each LST or Lactose broth tube (showing gas evolution) from the Presumptive test, transfer a loopful of each suspension to a tube of EC broth (a sterile wooden

applicator stick may also be used for these transfers). Incubate EC tubes 24 ± 2 h at 44.5°C and examine for gas production. If negative, reincubate and examine again at 48 ± 2 h. Use results of this test to calculate fecal coliform MPN.

MPN - Completed test for *E. coli*.

For the completed test for *E. coli*, gently agitate each gassing EC tube, remove a loopful of broth and streak for isolation on a L-EMB agar plate and incubate for 18-24 h at 35°C ± 0.5°C. Examine plates for suspicious *E. coli* colonies, i.e., dark centered and flat, with or without metallic sheen. Transfer up to 5 suspicious colonies from each L-EMB plate to PCA slants, incubate them for 18-24 h at 35°C ± 0.5°C and use for further testing.

(Identification of any 1 of the 5 colonies as *E. coli* is sufficient to regard that EC tube as positive; hence, not all 5 isolates may need to be tested).

Perform Gram stain. All cultures appearing as Gram-negative, short rods should be tested for the IMViC reactions below and also re-inoculated back into LST to confirm gas production.

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

Voges-Proskauer (VP)-reactive compounds. Inoculate tube of MR-VP broth and incubate 48 ± 2 h at 35°C± 0.5°C . Transfer 1 mL to 13 × 100 mm tube. Add 0.6 mL α-naphthol solution and 0.2 mL 40% KOH, and shake. Add a few crystals of creatine. Shake and let stand 2 h. Test is positive if eosin pink color develops.

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

Citrate. Lightly inoculate tube of Koser's citrate broth; avoid detectable turbidity. Incubate for 96 h at 35°C ± 0.5°C. Development of distinct turbidity is positive reaction.

Gas from lactose. Inoculate a tube of LST and incubate 48 ± 2 h at 35°C ± 0.5°C . Gas production (displacement of medium from inner vial) or effervescence after gentle agitation is positive reaction

Interpretation: All cultures that (a) ferment lactose with gas production within 48 h at 35°C, (b) appear as Gram-negative nonsporeforming rods and (c) give IMViC patterns of ++-- (biotype 1) or -+-- (biotype 2) are considered to be *E. coli*. Calculate MPN (see Appendix 2) of *E. coli* based on proportion of EC tubes in 3 successive dilutions that contain *E. coli*.

Membrane Filtration (MF) Method - coliforms Fish homogenates can easily clog filters, hence MF are most suitable for analysis of water samples for example in immediate adjoining area of fish harvest, water used or ice used for storing). MF may be used in the analysis of homogenized fish and fish products which do not contain high levels of particulate matter.

Staphylococcus aureus (Coagulase +ve)

The enumeration of *Staphylococcus aureus* in fish and fish products intended for human consumption by counting of colonies obtained on a solid medium (Baird-Parker medium) after incubation at 35ºC or 37ºC as specified in IS 5887 Part 8 (sec 1)/ ISO: 6888-1 / FSSAI – Microbiology of foods 2017.

Tools and Equipment's

Refer Annexure A: General tools and equipment needed for sample analysis/ analytical method for microbial analysis

Medium

- Baird Parker agar / VJ Agar
- Buffered peptone water 0.1%

Procedure

- Ascetically weigh fish and fish products sample in the ratio 1:9 i.e. 25 g of sample in 225 ml buffered peptone water 0.1% as primary dilution.
- Transfer 1 ml the initial suspension (1:10) on the surface of three small agar plates (90mm) prepare duplicate.
- Carefully spread the inoculum as quickly as possible over the surface of the agar plates using the spreader. Allow the plates to dry with their lids on for about 15 min at laboratory temperature.
- Invert the Petri plates and place them in the in the incubator at 35-37ºC for 48hours.

- Colonies of *S. aureus* are typically grey black to jet black, circular, smooth, convex, moist and 2-3 mm diameter. Frequently there is a light colored (off-white) margin, surrounded by opaque zone (precipitate) and frequently with outer clear zone; colonies have buttery to gummy consistency when touched with the inoculating needle.
- The typical colonies *S. aureus* are important to distinguishing which is catalase positive. The test is performed by flooding a nutrient agar slant with several drops of 3% hydrogen peroxide. The bubble formation is indicative of a positive reaction,
- On staining, *S. aureus* are Gram positive, cocci-shaped and tend to be arranged in grape like clusters.

Counting Colonies and Expression Results

- If a 1 ml inoculum was spread over three plates, treat these plates as one in all subsequent counting.
- Maximum total number of colonies (typical & atypical) present on a dish should not exceed 300 per dish & maximum number of counts for typical or presumptive colonies 150 per dish.
- Multiply the count obtained by corresponding sample dilution. Report as *S. aureus* per gram or ml of the sample.
- 4) calculate the number (N) of microorganism per g or milliliter of product using following equation;

$$\mathrm{N\ (cfu/g)} = \frac{\sum a}{\mathrm{V\ x\ d}}$$

Where

∑ a : The sum of total number of colonies identified on all the petri plates selected

V : Total volume (in milliliter) of inoculum taken for plating on all petri plates

d : The dilution from which inoculum taken for plating.

Example : Counting has produced the following results:

at the Initial suspension retained: 4 colonies in all plates and sample cultured 1 ml;

$$\text{N (cfu/g)} = \frac{4}{1\text{ml x } 10^{-1}} = 4 \text{ x } 10 = 40$$

If there were no colonies on plates from the initial suspension, the number of *S. aureus* per gram of product should be reported as less than 10.

The microbiological limits suggested for *E.coli* and *S.aureus* in fishes and seafood (ICMSF, 1986)

Product	Test	Case	Plan Class	no. of samples	no. of positive results	Limit per gram or per cm^2	
						m	M
Fresh and frozen fish	APC	1	3	5	3	$5 \text{ x } 10^5$	10^7
	E. Coli	4	3	5	3	11	500
Precooked breaded fish	APC	2	3	5	2	$5 \text{ x } 10^5$	10^7
	E. Coli	5	3	5	2	11	500
Frozen raw crustaceans	APC	1	3	5	3	10^6	10^7
	E. Coli	4	3	5	3	11	500
Frozen cooked crustaceans	APC	2	3	5	2	$5 \text{ x } 10^5$	10^7
	E. Coli	5	3	5	2	11	500
	S. aureus	8	2	5	0	10^3	-
Cooked, chilled, and frozen crabmeat	APC	2	3	5	2	10^5	10^6
	E. Coli	6	3	5	1	11	500
	S. aureus	9	2	5	0	10^3	-
Fresh and frozen bivalve molluscs	APC	3	2	5	0	$5 \text{ x } 10^5$	-
	E. Coli	6	2	5	0	16	

Part II: Food Safety Criteria

Salmonella spp.

Isolation of *Salmonella spp*. from various fish and fish products samples are carried out by the pre-enrichment, enrichment in selective broth followed by selective plating on different medium and its confirmation according to IS:5887 Part 3 / ISO 6579 / FSSAI Manual– Microbiology of foods 2017 as below.

Tools and Equipment's

Refer Annexure A: General tools and equipment needed for sample analysis/ analytical method for microbial analysis

Medium:

- Lactose broth
- Buffered peptone water
- Rappaport Vassiliadis broth
- Tetrathionate broth
- Selective / cystine medium
- Phenol red agar
- Xylose lysine deoxycholate (XLD) Agar
- Hektoen enteric agar (HEA)
- Bismuth sulphite agar (BSA)
- Brilliant green agar (BGA)
- Nutrient agar
- Triple sugar iron (TSI) agar
- Urea broth
- Phenol red dulcitol broth
- Phenol red lactose broth
- Phenol red sucrose broth
- Tryptone broth
- KCN broth
- Malonate broth
- Buffered glucose (MR-VP) medium

Analyst can use readymade media as given above or prepare the media as per protocol given in IS/ISO method.

Procedure

1) **Sampling** – 25 g of either fresh or frozen fish sample shall be used.
2) **Pre-enrichment -** Buffered peptone water or lactose broth is used for pre-

enrichment. Inoculate 25 g of fish or fish products sample in 225 ml pre-enrichment broth and incubate it for 16-20 hour at 35°C or 37°C.

3) **Enrichment -** Several selective broths are used for enrichment eg. selenite/cystine, tetrathionate and Rappaport-Vassiliadis (RV) broths. Gently shake the incubated sample of pre-enrichment and transfer the same in any one of below medium as per given protocol of transfer and inoculation.

Enrichment Media	Pre-enrichment transfer quantity	Incubation	
		Temperature (°C)	Time (Hrs.)
RV broth (10 ml)	1 ml	42	18 to 24
Tetrathionate broth (10 ml)	1 ml	37	18 to 24
Selenite/cystine broth (100 ml)	10 ml	35-37	24

Note: It is always recommended that to incubate enrichment for further 24 hours and second streaking can be done after 48 hours of total incubation of enrichment.

Selective Plating –

Several alternativee media are used for getting selective colonies. Below are the details of media with colony characteristics of bacteria.

a) XLD agar: Pink colonies with or without black centers.

b) Hektoen enteric agar: Blue green to blue colonies with or without black centers.

c) Brilliant green agar: Pink colony surround by red medium

d) Bismuth sulfite agar: Brown, grey or black colonies sometimes with metallic sheen.

e) Phenol red agar; Colour of the medium to change from pink to red

Streak the loopfull of culture on any two of above selective medias and incubate the plates for 35 or 37°C for 24 hours.

4) Streak the typical/suspected colonies on to the surface of pre-dried nutrient agar plates in a manner which will allow well isolated colonies to develop.

5) Incubate the inoculated nutrient agar plates at 35 or 37°C for 24 hours. Use this pure culture for biochemical and serological confirmation.

6) Steak loopfull of pure culture on triple sugar iron agar slants and incubate at 35 or 37°C for 24 hours.
7) Triple sugar iron agar slants showing alkaline slant (red) with gas formation and acid butts (yellow) with formation of H_2S (blackening of the agar) is treated as presumptive positive.

Biochemical identification of *Salmonella spp. from* Presumptive positive *Salmonella*

1) Using sterile needle inoculate a portion of the presumptive positive culture on TSI slant into the following broths. Incubate after inoculation at 35 or 37°C for the specified period given below and read for *Salmonella* typical reactions.

Broth/Media	Time of Incubation	Results
Urea broth	24+2h	**Negative (no change in yellow colour of medium)**
Lysine decarboxylation Medium	24 h	Positive (Purple colour)
Phenol red lactose broth	48+2h	*Negative for gas and/or acid reaction
Phenol red sucrose broth	48+2h	*Negative for gas and/or acid reaction
Phenol red dulcitol broth	48+2h	*Positive for gas and/or acid reaction
Tryptone broth	24+2h	Negative for indole test
KCN broth	48+2h	Negative (no turbidity)
Malonate broth	48+2h	*Negative (green color unchanged)
MR-VP medium	48+2h	Negative for VP test but positive for MR test.
***(Note : Majority of *S.arizonae* are atypical for these reaction)**		

2) If possible, for further identification of *Salmonella* spp., serological tests may be conducted.

The fish document comprises of the most important marine requirements a per industry norms.

Interpretation of results

1) Based on the presence of growth of colonies on selective media and further confirmed by biochemical testing, we can interpret about presence or absence of *Salmonella* Spp. in that particular batch of designated product.
2) Product with presence of *Salmonella* Spp. as per above interpretation may be retested for further reconfirmation (antibody based rapid tests can be used also, if desired).
3) Such product shall be treated as non-confirmatory product and shall be kept on isolated and kept on hold, stored separately and then disposed of in appropriate way.

   Investigations/root cause analysis, correction and corrective actions shall be taken for further improvements.

Expression of Results: Present / Absent per 25 g or ml

Listeria monocytogenes

This part of ISO 11290 specifies a horizontal method for the detection of *Listeria monocytogenes*. This part is applicable to products intended for human consumption and to environmental samples in the area of food production and food handling.

Tools and Equipment's

Refer Annexure A: General tools and equipment needed for sample analysis/ analytical method for microbial analysis

Medium

- Fraser broth
- Oxford agar
- PALCAM agar
- Tryptone soya yeast extract agar (TSYEA)

Analyst can use readymade media as given above or prepare the media as per protocol given in IS/ISO method.

Procedure

Primary and Secondary Enrichment

- Add 25 g of fish or frozen fish sample to 225 ml enrichment broth (Half-Fraser broth) and mix thoroughly.
- Incubate the initial suspension at 30ºC for 24±2 hours. (A black coloration may develop during the incubation).
- After incubation of the initial suspension (Half-Fraser broth), transfer 0.1 ml of the culture to a tube containing 10 ml of secondary enrichment medium (Fraser broth).
- Incubate the inoculated medium for 48±2 hours at 35°C or 37°C.

Plating out and identification

- From the primary enrichment (Half-Fraser broth) culture incubated for 24±2 hours at 30°C, take by means of a loop or glass rod, a portion of the culture and inoculate the surface of the first selective plating medium (Oxford agar) so that well-separated colonies are obtained.
- Proceed in the same way with the second selective plating-out medium (PALCAM agar).
- From the secondary enrichment (Fraser broth) medium incubated for 48±2 hours at 35°C or 37°C, repeat the procedure with the two selective plating-out media.
- Invert the Oxford agar dishes and place them in an incubator set at 35°C or 37°C. PALCAM agar plates are incubate aerobically at 35°C or 37°C.
- After incubation for 24 hours and for an additional 18 to 24 hours (if growth is slight or if no colonies are observed after 24 hours of incubation), examine the dishes for the presence of colonies presumed to be *Listeria* spp.
- **Oxford agar:** Typical colonies of *Listeria* spp. grown on Oxford agar for 24 hours are small (1 mm) greyish colonies surrounded by black halos. After 48 hours, colonies become darker, with a possible greenish sheen and are about 2 mm in diameter with black halos and sunken centres.
- **PALCAM agar:** For plates incubated micro aerobically, after incubation, expose the PALCAM agar plates to air for 1 hour to allow the medium to

regain its pink to purple colour. After 24 hours *Listeria* spp. grow as small or very small greyish green or olive green colonies 1.5 mm to 2 mm in diameter, sometimes with black centres but always with black halos. After 48 hours, *Listeria* spp. appear in the form of green colonies about 1.5 mm to 2 mm in diameter with a central depression and surrounded by a black halo.

Confirmation of *Listeria* spp.

For confirmation, take from each plate of each selective medium five colonies presumed to be *Listeria* spp. If on one plate there are fewer than five presumed colonies, take all of them for confirmation.

- Streak the selected colonies onto the surface of pre-dried plates of tryptone soya yeast extract agar (TSYEA) in a manner which will allow well-separated colonies to develop.
- Place the plates in the incubator set at 35ºC or 37ºC for 18 to 24 hours or until growth is satisfactory.
- Typical colonies are 1 to 2 mm in diameter, convex, colorless and opaque with an entire edge. If the colonies are not well separated, pick a typical *Listeria* spp. colony onto another TSYEA plate. Carry out the following tests from colonies of a pure culture on the TSYEA.
- **Catalase reaction:** The immediate formation of gas bubbles after adding 3% hydrogen peroxide solution on the colonies indicates a positive reaction.
- **Gram staining:** Gram-positive slim, short rods.
- **Motility test:** *Listeria* spp. are motile, giving a typical umbrella-like growth pattern in stab culture in motility medium.
- All *Listeria spp.*are small, gram-positive rods that demonstrate motility. They are catalase positive.

Confirmation of L. monocytogenes

Haemolysis test

a. If the morphological and physiological characteristics and catalase reaction are indicative of *Listeria* Spp., inoculate culture on the sheep blood agar plates to determine the haemolytic reaction.

b. Dry the agar surface well before use. Take a colony and plate and stab one space for each culture, using a wire. Simultaneously stab positive *(L. monocytogenes)* and negative control cultures *(L. irmocua)*.

c. After incubation at 35ºC or 37ºC for 24+2 hours, examine the test strains and controls.

L. monocytogenes show narrow, clear, light zones (Beta haemolysis). *L. innocua* show no clear zone around the stab. *L. seeligeri* show a weak zone of haemolysis. *L. ivanovii* usually show wide, clearly delineated zones of beta haemolysis. Examine the plates in a bright light to compare test cultures with controls.

Carbohydrate utilization

d. Inoculate using a loop each of the carbohydrate utilization broths with a culture from TSYEB. Incubate at 35ºC or 37ºC for up to 5 days. Positive reactions (acid formation) are indicated by a yellow colour and occur mostly within 24 to 48 h.

CAMP test

e. Streak each of the *S. aureus* and *R. equi* cultures in single lines across the sheep blood agar plate so that the two cultures are parallel and diametrically opposite. A thin, even inoculum is required. This can be obtained by using an inoculation loop or a wire held at right angles to the agar.

f. Streak the test stain in a similar fashion at right angles to these cultures so that the test culture and *S. aureus* and *R. equi* cultures do not touch but at their closest are about 1 mm to 2 mm apart. Several test strains may be streaked on the same plate.

g. Simultaneously, streak control cultures of *L. monocytogerres*, *L. irmocua* and *L. ivanovii*. If blood agar is used, incubate the plates at 35ºC or 37ºC for 18 to 24 h. If double-layer plates are used, incubate at 35ºC or 37ºC for12 to18 h.

h. An enhanced zone of beta haemolysis at the intersection of the test strain with each of the cultures of *S. aureus* and *R. equi k* considered to be a positive reaction.

i. The positive reaction with *R. equi* is seen as a wide (5 mm to 10 mm) “arrow-head” of haemolysis. The reaction is considered as negative if a small zone of weak haemolysis extends only about 1 mm at the intersection of the test strain with the diffusion zone of the *R. equi* culture. A positive reaction with *S. aureus* appears as a small zone of enhanced haemolysis extending only about 2 mm from the test strain

and within the weakly haemolytic zone due to growth of the *S. aureus* culture. Large zones of haemolysis do not occur in the area of S. *aureus* and *L. monocytogenes*.

Table: Reactions for the identification of *Listeria* spp.

Species	Haemolysis	Production of acid		CAMP test	
		Rhamnose	Xylose	*S. aureus*	*R. equi*
L. monocytogenes	+	+	-	+	-
L. innocua	-	V	-	-	-
L. ivanovii	+	-	+	-	+
L. seeligeri	(+)	-	+	(+)	-
L. welshimeri	-	V	+	-	-
L. grayi* subsp. *Grayi	-	-	-	-	-
L. grayi* subsp. *Murrayi	-	V	-	-	-
V: variable reaction; (+): weak reaction; +: >90 % of positive reactions; —: no reaction					

Expression of results

Report the presence or absence of *Listeria monocytogenes* in the test portion specifying the mass in grams of the sample tested.

Sulphite reducing Clostridia

The enumeration of Sulfite-reducing bacteria growing under anaerobic conditions. It is applicable to products intended for human consumption according to ISO 15213.

Tools and Equipment's

Refer Annexure A: General tools and equipment needed for sample analysis/ analytical method for microbial analysis

Medium:

- Buffered peptone water 0.1 %
- Iron sulfite agar

Procedure

- Aseptically weigh fish and fish products in the ratio 1:9 i.e. 25 g of sample in 225 ml of buffered peptone water as a primary dilution.
- Heat treatment of the initial suspension may be necessary to eliminate vegetative forms of spore-forming bacteria and/or non-spore-forming bacteria. Temperatures and heating times vary according to the actual need, from combination producing a definite pasteurization effect at a moderate heat activation effect (e.g 75^0 C for 20 min), to boiling for several minutes. In this case, results could be given as number of spores of sulfite- reducing bacteria growing under anaerobic conditions.
- Solution obtained by mixing a measured volume of the primary dilution with a ninefold volume of diluent and by repeating this operation with further decimal dilution series.
- Pipette 1 ml of the dilutions which have been selected for plating into Petri dish. Pour about 12 ml to 15 ml of the iron sulfite agar which has been cooled to 44 ^{0} C to 47 ^{0} C into each Petri dish. The time elapsing between the end of the preparation of the initial suspension and the moment when the medium is poured into dishes shall not exceeded 45 min.
- Carefully mix the inoculum with the medium by rotating the Petri dish by horizontal movement and allow the mixture to solidify.
- Incubate the Petri dishes in anaerobic jars at 37 +/- 1^0 C for 48 h.

Counting of the colonies

- Read the result after 48 hours. Black colonies, possibly surrounded by a black zone, are counted as sulfite-reducing bacteria.
  Count colonies of sulfite-reducing bacteria in each dish containing less than 150 typical colonies and less than 300 total colonies.

Method of calculation & result expression

- Count the colonies on at least one dish containing at least 10 colonies. Calculate the number *N* of microorganisms present in the test sample as a weighted mean from two successive dilutions using Equation.

$$N = \frac{\sum a}{V \times 1.1 \times d}$$

Where,

$\sum a$ is the sum of the colonies on the two dishes retained from two successive dilutions, at least one of which contains a minimum of 10 colonies;

V is the volume of inoculum placed in each dish, in millitres;

d is the dilution corresponding to the first dilution retained [*d* = 1 when the undiluted liquid product (test sample) is retained].

- Round off the calculated result to two significant figures. When doing this, if the third figure is less than 5, do not modify the preceding figure; if the third figure is greater than or equal to 5, increase the preceding figure by one unit.
- Report the result as the number *N* of microorganisms per gram of sample.

Example: Counting has produced the following results:

at the first dilution retained (10^{-2}): 0 colonies;

at the second dilution retained (10^{-3}): 0 colony;

$$N = \frac{0 + 0}{1 \text{ x } 1.1 \text{ x } 10^{-2}} = \frac{0}{1.1 \text{ x } 10^{-2}} = 0$$

Clostridium botulinum*

*Cl.botulinum** identification is important for thermally processed/canned fishes and fish products. Isolation and identification of *Clostridium botulinum* in food can be carried out according to IS 5887 (Part 4):1999

Tools & Equipment's

Refer Annexure A: General tools and equipment needed for sample analysis/ analytical method for microbial analysis

Medium

- Blood agar with neomycin
- Cooked meat medium
- Willis and Hobb's medium with neomycin
- Medium for *Cl. botulinum* type E

Procedure

Isolation

1) Preheat the sample at 80°C for 30 min and inoculate into cooked meat medium and the two solid media (Blood agar with neomycin and Wilfis and Hobb's medium with neomycin). The solid media are incubated anaerobically and all the three inoculated media are incubated at 37°C for 5 to 10 days.

2) **For the type E strains:** type E strains exhibit low thermal resistance and are missed in specimens which have been heated prior to inoculation. Inoculate the specimen in duplicate tubes of the medium (Medium for *Cl. botulinum type* E) and incubate at 30°C for 3 days.

3) In sterile test tubes take aliquots of 2 ml samples of growth and mix with equal volume of absolute ethanol. Let stand at 25°C for one hour with occasional mixing.

4) Streak onto Willis and Hobb's medium with neomycin and inoculate into medium for *Cl botulinum* type E. Incubate overnight at 37°C the solid medium being incubated in an anaerobic jar.

5) Examine the solid medium for presence of colonies with opalescence zones indicating growth of *Cl .botulinum* Type E. If such colonies are present, carry out test for toxin using the growth in medium for *Cl botulinum* type E inoculated with ethanol treated culture.

Identification

1) **Grams stain:** Test from liquid culture and solid media **-** Gram-positive-rods, large and stout with straight sides and rounded ends. Spores are oval, central or subterminal and distend the bacillary body.

2) **Colonial Characters:** On blood agar medium growth is associated with haemolysis which may not be larger than the colony. On Willis and Hobb's medium with neomycin, colonies produce opalescence and a pearly layer and are lactose negative.

3) ***In Vivo* Test for Toxin:** Grow suspect strain in cooked meat medium for 5 to 10 days. Obtain filtrate and divide into two portions, one of which is heated at 100°C for 10 min. Use three guinea pigs for intraperitoneal injection with filtrate as follows:

a) One animal is protected with polyvalent botulinum antitoxin and injected with 2 ml of unheated filtrate;

b) One animal as injected with 2 ml of unheated filtrate and is unprotected: and

c) One animal is injected with 2 ml of heated filtrate.

4) Death with paralytic symptoms of the unprotected animal receiving unheated filtrate and survival of the other two animals diagnose the presence of botulinum toxin.

Demonstration of Toxin of *Cl. botulinum* Type E

The procedure as in *In Vivo Test for Toxin* may fail to demonstrate toxin of *Cl. botulinum* Type
E. For such strains the procedure shall be as follows.

1) To filtrate from growth in medium as obtained after procedure described above trypsin is added to a final concentration of 0.1 percent. Incubate at 37°C for 60 min.

2) Dilute specific type E antitoxin 1 in 5 with 0.1 M phosphate buffer of pH 6.5 containing
0.2 percent gelatin.

3) To 1.5 ml of diluted antitoxin, add equal volume of trypsinized filtrate mix and keep at room temperature for 30 min.

4) Inject 1 ml of the mixture intra-peritoneally into a pair of white mice. Also inject a pair of mice with 0.5 ml of the filtrate heated at 100°C for 10 min and another pair of mice with 0.5 ml of unheated trypsinized filtrate.

5) Observe the mice up to 96 h. Death of the unprotected mice and survival of the mice receiving neutralized toxin and the heated toxin diagnose toxin of Cl. *Botulinum*-Type E.

In addition to the above analytical tests for *Vibrio* Sp can be carried out if considered desirable.

Chapter 7 – Analysis and Interpretation of Results and Record Keeping

Analysis and Interpretation of Results

FSSAI Microbiological standards for Fish and fish products shall be referred as guidance document on interpreting results for the microbiological examination for pathogenic microorganisms and for process hygiene/indicator microorganisms. The limits apply to different kind of fish and fish products as mentioned in FSSAI standards at different stages in food chain (Process Hygiene Criteria Indicators - indicate the acceptable functioning of the production process; Food Safety Criteria - define the acceptability of a batch/lot till the shelf life). The following are the microbiological standards for Fish and fish products:

Table1: Microbiological Standards for Fish products and sampling plans

Sr. No.	Product Category*	Aerobic Plate Count				Coagulase positive Staphylococci				Yeast And mold count			
		Sampling Plan		Limits		Sampling Plan		Limits		Sampling Plan		Limits	
		n	c	m	M	n	c	m	M	n	c	m	M
1.	Raw/Chilled/Frozen Finfish	5	3	$5x10^5$ cfu/g	10^7 cfu/g	-	-	-	-	-	-	-	-
2.	Raw/Chilled/Frozen Crustaceans	5	3	10^6 cfu/g	10^7 cfu/g	-	-	-	-	-	-	-	-
3.	Raw/Chilled/frozen Cephalopods	5	2	10^5 cfu/g	10^6 cfu/g	-	-	-	-	-	-	-	-
4.	Chilled/Frozen Bivalves	5	2	10^5 cfu/g	10^6 cfu/g	-	-	-	-	-	-	-	-
5.	Frozen cooked crustaceans/ Frozen heat shucked Mollusca	5	2	10^5 cfu/g	10^6 cfu/g	5	2	10^2 cfu/g	10^3 cfu/ g	-	-	-	-
6.	Dried/ Salted and dried fishery Products	5	0	10^5cfu/g		-	-	-	-	100 cfu/gm	0	100cfu/g	
7.	Thermally processed fishery products	Commercially Sterile				-	-	-	-	-	-	-	-
8.	Fermented fishery products	-	-	-	-	5	1	10^2 cfu/g	10^3 cfu/g	100 cfu/ gm5	0	100cfu/g	
9.	Smoked fishery products	5	0	10^5cfu/g		5	2	10^2 cfu/g	10^3 cfu/g	-	-	-	-
10.	Accelerated Freeze Dried fishery products	5	0	10^4 cfu/g		5	0	100cfu/g		-	-	-	-
11.	Fish Mince/Surimi and analogues	5	2	10^6 cfu/g	10^6 cfu/g	5	2	10^2 cfu/g	10^3 cfu/g	-	-	-	-

Table2: Microbiological requirements for Fish and fishery products-hygienic indicator microorganism

Product Category*	Aerobic Plate Count				Coagulase positive Staphylococci			Yeast & mold Count				
	Sampling Plan		Limits		Sampling Plan		Limits	Sampling Plan			Limits	
	N	c	m	M	n	c	m	M	N	c	m	M
Fish Pickle	5	0	10^3 cfu/g		5	1	10^2 cfu/g	10^3 cfu/g	5	0	100cfu/g	
Battered and breaded fishery products	5	0	10^5 cfu/g	10^7 cfu/g	5	1	10^2 cfu/g	10^3 cfu/g	5	0	100cfu/g	
Convenience fishery products	5	2	$5x10^3$ cfu/ g	$5x10^4$ cfu/ g	5	2	10^2 cfu/g	10^3 cfu/g	-	-	-	
Powdered fish-based products	5	2	10^4 cfu/g	10^5 cfu/g	5	2	10 cfu/g	10^2 cfu/g	5	0	100 cfu/g	

Table 3: Microbiological requirement for Fish and Fishery products- Safety Indicator Organisms

Product Category*	*Escherichiacoli*				*Salmonella*				*Vibriocholerae* (O1 and O139)				*Listeria monocytogenes*				*Clostridium Botulinum*			
	Sampling Plan		Limits		Sampling Plan		Limits		Sampling Plan		Limits		Sampling Plan		Limits		Sampling Plan		Limits	
	n	c	M	M	n	c	m	M	n	c	m	M	n	c	m	M	n	C	m	M
Fermented fishery products	5	2	4 MPN/g	40 MPN/g	10	0	Absent/25g		-	-	-	-	-	-	-	-	Absence of viable spores or vegetative cells of *Clostridium botulinum* and absence of botulinum			
Smoked fishery products	5	3	11 MPN/g	500 MPN/g	5	0	Absent/25g		5	0	Absent/25g		5	0	Absent/25g		-	-	-	-
Accelerated Freeze Dried Fishery Products	5	0	20 cfu/g		5	0	Absent/25g		5	0	Absent/25g		5	0	Absent/25g		-	-	-	-
Fish Mince/Surimi and analogues	5	0	20 cfu/g		5	0	Absent/25g		5	0	Absent/25g		5	0	Absent/25g		-	-	-	-
Fish Pickle	5	0	20 cfu/g		5	0	Absent/25g		-	-	-		-	-	-		-	-	-	-
Battered and breaded fishery products	5	2	11 MPN/g	500 MPN/g	5	0	Absent/25g		5	0	Absent/25g		5	0	Absent/25g					
Convenience fishery products	5	2	1 MPN/g	10 MPN/g	5	0	Absent/25g		5	0	Absent/25g		5	0	Absent/25g					
Powered Fish based products	-	-	-	-	5	0	Absent/25g		-	-	-		-	-	-		-	-	-	-

For more information, please refer to FSSAI Gazzeted Notification dated 13th February 2017, Fish and Fish Products, F.No1-10/7/Standards/Fish.FSSAI-2013

biological Standards– Food Safety Criteria

The Microbiological requirements and guidelines laid down for fish and fish products by FSSAI, can be found in FSSAI Gazzeted Notification dated 1st January 2018, related to Food Safety and Standards (Food Products Standards and Food Additives) Tenth Amendment Regulations, 2016.

Kindly refer FSSAI Food Safety and Standards (Food Product Standards and Food Additives) Third Amendment Regulation, 2017 related to microbiological standards for fish and fish products, APPENDIX B of the Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments in it thereafter for interpretation of results and appropriate utilization of analyzed and interpreted results for further information.

Interpretation of results should also be based on knowledge of the food product and the production process. Care must be taken when interpreting results obtained in the absence of this information.

Record Keeping

1) Record keeping is developed to control and maintain records that are appropriate to Food Safety Management System like processing / preparation, production, storage, distribution, service, product quality, laboratory test results, cleaning and sanitation, pest control and product recall etc.
2) Process is placed to ensure confidentiality and effective storage, protection, retrieval and disposition of records
3) Record retention time period designated or as required by local regulation or customer's requirement, and it should not be less than the shelf life of the product.
4) The QA department follows a definite procedure to identify the inspection and test status of the samples by either labelling them or keeping in separate place. These samples are generally segregated in following categories (samples received but not tested, samples under testing, samples approved by laboratory, samples rejected, samples kept for further reference, *etc.*)

Chapter 8 – Actions to be initiated in response to results

1) In case of non-compliance in respect of process hygiene criteria, the processor shall check and improve process hygiene by implementation of guidelines in Schedule 4 (Part IV) of FSS (Licensing and Registration of Food Businesses) Regulations
2) In case of Food Safety Criteria, ensure that all food safety criteria are complied with before releasing the product batch/lot in the market. In case of Noncompliance in respect to Food Safety criteria, product shall be treated as non-conformity product and the lot/batch is rejected.
3) Different organism wise actions can be initiated considering below guidelines for further improvement.

Microorganism	Likely cause	Actions
Aerobic Plate Count	Inadequate temperature control and poor personal hygiene and other hygienic practices.	Food handling practices should be investigated to: ensure all practicable measures are being undertaken by food handlers to prevent cross contamination and maintain hygienic practices. ensure good levels of personal hygiene review temperature and time controls Proactive investigation to ensure hygiene practices and temperature controls are effectively implemented.
Yeast and Mold	Inadequate temperature control, inadequate air filtration and poor hygienic practices.	Food handling practices should be investigated to: ensure all practicable measures are being undertaken to maintain hygienic practices by food FBO review temperature and time controls. air handling units shall be cleaned properly, and frequent air and food contact equipment shall be tested to reduce incidence of Y and M. Proactive investigation to ensure hygiene practices and temperature controls are effectively implemented.

Escherichia coli	. Contamination of faecal origin from poor hygienic practices (cross contamination from food contact surfaces, raw foods or food handlers) or there has been inadequate	Review: Check working efficiency of processing equipment's, area and evisceration machines to reduce contamination. Control at primary production like good harvest practices, water quality and other inputs as appropriate. Effective cleaning and sanitation of harvested fishes
	Processing b. Contamination from the primary production environment should be considered. Poor hygienic practices.	Cleaning and sanitizing practices for premises and equipment. Food handler personal hygiene Time and temperature control The processing controls used (such as cooking temperatures) Additional food or environmental samples may be required for investigation and testing for enteric pathogens considered if appropriate. Proactive investigation to ensure processing and hygiene controls are being implemented.
Staphylococcus aureus	Inadequate temperature control and poor personal hygiene and other hygienic practices.	Food handling practices should be investigated to: ensure all practicable measures are being undertaken by food handlers to prevent cross contamination and maintain hygienic practices. ensure good levels of personal hygiene review temperature and time controls. food handling practices should be investigated by frequent collection of swabs Proactive investigation to ensure personal hygiene practices and temperature controls are effectively implemented.

***Salmonella* spp.**	a. Inadequate control at primary production b. Inadequate processing of raw products, cross contamination or contaminated raw materials. c. Inadequate sanitation of processed fish, gross unsanitary conditions of areas used for storing harvested fishes, contaminated ice and water used. d. Poor time and temperature control are important contributing factors for multiplication.	Products should be treated as non-conforming and kept on hold and isolated and then product disposition action to be taken in appropriate way. An investigation should be undertaken to assess: raw material suitability the adequacy of processing used (e.g. cooking) the adequacy of measures implemented to prevent the likelihood of cross contamination the effectiveness of cleaning and sanitizing equipment. the adequacy of time and temperature controls used. The adequacy of health and hygiene practices may also require investigation if an infected food handler is suspected.
Listeria monocytogenes	Post-processing or post- harvest contamination or inadequate process control.	Products should be treated as non-conforming and kept on hold and isolated and then product disposition action to be taken in an appropriate way. An investigation should be undertaken of: a. the raw materials used
	Higher levels in product in the marketplace may be due to poor temperature control during storage and/or distribution or inappropriate length of shelf life.	b. adequacy of cleaning and sanitizing of premises and equipment, particularly of preferred sites such as drains c. adequacy of construction and maintenance of premises d. the effectiveness of processing controls e. the adequacy of process flow. f. Increased sampling, including environmental monitoring sampling may be done.

Annexures

Annexure A: General tools and equipment 's needed for Sample analysis/ Analytical method for microbial analysis

A. Weighing balance sensitive to 0.1 g
B. Autoclave
C. Colony counter
D. Dilution and media storage bottles
E. Glass test tubes
F. Hot air ovens
G. Incubators
H. Petri plate
I. Laminar flow chamber
J. Sterile forceps and scissors
K. Pipettes
L. pH meter
M. Refrigerator and deep freezer
N. Blenders with steel jar and lid / Stomacher
O. Test tube racks and baskets to hold test tubes
P. Durham's tubes
Q. Microscope
R. Conical flasks
S. Inoculation loops
T. Spreaders (L-shape)
U. Glass slides
V. Measuring cylinder
W. Anaerobic jar
X. Anaerobic/Microaerobic Sachet

Annexure B: Microbiological Sampling -Toolbox

Sr. No.	Tools in sampling box	Verification
1	Sterile Forceps, Scissors and Spatula	
2	70% Alcohol or other suitable sanitizer and Cotton	
3	Clean, dry, leak proof, insoluble, non-absorbent and sterile containers / bag of a size suitable for sample.	
4	Labels, markers, lab sample sheet/ forms / notebook to record product information.	
5	Sterile swabs (If testing by swabbing)	
6	Suitable Diluent – BPW / 0.85% NaCl (If testing by rinse method)	
7	Cooler with ice packs	
8	Appropriate Clothing – Hair net, Mask, Hand Gloves etc.	
9	Sterile template for area measurement (If surface swabbing)	
10	Others, if required	

Annexure C: Precautions during sampling and transportation.

Sr. No.	Particular	Verification
I	**During sample receiving and analysis.**	
1	Wash your hands before you start with the sample collection	
2	Wear appropriate Clothing – Hair net, Mask, Hand Gloves etc.	
3	Collect all samples aseptically so as to not contaminate the sample.	
4	Collect a representative sample and the number of units that comprise a representative sample from a designated batch/lot of a food product must be statistically significant.	
5	Separate sampling units and sufficient quantity shall be taken for each type of test (e.g. Chemical, Microbiological and Physical).	
6	Place each sample into a separate clean, dry, leak proof, insoluble, non-absorbent and sterile containers/bag of a size suitable for sample.	
7	Make sure that the top of the container/bag is adequately closed.	
8	Label the outside of the bag with detailed information that will identify each sample.	
9	The sampling equipment and container shall not influence the microflora of the product.	
10	Use new sample container/bag for each sample unit.	
11	Sampling units shall be sent to the laboratory as quickly as possible after sampling, during which time they shall be maintained at the temperature at which the product concerned should be stored.	
12	Sample must be submitted in original and in sealed condition.	
13	Dry fish products like fish powders should be stored in temperatures not more than 5^0C	
14	Make sure that all the fields on the sample sheets are filled-out and the adequate information is provided.	

Annexure D: Precautions to be taken during sample receipt and analysis

Sr. No.	Particular	Verification
II	**During sample receiving and analysis.**	
1	Receive the samples with all the necessary information for the product identification.	
2	Note down the sample nature, condition, temperature etc.	
3	Samples shall be stored at the prescribed temperature, protected from direct sunlight or other sources of heat on receipt and examine them within 24 h.	
4	Store the swab sample in the refrigerator on receipt. The samples shall be examined as soon as possible, but in any case, on the day of receipt.	
5	Ensure that the working area is clean and draught-free; do not subject samples to direct sunlight.	
6	Clean the work surface with a suitable disinfectant before and after testing	
7	Sterilize apparatus and instruments for handling and opening sample container/bag in advance.	
8	Disinfect the packages over such a part of the exterior that contamination is avoided on opening (packaging material is very thin and could be damaged by the cleaning process this procedure shall be omitted).	
9	Do not proceed the samples with damaged packaging and product exposed outside.	
10	Sample analysis should be in the aseptic condition and necessary clothing and other measures should be taken to avoid the contamination.	

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